

Original article

Early markers of allergic disease in a primary prevention study using probiotics: 2.5-year follow-up phase

Background: We previously reported that a *Lactobacillus acidophilus* probiotic strain (LAFTI® L10/LAVRI-A1) given for the first 6 months of life increased the risk of allergen sensitization at 1 year of age.

Methods: To assess the effects on subsequent allergic outcomes, 153 children from the initial prevention cohort ($n = 178$) were reviewed at 2.5 years of age. Clinical outcomes were assessed in relation to (i) probiotic supplementation; and (ii) immune function previously assessed at 6 months of age.

Results: Supplementation with this probiotic did not reduce the risk of dermatitis at 2.5 years (31/74, 42%) compared with that in placebo group (25/76, 34%). There was no significant reduction in any other allergic disease or allergen sensitization. Inhalant sensitization at 2.5 years ($n = 29$) was associated with higher proportions of circulating CD4+ CD25+ regulatory T-cell populations ($P = 0.005$) and higher allergen-induced FOXP3 levels ($P = 0.003$) at 6 months. This was also seen in children with dermatitis. Children with dermatitis at 2.5 years also had significantly lower toll-like receptor 4 lipopolysaccharide responses at 6 months of age (IL-12 $P = 0.04$, IL-6 $P = 0.039$) and lower polyclonal (PHA) responses (IFN- γ $P = 0.005$, IL-10 $P = 0.001$, and IL-6 $P = 0.001$). Children who had previously received the probiotic had fewer gastrointestinal infections in the preceding 18 months ($P = 0.023$).

Conclusion: The LAFTI® L10 probiotic strain did not have any significant effect on allergy outcomes. Allergic children showed a number of early differences in immune function including altered regulatory T-cell markers and innate immune function.

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The therapeutic properties of probiotic bacteria are of major interest in the context of rising rates of allergy and concerns that more 'hygienic' environments may contribute to early immune dysregulation. Probiotic strains are derived from normal intestinal flora with recognized human health benefits. At least some of these beneficial effects may be caused by immunoregulatory properties that promote tolerance and inhibit inappropriate systemic immune responses (reviewed in (1)). Several studies suggest that specific probiotics may be of benefit in the treatment of allergic disease (namely dermatitis) in early childhood (2–6). The role in primary disease prevention is less clear and is likely to depend on the probiotic strain used, the timing and duration of supplementation, and other host factors. The first studies to suggest a role in allergy prevention used the *Lactobacillus rhamnosus* LGG strain, beginning in late pregnancy 2–4 weeks before delivery. This was reported to reduce the incidence of eczema at 2 years by around 50%, but there was no reduction in respiratory allergy, IgE levels or allergic sensitization (7). Other studies have also showed a reduction in dermatitis (8) or IgE-mediated disease

(8, 9), although one of these used probiotic strains in combination with prebiotic galacto-oligosaccharides which could have independent effects on colonization (8). So far none of these studies has shown long-term benefits on other allergic diseases (such as asthma and allergic rhinitis).

Our own studies used a *Lactobacillus acidophilus* LAFTI® L10 [DSM Food Specialties, Moorebank, NSW, Australia, also known as LAVRI-A1 (10)]. This strain failed to demonstrate any health benefits in the first year of life (10) despite previously reported favorable effects in humans (11) and in animal models (11–13). Rather, there was a concerning increase in sensitization and in IgE-associated atopic eczema (10).

The main purpose of this follow-up study was to determine the subsequent effects of early supplementation with this strain, particularly on persistent dermatitis and food sensitization in addition to emerging inhalant sensitization and early symptoms of respiratory disease in this population.

Another key purpose of the study was to examine the relationship between early measures of immune function

(already assessed at 6 months of age) and subsequent allergic outcomes in this cohort.

Methods

Study design

This was a follow-up study of a previously recruited cohort (10). The original design was a randomized double-blind, placebo-controlled study to assess the effect of probiotic supplementation from birth to 6 months on allergic outcomes. These were previously assessed at 12 months of age (10), and this study followed the same infants at 30 months (2.5 years) of age. The subjects and the investigators performing the clinical assessments remained blind to the original intervention.

