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Clinical effects of probiotics are associated with increased interferon-γ responses in very young children with atopic dermatitis

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Summary

Background We recently demonstrated that administration of probiotics resulted in significant clinical improvement in very young children with moderate-to-severe atopic dermatitis (AD). The purpose of this study was to determine the underlying immunological effects that are associated with these apparent clinical benefits.

Methods Peripheral blood mononuclear cells (PBMC) were isolated from children ($n = 53$) at baseline and at the end of an 8-week supplementation period during which they received a probiotic (Lactobacillus fermentum PCC^{TM}) ($n = 26$) or a placebo ($n = 27$). A further sample was collected at 16 weeks (8 weeks after ceasing the supplement). Cytokine (IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α) responses to allergens (egg ovalbumin (OVA), beta lactoglobulin (BLG), house dust mite (HDM)), vaccines (tetanus toxoid (TT)), diphtheria toxoid (DT)), intestinal flora (heat-killed Lactobacillus (HKLB)), heat-killed *Staphylococcus aureus* (HKSA), *Staphylococcus aureus* enterotoxin B (SEB) and mitogen (phytohaemaglutinin (PHA)) were compared.

Results The administration of probiotics was associated with a significant increase in T-helper type 1(Th1-type) cytokine IFN-γ responses to PHA and SEB at the end of the supplementation period (week 8: $P = 0.004$ and 0.046) as well as 8 weeks after ceasing supplementation (week 16: $P = 0.005$ and 0.021) relative to baseline levels of response. No significant changes were seen in the placebo group. The increase in IFN-γ responses to SEB was directly proportional to the decrease in the severity of AD ($r = -0.445$, $P = 0.026$) over the intervention period. At the end of the supplementation period (week 8) children receiving probiotics showed significantly higher TNF-α responses to HKLB ($P = 0.018$) and HKSA ($P = 0.011$) but this was no longer evident when supplementation ceased (week 16). Although IL-13 responses to OVA were significantly reduced in children receiving probiotics after 8 weeks ($P = 0.008$), there were no other effects on allergen-specific responses, and this effect was not sustained after ceasing supplementation (week 16). There were no effects on vaccine-specific responses, or on responses to any of the stimuli assessed.

Conclusion The improvement in AD severity with probiotic treatment was associated with significant increases in the capacity for Th1 IFN-γ responses and altered responses to skin and enteric flora. This effect was still evident 2 months after the supplementation was ceased. The lack of consistent effects on allergen-specific responses suggests that the effects of probiotics may be mediated through other independent pathways, which need to be explored further.

Keywords allergy, cytokines, food allergy, infantile atopic dermatitis, probiotics

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Introduction

While the recent rise in T-helper cell type 2 (Th2)-mediated allergic disease is likely to be multi-factorial, one of the most plausible candidates has been the apparent decline in microbial burden in early life. Early bacterial exposure during this period is essential for the development of antigen-presenting cell (APC), Th1 and T regulatory T cell function, as well as local airway immune networks. It has been proposed that early encounter with bacteria may inhibit the development of Th2-mediated allergic responses ([1, 2] and others) during early life when immune maturation is critical. Probiotic intestinal flora is arguably the most abundant source of early immune stimulation, and contribute significantly to 'microbial burden' in early life. There is good evidence from germ-free animal models that bacterial gut colonization is essential for maturation of immune function and induction of oral tolerance [3]. This has lead to interest in probiotic bacterial products in the treatment and prevention of disease.

The gastrointestinal tract makes up a critical part of the integrated 'common mucosal immune system' which is recognized as a distinct functional entity [4, 5]. Collectively, the mucosal associated lymphoid tissue (MALT) is the

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largest immune network in the body. Events at mucosal surfaces are integrated across anatomically remote locations (namely the gastrointestinal tract and respiratory tract), and it is well recognized that mucosa-homing IgA-producing B cells and effector T cells mature in the gut mucosa before seeding to distal mucosal sites in the respiratory tract [4, 5]. This provides one logical mechanism by which intestinal microflora could directly influence the maturation of immune responses in other tissues. Alteration in microflora or events that lead to gut inflammation in the gut could logically modify the local milieu and the rate and pattern of precursor maturation. This is supported by observations that infants who develop allergic disease have differences in very early colonization patterns [6–9]. Recent animal studies suggest that intestinal flora (probiotic supplementation) can induce regulatory populations [10] and human studies have also demonstrated an increase in the *in vitro* production of both Th1 IFN-γ [11] and regulatory cytokines (IL-10) after probiotic ingestion [12].