Participants

The original cohort of 231 pregnant, atopic women was recruited in Perth, Western Australia as previously described (10). Maternal atopy was defined as a doctor diagnosed clinical history of asthma, allergic rhinitis or eczema plus a positive skin prick test (SPT) to one or more common allergens (house dust mite; grass pollens; cat; dog; feathers; moulds; and cockroach extracts; Hollister-Stier Laboratories, Spokane, WA, USA). Women were ineligible for the study if they smoked; had other medical problems or pregnancy complications; delivered prior to 37 weeks gestation; or were already taking probiotic supplements.

The supplementation

The intervention commenced within 48 h of delivery, and was given only to the baby, independent of feeding methods. Infants in the probiotic group received daily 3×10^9 *L. acidophilus* LAFTI® L10 in maltodextrin (sourced from DSM Food Specialties, Moorebank, NSW, Australia and supplied as LAVRI-A1 Probiomics, Australia), while those in the control group received maltodextrin alone. Supplements were supplied as stable freeze dried powder (in sachet packets), dissolved in 1–2 ml sterile water, and administered orally on a daily basis from birth to 6 months. *Lactobacillus 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) (14, 15), as tested and verified by the commercial entity Probiomics.

Probiotic and placebo supplements were image-matched and participants, research scientists, and pediatrician 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 allocation process. Compliance was monitored by the use of a dose chart (completed by parents) and dose counts (returned sachet packets counted by the pharmacy department).

Clinical outcomes

Infants were clinically evaluated at 2.5 years of age, which included a detailed history and examination by the same pediatrician (Prof. Susan Prescott). The diagnostic criteria conformed to the recently published clinical guidelines (16). A diagnosis of atopic dermatitis was made in infants with typical skin lesions responsive to topical

steroids (17). The severity of dermatitis was determined using the SCORAD index as previously described (18). IgE-mediated food allergy was defined as a history of immediate (within 60 min) symptoms following contact with and/or ingestion of food (such as egg, dairy, nut, etc.) and a positive SPT to the implicated food. Symptoms of acute food allergy included skin reactions (hives, rash or swelling) and/or respiratory symptoms (cough, wheeze, stridor) and/or gastrointestinal symptoms (abdominal pain, vomiting, loose stools) and/or cardiovascular symptoms (collapse). Asthma was defined as recurrent wheezing (three or more episodes, at least one confirmed by a pediatrician or general practitioner with evidence of responsiveness to bronchodilator therapy).

Other symptoms were also assessed, based on as parental recording (on prospective monthly diary cards). All parents who noted 'noisy breathing' of any kind were asked to give detailed descriptions of this to the pediatrician (if not already confirmed by another physician). This was then used to determine if the symptoms were likely to be 'wheeze', 'stridor' or because of secretions in the upper airway. Only children with a convincing history of 'wheeze' (or physician documented wheeze) were classified in this category. Children were recorded as having 'upper respiratory infections' if they had infections that were limited to coryzal symptoms in the absence of significant chest symptoms. Doctor-diagnosed gastrointestinal infections were also recorded.

Allergen SPT

Skin prick test was performed with common allergen extracts [milk, peanut, house dust mite, cat, grass, mould (Hollister-Stier Laboratories); and whole egg] applied to the volar surface of the forearm. Separate sterile lancets were used for each allergen tested. Histamine was used as a positive control and glycerine as a negative control. A wheal diameter of ≥ 3 mm was considered positive.

Assessment of potential confounding factors and interpretation of clinical data

Extensive data were collected on environmental factors that could confound or influence the relationship between probiotics and allergic disease. Most of this data were collected prospectively (diary cards) and included information about other clinical disease and common exposures (vaccination, infection, day care, diet, and medication use including antibiotics) as well as the home environment (including carpeting, sibship, and pets).

Sample collection and initial processing

Blood samples were available from 103 of the 151 subjects. These were collected into heparinized tubes and peripheral blood mononuclear cells (PBMC) were isolated within 2 h using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis.

Flow cytometric analysis of regulatory T-cell phenotype

Thawed PBMC were washed and stained with a commercial three-colour antibody kit (BD Biosciences, Sydney, NSW, Australia) containing fluorescein isothiocyanate-conjugated CD4, CD25-phycoerythrin, and CD3-PerCpCy5.5. Intracellular staining for CTLA4 was performed with allophycocyanin-conjugated CTLA4 using Cell Fix/Perm (BD Biosciences). Stained cells were resuspended in PBS/0.2% BSA/0.05% sodium azide and analyzed immediately on a

FACSCalibur flow cytometer (Becton Dickinson). At least 100 000 mononuclear cells were acquired on the flow cytometer to obtain sufficient numbers of regulatory T cells for accurate enumeration. Data analysis was performed with the FLOWJO Software (TreeStar, Ashland, OR, USA).

Cell culture for allergen and mitogen responses

The technique used to culture infant cells is described in detail elsewhere (19). Briefly, 2×10^6 PBMC/ml were cultured in duplicate in 96-well round-bottom plates in AIM V (Gibco; Life Technology Ltd, Paisley, UK) serum-free medium for 48 h with or without (control) whole dust mite extract (HDM) 20 µg/ml (CSL, Melbourne, Australia), ovalbumin (OVA) 100 µg/ml (Sigma, Castle Hill, Australia), or phytohaemagglutinin (PHA) mitogen 1 µg/ml (HA16; Murex; Biotech Ltd, Dartford, UK). For PHA stimulation ($1 \mu\text{g/ml}$) 10^6 PBMC/ml were used. All plates were then placed in a 37°C 5% CO₂ incubator for 48 h when supernatants were collected for cytokine detection (below).

Cell culture for TLR responses

To assess functional toll-like receptor (TLR) responses, we measured the pattern and magnitude of cytokine responses following activation with specific microbial ligands for TLR2 (Pansorbin) (20) and TLR4/CD14 [lipopolysaccharide (LPS)]. PBMC (1×10^6 /ml) were cultured in duplicate in 96-well round-bottom plates in RPMI plus 10% fetal calf serum (not heat inactivated) (Australian Bioscience, Australia) either alone or with optimized doses of Pansorbin (0.1%; Calbiochem®, EMD Biosciences, San Diego, CA, USA) or LPS (*Escherichia Coli* LPS; Sigma, Castle Hill, Australia; 10 ng/ml) for 24 h at 37°C with 5% CO₂. After 24 h, the supernatants were collected, and stored at -20°C for cytokine analysis by enzyme linked immunosorbent assay (ELISA) or time resolved fluorometry (TRF) as outlined below.

mRNA isolation and analysis

The mRNA was isolated from the cell pellets of allergen (OVA and HDM) and control cultures. Briefly, total RNA was isolated using the RNeasy 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. Reverse transcription was performed using the Omniscript kit (Qiagen) according to the manufacturer's protocol with oligo-dT (Promega, Madison, WI, USA) and RNasin® Ribonuclease Inhibitor (Promega). Reverse transcribed RNA samples were diluted 1/5 and FOXP3 expression was quantitated by real-time PCR using QuantiTect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems, Uppsala, Sweden). The FOXP3 primer sequence used was produced commercially by Prologo (Sigma Aldrich, St Louis, MO, USA; forward primer: GAA ACA GCA CATT CCC AGA GT; reverse primer: ATG GCC CAG CGG ATG AG). Relative standard curves were prepared from serial diluted RT-PCR products or plasmid standards and normalized to the reference housekeeping gene (HKG) UBE2D2 (21). Data are expressed as expression level above background (level in stimulated cells minus level in unstimulated cells) and multiplied by a scaling factor to obtain whole numbers.