Preliminary studies have noted encouraging results using probiotics early in life for both primary prevention [13] and treatment of early allergic disease, namely atopic dermatitis (AD) [14–17]. Changes in fecal microbial counts following supplementation confirm that these supplements can achieve changes in microbial flora [16]. We recently demonstrated that young children (aged 6–18 months) with moderate-to-severe AD showed significant clinical improvement in the severity and extent of their disease as assessed by the SCORAD index [17]. The purpose of the present study was to assess effects on underlying immune function and relate these to the clinical improvement using samples collected during the clinical study.

Methods

These analyses were performed on peripheral blood mononuclear cell (PBMC) samples collected during the course of a randomized controlled clinical trial [17] which noted a clinical benefit in young children (6–18 months) treated with probiotics ($n = 26$) or a placebo ($n = 27$) for 8 weeks. PBMC were isolated from blood samples collected at baseline (week 0; prior to supplementation) and the end of the supplementation period (week 8) and a further 8 weeks after ceasing supplementation (week 16).

Participants

These details are published elsewhere [17]. Briefly, children with moderate or severe AD ($n = 56$) were recruited between April and November 2003 from the general community or outpatient clinics. Of these, only 53 children completed the study and provided blood samples at each time-point. All children diagnosed with AD met the Hanafen and Raijka criteria for AD and had a modified SCORAD $\geqslant 25$ [18, 19]. Children were ineligible for the study if they had any other major medical problems and were not already taking probiotics or other supplements.

Supplementation

Participants in the probiotic group received 1×10^9 *Lactobacillus fermentum* VRI 003 PCC™ (Protract Probiomics, Eveleigh, NSW, Australia) freeze-dried powder twice daily for 8 weeks. The control group received maltodextran without probiotic twice daily for the same duration. Both were dispensed as stable powder in identical individual 1 g sachets, reconstituted by parents with 5–10 mL of water and administered orally as a suspension. The trial protocol was approved by the Ethics Committees at St John of God Hospital and Princess Margaret Hospital and all women gave informed consent.

Clinical assessments and previously reported clinical findings

Participants had clinical assessments at week 2, week 4, at the end of intervention week 8, and final assessment at week 16. A detailed history was obtained by interview questionnaire, and an SCORAD assessment [20] was also performed by a clinician. This tool was used to assess the severity of AD by combining evaluation of extent, intensity of lesions and subjective symptoms (pruritus and sleep loss) [20]. To ensure consistency, a single investigator performed all SCORAD assessments.

The details of the study outcomes are also published elsewhere [17]. In summary, we observed a significant reduction in SCORAD index over time in the probiotic group ($P = 0.03$) but not the placebo group. Furthermore, significantly more children receiving probiotics ($n = 24$, 92%) had a SCORAD index that was better than baseline at week 16 compared with the placebo group ($n = 17$, 63%) ($P = 0.01$). Finally, at the completion of the study more children in the probiotic group had mild AD ($n = 14$, 54%) compared with placebo ($n = 8$, 30%). The laboratory parameters measured in this study were examined in relation to these clinical outcomes.

Assessment of sensitization

All children had either a RAST (using commercial Pharmacia CAPSystem, Uppsela, Sweden) or a SPT (Hollister-Stier Laboratories, Spokane, WA, USA) to assess for evidence of sensitization (specific IgE antibodies) to common allergens (hen's egg, cows milk, peanut, HDM, cat and grass pollens). A RAST of $\geqslant 0.35$ kU/L was considered positive.

Lymphocyte responses to allergen, microbial and mitogen stimulation

Peripheral blood samples were collected into heparinized tubes. Plasma was collected (for RAST testing as above) and the remaining sample was reconstituted with RPMI (Life Technology, Paisley, UK) tissue culture medium. Mononuclear cells (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. For cytokine analysis 2×10^9 PBMC/L were cultured in AIM V (Gibco, Life Technology, Paisley, UK) serum-free medium [21] for 48 h without stimuli (for spontaneous production of cytokines) or with (a) allergens, including HDM extract 20 μg/mL (CSL, Melbourne, Australia), ovalbumin (OVA) 200 μg/mL (Sigma, Castle Hill, Australia), β lactoglobulin (BLG) 100 μg/mL (Sigma); (b) vaccine antigens, including tetanus toxoid

(TT) 0.5 fL and diphtheria toxoid (DT) 0.5 fL; (c) *Staphylococcus* antigens, including SEB 200 ng/mL and heat-killed *S. aureus* 10^7 CFU/mL; (d) heat-killed *L. fermentum* 10^7 colony forming units (CFU)/mL; (e) phytohemaglutinin (PHA) mitogen 1 μg/mL (HA16, Murex, Biotech Ltd, Dartford, UK). For PHA stimulation (1 μg/mL) 10^9 PBMC /L were used.