Cytokine assays

Supernatants from the assays outlined above were analyzed for IL-5, IL-6, IL-10, IL-12p70, IL-13, INF-γ, and TNF-α with an in-house ELISA using a time resolved fluorometry detection system

(DELPHIA, PerkinElmer; Life Sciences, Boston, 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; Life Sciences). Standard curves generated using serial dilutions of recombinant human IL-5, IL-6, IL-10, IL-13, INF-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). IL-12p70 was determined using a commercial ELISA kit (OptEIA; Pharmingen) according to the manufacturer's instructions. The limit of detection was 3 pg/ml for all cytokines. Cytokine data are shown as 'stimulated' production (i.e. the level above the parallel background (unstimulated) control cultures).

Statistical analysis

Statistical analysis was performed using SPSS software (version 11.0 for Mac OS X; SPSS Inc., Chicago, IL, USA). Categorical data were determined by the chi-squared, Fisher's exact tests or logistic regression. Continuous data were compared using non-parametric tests (Mann-Whitney *U*-test) and displayed as median, interquartile range, and 95% confidence intervals. Correlations were assessed using Spearman or Kendall's tau (τ) b (where a proportion of the variables of interest shared 'zero' values) to avoid the problems associated with 'ties' within the data. Logistic regression was used to determine the relationships between clinical outcomes (binary dependent variables) and supplementation or immune parameters and to assess the effect of potential confounding factors on these relationships. A *P*-value of <0.05 was considered statistically significant for all analyses.

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 (SJOG) and Mercy Hospital ethics committees, and all women gave informed consent.

Results

Clinical characteristics

Of the original 178 children that completed the supplementation, 153 were reviewed at 2.5 years of age including 77 in the probiotic group and 76 in the placebo group. The clinical characteristics are shown in Table 1.

There were no differences in the rate of allergic disease (food allergy, dermatitis or asthma) (Table 1) or allergen sensitization at 2.5 years (Table 2), although there was a trend for less sensitization to cow's milk in the probiotic group (*P* = 0.098, Fischer's exact test). All of the children who newly developed sensitization to milk (since their assessment at 1 year of age) were in the placebo group (with none of the probiotic group developing new sensitization).

Of children who were previously sensitized (at 1 year), those in the probiotic group were less likely (1/29, 3%) than the placebo group (4/15, 27%) to still have milk allergy at 2.5 years (*P* = 0.036). In this subgroup, there

Table 1. Clinical characteristics: probiotic compared to placebo group

Characteristics	Control <i>n</i> (%)	Probiotic <i>n</i> (%)	<i>P</i> -value
<i>2.5 years of age (n = 153)</i>	<i>n</i> = 76	<i>n</i> = 77	
Diagnosed allergic disease (any)	34/76 (46)	41/77 (42)	0.421
Sensitization (SPT +ve to any allergen) (see Table 2 for details)	20/69 (29)	24/71 (34)	0.588
Atopic dermatitis (AD)			
Any doctor-diagnosed AD	25/76 (34)	31/74 (42)	0.402
SPT +ve AD	12/72 (17)	17/75 (23)	0.411
SCORAD at 2.5 years	11.94 (1.71)	13.6 (1.92)	0.521
SCORAD at worst in last year (mean ± SE)	22.01 (2.30)	24.00 (2.39)	0.551
Food allergy (IgE mediated)	11/74 (14)	11/76 (14)	1.000
Recurrent cough (without cold)	21/74 (28)	22/76 (29)	1.000
Diagnosed asthma	7/77 (9)	5/74 (7)	0.766
Wheeze			
In the last year	13/74 (18)	18/76 (24)	0.422
Ever	18/74 (24)	22/76 (29)	0.582
Recurrent wheeze ≥2 episodes	10/74 (14)	12/76 (16)	0.818
Chest infection	37/74 (50)	46/76 (60)	0.250
Ear infection (diagnosed)	31/74 (42)	30/76 (40)	0.868
Gastrointestinal infections	20/74 (27)	9/76 (12)	0.023*
<i>Features of same children at 1 year of age (n = 153)</i>	<i>n</i> = 76	<i>n</i> = 77	
Diagnosed allergic disease (any)	32/73 (44)	40/76 (53)	0.283
Sensitization (SPT +ve to any allergen)	15/72 (21)	41/76 (34)	0.009**
Atopic dermatitis			
Any doctor-diagnosed AD	29/73 (40)	36/76 (47)	0.347
SPT +ve AD	9/72 (12)	21/76 (28)	0.022*
Food allergy (IgE mediated)	9/73 (12)	12/76 (16)	0.544
Recurrent cough (without cold)	23/73 (32)	18/76 (24)	0.285
Wheeze			
Ever in the in the first year	11/73 (15)	22/76 (29)	0.041*
Recurrent wheeze ≥2 episodes	3/73 (4)	4/76 (5)	0.739
Chest infection	42/73 (58)	45/76 (59)	0.836
Ear infection (diagnosed)	14/72 (19)	22/76 (29)	0.178
Gastrointestinal infections	16/72 (22)	18/76 (24)	0.833

Significant differences between the groups were determined by Pearson's chi-square test for all nominal data.

P* < 0.05 was considered a significant difference (shaded); *P* ≤ 0.01.

was also a trend for less grass pollen allergy in the probiotic group (3/27, 10%) at 2.5 years compared with that of placebo group (4/15, 27%; *P* = 0.154). Of children who were *not* previously sensitized at 1 year of age, those in the probiotic group tended to be less likely (0%) to have HDM allergy at 2.5 years compared with the placebo group (5/52, 10%; *P* = 0.05).

Twenty-five children were lost to follow-up after the previously published 12 month assessment (10). Although the characteristics of these children are not known, the characteristics of the remaining 153 children are also shown at 12 months in both Tables 1 and 2. Consistent with the previous report (10), this analysis also clearly shows that children in the probiotic group had higher rates of sensitization (*P* = 0.009) and SPT ± dermatitis (*P* = 0.02) at 1 year. This was no longer evident at 2.5 years in the same children, indicating that this change was not because of study drop-outs. We also noted that

Table 2. Sensitization characteristics in the first and third year of life: probiotic compared to placebo group (in children followed to 2.5 years)

	Control <i>n</i> = 69 <i>n</i> (%)	Probiotic <i>n</i> = 71 <i>n</i> (%)	<i>P</i> -value
Sensitization in third year			
Positive skin prick test (any), <i>n</i> (%)	20/69 (29)	24/71 (34)	0.568
Food (any)	12/69 (17)	14/71 (15)	0.528
Egg (whole fresh egg)	8/69 (12)	11/71 (16)	0.142
Cow's milk	5/69 (7)	1 (1)	0.098†
Peanut	6/69 (9)	11/79 (16)	0.302
Inhalant (any)	18/69 (26)	20/71 (28)	0.850
House dust mite	16/69 (23)	14/71 (20)	0.677
Grass mix	6/69 (9)	3/71 (4)	0.322
Mould mix	2/69 (3)	4/71 (6)	0.681
Cat pelt	5/69 (7)	8/71 (11)	0.563
Rye grass	3/69 (4)	2/71 (3)	0.678
Sensitization in first year	Control <i>n</i> = 72 <i>n</i> (%)	Probiotic <i>n</i> = 76 <i>n</i> (%)	<i>P</i> -value
Positive skin prick test (any), <i>n</i> (%)	15/72 (21)	41/76 (34)	0.009**
Food (any)	14/72 (19)	28/76 (37)	0.015*
Egg (whole fresh egg)	13/72 (18)	24/76 (32)	0.043*
Cow's milk	1/72 (1)	4/76 (1)	0.201
Peanut	6/72 (8)	12/76 (16)	0.128
Inhalant (any)	7/72 (10)	10/76 (13)	0.347
House dust mite	3/72 (4)	3/76 (4)	0.946
Grass mix	1/72 (1)	0/76 (0)	0.486
Mould mix	1/72 (1)	1/76 (1)	0.969
Cat pelt	2/72 (3)	7/76 (9)	0.097†
Rye grass	0/72 (0)	0/76 (0)	—

The groups were compared with Pearson's chi-squared test or Fischer's exact test for all nominal data.