The *S. aureus* strain was originally isolated from an infant with AD, and had been previously identified using three tests; latex agglutination assay (Staphatech®, Oxoid, Basingstoke, UK), DNase assay (Oxoid) and coagulase activity assay (Oxoid). *S. aureus* was cultured overnight in LB medium (Oxoid), washed twice with sterile PBS and CFU per millilitre quantified by spread plating 10-fold serial dilutions on LB agar (Oxoid) and incubating for 24 h at 37 °C. Bacteria were heat-killed at 60 °C for 60 min and stored in aliquots at −80 °C until use. An aliquot was checked for sterility by plating on LB agar for 48 °C.

PCC® *L. fermentum* VRI 003 (Probiomics Ltd, Eveleigh, NSW, Australia) was cultured overnight in De Man, Rogosa, Sharpe (MRS) broth (Oxoid) and the cells washed twice in phosphate-buffered saline (PBS) prior to enumeration of the viable cells by 10-fold serial dilution, and spread plating on MRS agar. CFU per millilitres of suspension were enumerated after anaerobic incubation at 37 °C for 24 h. The bacterial suspension was heat treated at 75 °C for 45 min and aliquots stored at −80 °C until use. An aliquot was checked for sterility by plating on MRS agar.

Supernatants were analysed for IL-5 using an in-house ELISA technique as previously described [22]. The limit of detection was 3 pg/mL. Transforming growth factor-β (TGF-β1) was determined using an ELISA kit (R&D Systems Europe, Abingdon, UK) for the detection the latent TGF-β. The concentration in many stimulated cell culture supernatants were reduced compared with the controls and this was reported as percentage suppression determined by the formula $(100 - [\text{control} - \text{stimulated}/\text{control}]) \times 100$. IL-6, IL-10, IL-13, TNF-α and IFN-γ were quantified by an in-house ELISA using a time resolved fluorometry (TRF) detection system (DELPHIA, PerkinElmer, Life Sciences, MA, USA) as previously described [23]. Briefly, the ELISA method was followed using paired antibodies (Pharmingen, Sydney, NSW, Australia) and the biotinylated antibody was detected using Europium-labelled streptavidin and fluorescence was quantified using a fluorometer (WALLAC VICTOR^2, PerkinElmer, Life Sciences). Standard curves, generated using serial dilutions of recombinant human IL-6, IL-10, IL-13, TNF-α or IFN-γ (Pharmingen), were linear between 5 and 30 000 pg/mL. Cytokine data was expressed as the difference between the stimulated culture and the control (pg/mL) and as detected (>5 pg/mL) or not detected.

Cytokine data are shown as spontaneous or stimulated production as indicated. The responses to various stimuli are shown as the level above the parallel background (unstimulated) control cultures. TGF-β1 is spontaneously produced in high levels in PBMC cultures and is generally down-regulated with activation, the data for this cytokine were defined in terms the percentage suppression. This is the level of down-regulation (background minus stimulated) as a percentage of the spontaneous production [24].

Analysis

Cytokine data were analysed as (a) continuous data, described by the geometric mean and 95% confidence intervals, and (b) dichotomous data (detected or non-detected). Where the data could be normalized with logarithmic transformation they are displayed as geometric mean and 95% confidence intervals, and groups compared by paired (for within-groups changes) or unpaired (for between-group comparisons) Student's *t*-tests. Where this was not possible the data are displayed as median and interquartile range and compared using nonparametric tests (Mann–Whitney *U*-test for between group comparisons and Wilcoxon's test for within group changes). For measures of correlation parametric tests (Pearson's correlation) were used where both variables where normally distributed. Nonparametric correlations were calculated (Spearman's or Kendall's (τ) b) where one or both valuables were not normally distributed. The Kendall's (τ) b-test was used in cases where a proportion of the variables of interest shared 'zero' values, in order to avoid the problems associated with 'ties' within the data. All statistical analyses were performed using SPSS software (Version 11 for Macintosh). A P-value <0.05 was considered statistically significant for all analyses.

Table 1. Characteristics of children in the probiotics and placebo groups

	Probiotic group ($n=26$)	Placebo group ($n=27$)
Gender		
Male	13 (50)	16 (59.3)
Female	13 (50)	11 (40.7)
Age (mean+SD) months	11.6 (4.3)	10.4 (3.32)
Total IgE (mean+SD) (kU/L)	29.7 (4.3)	37.3 (5.9)
RAST to food allergens		
Positive (>0.35 kU/L)	18 (70)	19 (70)
Negative	8 (30)	8 (31)
Immediate hypersensitivity reactions to foods		
Yes	7 (26.9)	9 (33.3)
No	19 (73.1)	18 (66.7)
Food avoidance (for immediate or delayed symptoms)		
Yes	14 (53.8)	13 (48.1)
No	12 (46.2)	14 (51.7)
Regularly eats yoghurt		
Yes	14 (53.8)	11 (40.7)
No	12 (46.2)	16 (59.3)
Has pets at home		
Yes	13 (50)	15 (55.6)
No	13 (50)	12 (44.4)
Antibiotics during study period		
Yes	10 (38.5)	11 (40.7)
No	16 (61.5)	16 (59.3)
Attendance at daycare		
Yes	5 (19.2)	7 (26)
No	21 (80.8)	20 (74)

Unless otherwise stated, values shown are for numbers of children (and percentages).
There were no significant differences between the groups.

Results

As previously published [17], there were no significant differences between the probiotic and placebo group in any of the baseline characteristics including age, gender, initial SCORAD score, use or potency of topical steroids, IgE levels or patterns of sensitization, and the presence of other allergic diseases. Group characteristics are shown in Table 1. Although around 40% of the children on this study received antibiotics at some stage during the study, the probiotic was resistant to all antibiotics prescribed (mainly penicillin and cephalosporin derivatives).

Probiotic supplementation increases polyclonal T-helper type 1 interferon-γ immune responses

Changes in IFN-γ responses were examined over the course of the study (weeks 0, 8,16) in response to allergens, mitogens, vaccines and microbial antigens. The children who received probiotics showed a significant increase in IFN-γ responses to mitogens and *Staphylococcal* SEB toxin following supplementation. Specifically, there was a significant increase in SEB-induced IFN-γ responses to mitogens between week 0 and the end of supplementation (week 8) in the probiotic group ($P = 0.046$). These levels of response were sustained at week 16 (with no subsequent decline to baseline responses) 8 weeks after ceasing supplementation (Fig. 1a). There were no significant changes in the placebo group, although the baseline levels (week 0) were higher in this group prior to the intervention ($P = 0.032$). IFN-γ responses to PHA also increased during supplementation with probiotics but not placebo (Fig. 1b), and this was statistically significant both at the end of the supplementation period (week 8; $P = 0.004$) as well as 8 weeks after ceasing supplementation (week 16; $P = 0.005$). However, there were no differences between the groups at weeks 8 and 16.

Probiotic supplementation was not associated with any other effects on IFN-γ responses to other specific stimuli (to allergens, whole killed bacteria or vaccines).

The effects of probiotics on pro-inflammatory cytokine responses

After supplementation with probiotics (week 8) children had significantly higher TNF-α responses to heat-killed bacteria compared with the placebo group (Fig. 2), including responses to both the intestinal species used for the supplementation (heat-killed Lactobacillus (HKLB) $P = 0.018$) and colonizing skin flora (heat-killed Staphylococcus aureus (HKSA) $P = 0.011$). This effect was no longer significant at 16 weeks. Probiotic supplementation did not alter IL-6 responses or TNF-α responses to any other stimuli.

The effects of probiotics on T-helper type 2 cytokine responses and allergen-specific responses

Although there were no consistent effects of probiotics on allergen-specific responses, we observed that IL-13 responses to OVA food allergen decreased significantly ($P = 0.008$) during the supplementation period (week 8). However this effect was not sustained after ceasing supplementation (week 16) as shown in Table 2. There were no other effects of