P* < 0.05 was considered a significant difference (none detected); *P* ≤ 0.01.

†Relationship of interest (shaded).

wheezing was more common in the first year of life in the probiotic group but that this was no longer evident in the third year of life.

Children who had previously received the probiotic had significantly fewer gastrointestinal infections in the preceding 18 months compared with the placebo group (*P* = 0.023). This relationship remained evident when allowing for variations in exposure to other children (in day care and play groups).

There were no differences in the key environmental exposures assessed including attendance at childcare facilities, household pets, older siblings or prescribed antibiotics. In this study, there was no relationship between the duration of breastfeeding (or the age at starting solid foods) and allergic outcomes.

Associations between early immune measures and allergic outcomes

We have previously reported the effect of perinatal probiotic supplementation in immune function at 6 months of age (22–24), without major effects on regulatory cell markers (23), innate immune function (22) or allergen-specific responses (24). Here, we

examined the relationship between these early measures of immune function (at 6 months of age) and subsequent allergy outcomes at 2.5 years of age (using blood samples from 103 children).

Regulatory markers. Early regulatory T-cell markers were compared in children with and without evidence of allergy at 2.5 years of age. Data on CD4+ CD25+ CTLA4+ Treg populations were collected on 74 children (with sufficient samples for this). Those with an allergic phenotype had significantly higher proportions of circulating CD4+ CD25+ CTLA4+ Treg populations (as a proportion of total CD4+ T cells) at 6 months of age compared with nonallergic children. This was seen for multiple outcomes including dermatitis ($P = 0.038$), food allergen sensitization ($P = 0.007$) and inhalant sensitization ($P = 0.005$) as shown on Fig. 1. Each of these outcomes has been considered separately and independently of other outcomes (i.e. the comparison of inhalant sensitization in Fig. 1 includes all children with available samples regardless of food sensitization or atopic dermatitis).

FOXP3 data were available on 102 children (although only 94 of these had SPT data for comparison). The

development of inhalant sensitization at 2.5 years ($n = 29$) was also associated with significantly higher allergen-induced FOXP3 levels, to both OVA ($P = 0.003$) and HDM ($P = 0.009$). OVA induced FOXP3 expression was higher in children with dermatitis at 2.5 years ($n = 41$, $P = 0.013$) and children with food sensitization at 2.5 years also had higher OVA ($P = 0.02$) and HDM ($P = 0.06$) induced FOXP3 expression. These data suggest that the allergic phenotype is associated with increased early expression of regulatory T-cell markers. It was not possible to assess the function of these regulatory populations in this study.

It is possible that Treg may be induced because of a chronic inflammatory status such as eczema. Therefore, a sub-analysis was conducted to exclude infants ($n = 38$), who already had developed eczema. The remaining children with no active disease at 6 months but subsequent sensitization to inhalant or food allergens at 2.5 years had higher proportions of CD4+ CD25+ CTLA4+ Treg in peripheral blood at 6 months ($P = 0.009$ and $P = 0.038$, respectively). This suggests that the differences in circulating proportions of CD4+ CD25+ CTLA4+ Treg are not induced secondary to the inflammation of active dermatitis (as these remain