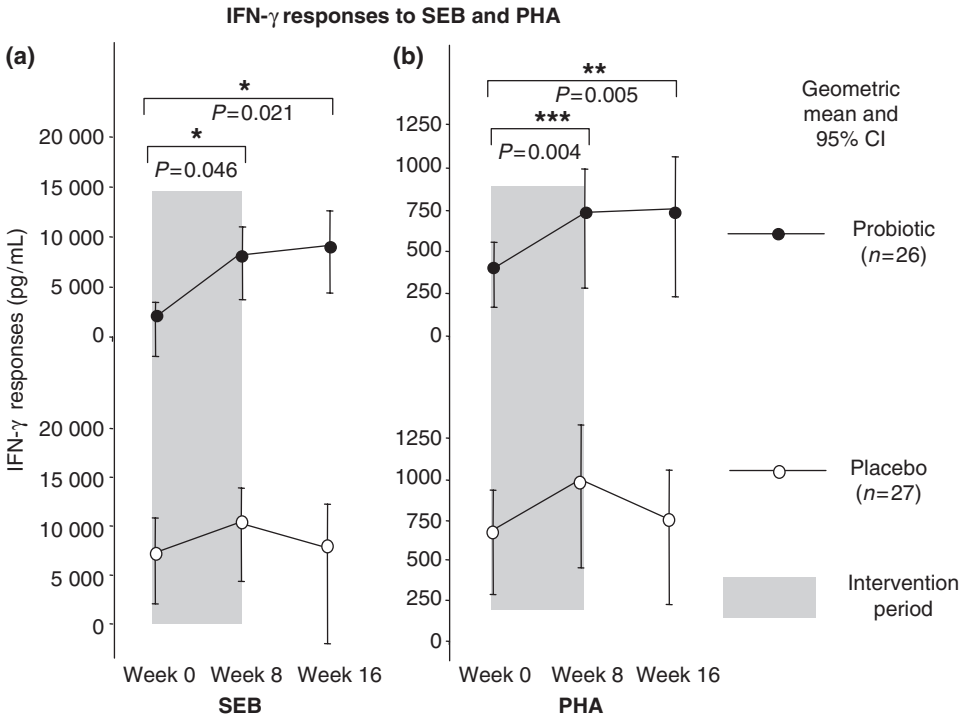

Fig. 1. Interferon-γ (IFN-γ) responses to (a) Staphylococcus enterotoxin B and (b) phytohaemaglutinen. The changes in IFN-γ responses to these stimuli are shown for the probiotic group (dark circles) and the placebo group (open circles) during the study period. The intervention period is denoted by the shaded area. All cytokine levels are shown as the level above the unstimulated control cultures and data are displayed as geometric mean and 95% confidence intervals. The data were analysed using paired Student's *t*-tests (for within-groups comparisons) or unpaired tests for between-group comparisons. $P < 0.05$ was considered statistically significant. Significance levels are indicated and denoted $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.005$.

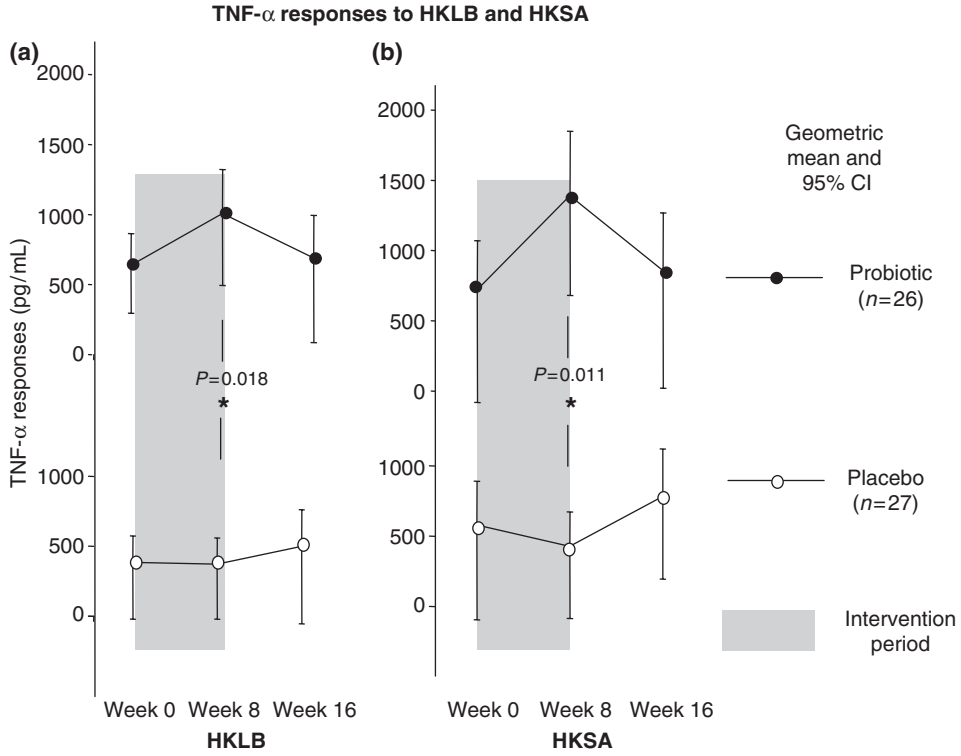

Fig. 2. Tumour necrosis factor alpha (TNFα) responses to (a) heat-killed Lactobacilli and (b) heat-killed Staphylococcus aureus. The changes in TNFα responses to these stimuli are shown for the probiotic group (dark circles) and the placebo group (open circles) during the study period. The intervention period is denoted by the shaded area. All cytokine levels are shown as the level above the unstimulated control cultures and data are displayed as geometric mean and 95% confidence intervals. The data were analysed using paired Student's *t*-tests (for within-group comparisons) or unpaired tests for between-group comparisons. $P<0.05$ was considered statistically significant. Significance levels are indicated and denoted $^{*}P<0.05$.

Table 2. Comparison of allergen-specific responses (to house dust mite (HDM), ovalbumin (OVA) and beta lactoglobulin (BLG) in children receiving probiotics or placebo

	PROBIOTIC GROUP (n = 26) median (interquartile ranges) pg/ml / % responders						PLACEBO GROUP (n = 27) median (interquartile ranges) pg/ml / % responders					
	Week 0		Week 8		Week 16		Week 0		Week 8		Week 16	
IL-10												
HDM	7.3 (<3.0–13.8)	54%	<3.0 (<3.0–9.4)	48%	11.3 (<3.0–21.5)	61%	5.9 (<3.0–11.5)	56%	8.5 (<3.0–15)	67%	11.7 (<3.0–18.9)	69%
OVA	25.3 (9.3–54.2)	85%	15.8 (7.3–34.9)	80%	30.7 (16.1–93.1)	91%	29.0 (11.2–61.7)	89%	29.7 (11.5–56)	85%	37.5 (17.9–55.7)	92%
BLG	<3.0 (<3.0–9.5)	39%	<3.0 (<3.0–6.9)	32%	<3.0 (<3.0–7.4)	30%	7.1 (<3.0–9.8)	59%	6.6 (<3.0–11)	58%	5.0 (<3.0–10.9)	50%
IL-13												
HDM	19.5 (9.7–100.3)	85%	28.0 (8.5–81.6)	84%	46.9 (17.6–248.7)	83%	17.9 (<3.0–59.4)	74%	19.5 (7.8–121)	82%	#29.9 (8.3–115.2)	81%
OVA	18.9 (<3.0–60.6)	73%	*9.3 (<3.0–39)	60%	16.3 (<3.0–76.9)	70%	16.0 (<3.0–37.6)	70%	13.8 (<3.0–37)	70%	16.1 (6.4–39.5)	77%
BLG	3.9 (<3.0–17.5)	50%	<3.0 (<3.0–9.4)	36%	<3.0 (<3.0–15.1)	48%	<3.0 (<3.0–10.2)	37%	<3.0 (<3.0–11)	31%	<3.0 (<3.0–14.7)	46%
IFNγ												
HDM	<3.0 (<3.0–24)	44%	<3.0 (<3.0–27)	40%	<3.0 (<3.0–61.4)	44%	<3.0 (<3.0–11.4)	37%	*13.1 (<3.0–89)	54%	6.0 (<3.0–25.2)	58%
OVA	<3.0 (<3.0–28.7)	44%	<3.0 (<3.0–23.7)	38%	8.1 (<3.0–52.2)	52%	7.0 (<3.0–33.7)	52%	15.9 (<3.0–99)	54%	13 (<3.0–73)	54%
BLG	<3.0 (<3.0–<3.0)	12%	<3.0 (<3.0–<3.0)	20%	<3.0 (<3.0–<3.0)	22%	<3.0 (<3.0–<3.0)	19%	<3.0 (<3.0–5)	24%	<3.0 (<3.0–<3.0)	12%
TNFα												
HDM	<3.0 (<3.0–5)	26%	<3.0 (<3.0–<3.0)	9%	<3.0 (<3.0–<3.0)	18%	<3.0 (<3.0–<3.0)	0%	<3.0 (<3.0–3)	9%	<3.0 (<3.0–<3.0)	9%
OVA	34.1 (15.5–96.1)	91%	37.9 (19.2–80.3)	96%	35.8 (22.3–67.4)	86%	23.1 (8.6–53.8)	79%	24.5 (6.7250)	83%	24.3 (8.0–57.9)	87%
BLG	<3.0 (<3.0–<3.0)	9%	<3.0 (<3.0–<3.0)	4%	<3.0 (<3.0–<3.0)	14%	<3.0 (<3.0–<3.0)	8%	<3.0 (<3.0–3)	9%	<3.0 (<3.0–<3.0)	4%

*Denotes a significant change in response within a group between week 0-8 ($P<0.01$);
# Denotes a significant change in response within a group between week 0–16 ($P<0.01$);
There was no significant difference between the groups

probiotic supplementation on allergen-specific responses. Although there was an age-related increase in IL-13 responses to HDM in both groups, this was only significant ($P = 0.008$) in the placebo group (Table 2).

Probiotic supplementation was not associated with any effects on Th2 responses to any of the other stimuli tested.

The effects of probiotics on cytokine-10 and transforming growth factor-β regulatory cytokine responses

In this study we did not see any effects of probiotic supplementation on the production of either of these cytokines in response to any of the stimuli assessed.

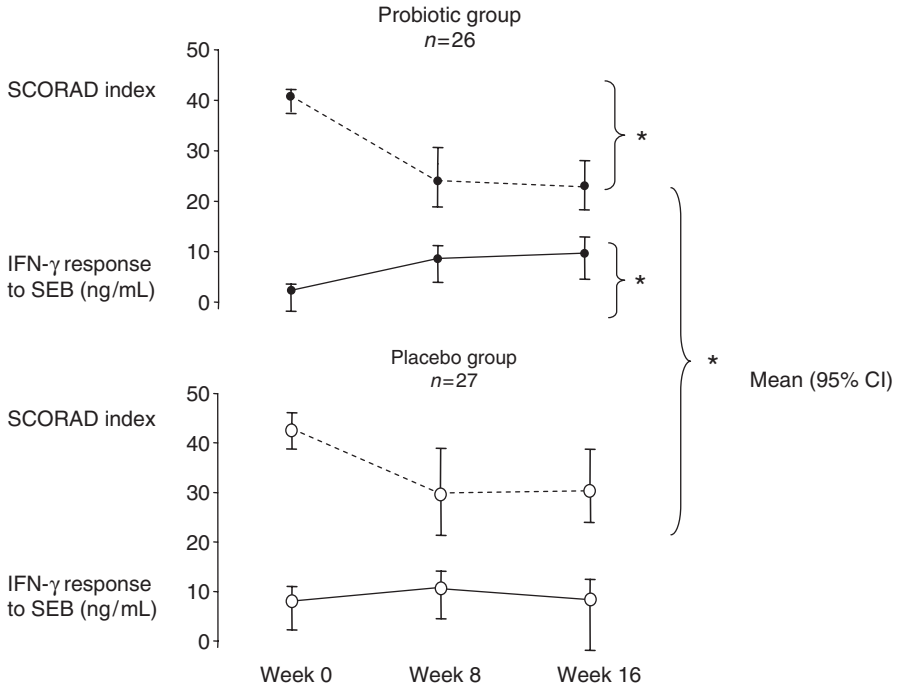

Fig. 3. Changes in dermatitis severity (SCORAD index) in relation to changes in polyclonal Interferon-γ (IFN-γ) responses. The changes in IFN-γ responses (ng/mL) to Staphylococcus enterotoxin B (uninterrupted lines) are shown in relation to the change in SCORAD index (dashed lines) for the probiotic group (dark circles) and the placebo group (open circles) during the study period. All cytokine levels are shown as the level above the unstimulated control cultures. Data are displayed as geometric mean and 95% confidence intervals. The data were analysed using paired Student's *t*-tests (for within-group comparisons) or unpaired tests for between-group comparisons. $P<0.05$ was considered statistically significant, difference between the groups or changes from baseline (week 0) are denoted $^*P<0.05$.

The relationship between clinical effects and cytokine responses

The infant immune responses were examined in relation to clinical effects of probiotic supplementation. The increase in polyclonal IFN-γ response was associated with the previously documented [17] clinical improvement in the children receiving probiotic as shown by the significant decrease in SCORAD index in Fig. 3 (also see information on participants in 'Methods'). The *increase* in IFN-γ responses to SEB (week 8) was directly proportional to the percentage improvement (*decrease* in the severity) of AD ($r = -0.445$, $P = 0.026$) over the 8-week intervention periods. This trend was no longer seen 2 months after the supplementation (week 16; $r = -0.13$, $P = 0.53$). There were no other significant correlations with the change in SCORAD over the study period.

Discussion

This study provided an opportunity to further examine the immunologic effects of probiotics in a population of children with AD who have previously been shown to have clinical improvement with supplementation. The principal finding was that probiotic administration was associated with maturation of Th1 IFN-γ responses, as measured by 'non-specific' polyclonal responses (to SEB and PHA), and this was related to the clinical improvement. Although these stimuli elicit T cell stimulation by different pathways, they are useful measures of the functional capacity and maturational status of Th1 pathways [25], as they result in higher-level stimulation compared with antigen-specific methods of stimulation. These results are consistent with a recent report by Pohjavuori et al. [11] in which children with cow's milk allergy ($n = 12$) and AD ($n = 14$) showed increases *in vitro* polyclonal IFN-γ responses after 4 weeks of supplementation with *Lactobacillus rhamnosus* GG. Of note, in this study we observed that differences in Th1 IFN-γ responses were not seen in response to 'specific' stimuli such as allergens and vaccines. This may be because of more subtle effects not detectable here, of which the effects of probiotics are independent of these pathways. In support of the latter, we did not see any consistent effects on allergen-specific responses, with the exception of a transient decrease in IL-13 responses to OVA during probiotic supplementation (Table 2). There were no effects on any other allergen/antigen responses, and the potential long-term effects on development of these immune responses remain to be determined.

We did note that the probiotic group had lower Th1 IFN-γ responses to SEB at baseline compared with the placebo group. This could not have been anticipated or avoided at randomization as the groups were otherwise carefully matched for all relevant characteristics (such as disease severity and medication usage). It remains relevant that there were statistically significant changes within the probiotic group and no changes in the placebo group. This effect could not be accounted for by changes in any other exposures, or other differences between the groups (Table 1).

While allergens and other specific antigens stimulate APC-driven specific T cell responses, microbial stimuli also stimulate APC through innate pathways including ligation

of Toll-like receptors (TLR). As a key part of host defence these pathways evoke potent Th1 responses. The same microbial elements also stimulate natural killer T (NKT) cells that also produce large amounts of IFN-γ and TNF-α. It is likely that these pathways are contributing significantly to the HKSA- and HKLB-induced production of these cytokines in this study. We have previously noted that TNF-α is deficient in AD and may play a role in disease expression, with significantly lower TNF-α responses to allergens, vaccines and bacteria (including both HKSA and HKLB). Here we demonstrate that probiotics increase TNF-α responses to both HKSA and HKLB (but not other stimuli) suggesting effects, on innate pathways but not allergen-specific pathways, by which probiotics may mediate an effect in AD. This needs to be investigated further.

Thus, we have demonstrated that the administration of probiotic gut flora is associated with changes in systemic T cell responses as assessed using circulating PBMC, and that this was associated with a clinical improvement in cutaneous disease. Some of these effects *persist* after the probiotics ceased (such as the increased polyclonal IFN-γ responses) whereas other effects were only seen *transiently* during the period of supplementation (such as lower IL-13 responses to OVA and increased TNF-α responses to bacteria). The mechanisms by which events in the gut can affect the systemic immune system and local inflammation in remote tissues such as the skin and the respiratory tract remain to be determined. We propose that intestinal flora (including probiotic strains) influence the maturation of a large pool of immature precursor cells that circulate through the gut and subsequently home to tissues throughout the body [4, 5], particularly to other mucosal surfaces where they develop their mature functional attributes. These precursors can develop into a diverse range of lymphocytes (including regulatory cells) depending on ambient maturational signals and this could logically explain the apparently diverse effects of intestinal microbiotica. Alteration in microflora or events that lead to gut inflammation in the gut could logically modify the local milieu and the rate and pattern of precursor maturation. This could also explain how events in the gut mucosa can influence local immune development in remote tissues. Animal studies suggest that probiotic supplementation can induce regulatory populations [10]. While we did not measure T regulatory cell function directly in this study, we did not see any effects on regulatory cytokines (IL-10 or TGF-β). Our findings are also at odds with human studies that reported an increase in the *in vitro* production of regulatory cytokines (IL-10) after probiotic ingestion [12]. It is possible that this reflects noted species-specific differences in immunologic effects [11, 27]. Further studies are still needed in humans to address the effects on regulatory cell populations directly.

It has been more difficult to explain remote effects of gut microflora on immune cells of bone marrow origin (including monocytes and other APC) that do not directly circulate through the gut before migrating to peripheral tissues. We did not examine the effects on specific cell populations in this study, but others have noted that peripheral blood monocytes in infant animals mature at significantly different rates depending on enteric microflora exposure [28]; with two-fold lower function in germ-free animals [29]. Although dendritic cells (DC) derived from murine bone marrow are activated *directly* by probiotics *in vitro* to produce strong IL-12 and TNF-α responses [30] this is unlikely to be relevant i*n vivo* except for DC which ultimately home to the gut. This suggests other *indirect* influences between events in the gut and developing bone marrow populations. Preliminary studies in humans suggest that this effect could be directly on the bone marrow [31]. The study in question found that oral probiotic supplementation was associated with significant changes ($P<0.001$) in the numbers of circulating ($CD34^+$) bone marrow precursor cells in peripheral blood [31]. This suggests that probiotics may act through a number of pathways in addition to the effects on T cell function that we have shown here. Further studies are needed to examine this in more detail. A better understanding of the underlying mechanisms of action may help optimize the therapeutic potential of probiotics in allergic disease. As many children with AD are at risk of developing inhalant sensitization and related respiratory disease, this early period may also offer a window of opportunity to prevent this 'atopic march', and further prospective studies are also needed to examine the potential of probiotics in this regard.

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