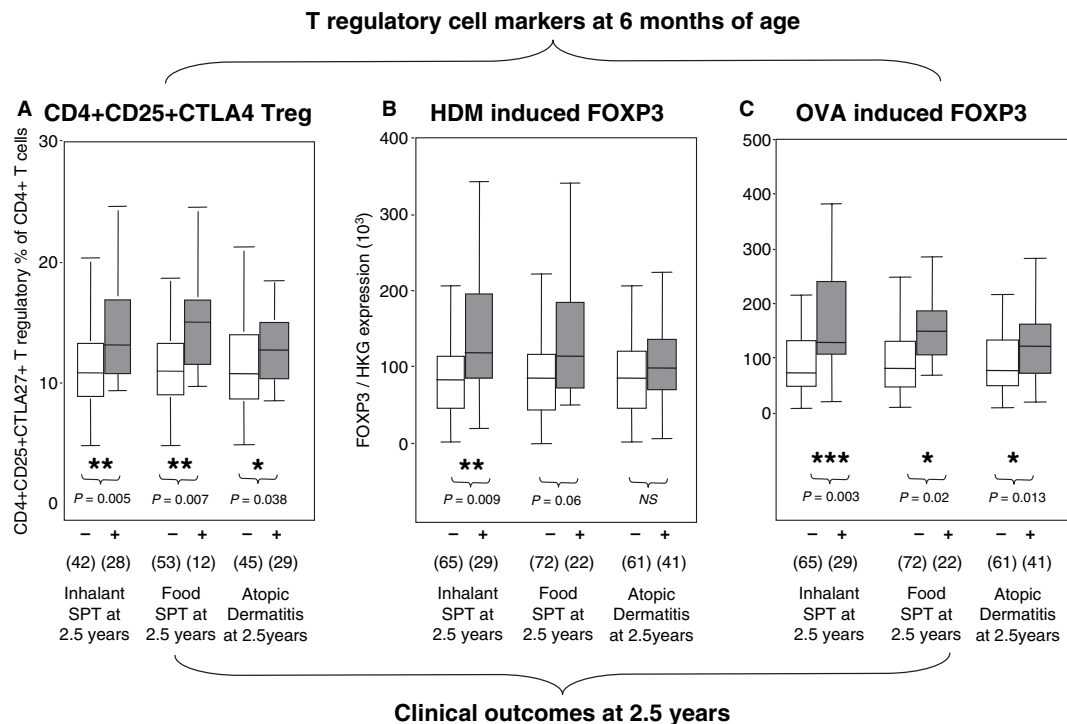


Figure 1. Relationship between early regulatory markers (CD4+ CD25+ CTLA4+ Treg and FOXP3 expression) at 6 months and subsequent allergic outcomes: (A) Using flow cytometry, CD4+ CD25+ CTLA4+ T regulatory cells were enumerated at 6 months of age and expressed as a percentage of CD4+ T cells comparing children with subsequent allergic outcomes (shaded bars) with children who remained nonallergic at 2.5 years of age (clear bars). Quantitative gene expression was assessed with real-time RT-PCR and the mRNA level is shown as FOXP3 gene expression relative to the housekeeping gene UBE2D2 (HKG). The levels shown are the difference between the levels in the (B) house dust mite (HDM) stimulated and (C) ovalbumin (OVA) and unstimulated cultures, again for the allergic (shaded bars) and nonallergic (clear bars) groups as above and denoted, if $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

regardless of disease status at 6 months). This was confirmed in regression models, which showed that CD4+ CD25+ CTLA4+ Treg remained strongly related to subsequent sensitization (to inhalants: $\beta = 0.34$, $P = 0.009$ and foods: $\beta = 0.27$, $P = 0.026$). after adjusting for the presence of dermatitis at 6 months of age (which was not independently related to Treg).

In contrast, differences in FOXP3 expression were not seen when children with dermatitis at 6 months were excluded, and this marker was shown in regression models (below) to be more strongly related to current dermatitis ($\beta = 0.27$, $P = 0.012$) than subsequent sensitization ($\beta = 0.16$, $P = 0.10$) and could therefore reflect activation of compensatory mechanisms.

Innate responses. Innate responses (to TLR2 and TLR4 ligands) were also compared in this population. Children with evidence of allergic disease (specifically dermatitis) at 2.5 years of age had attenuated TLR4-mediated responses, namely with lower IL-12 ($P = 0.042$) and IL-6 ($P = 0.039$) responses, compared with children with no evidence of disease (Fig. 2). The production of IL-5 was significantly higher in the allergic group ($P = 0.007$). There were no differences in TLR4 mediated IL-10 responses, or in the production of other cytokines (IFN- γ or TNF- α). There were no significant differences in TLR2 responses in allergic and nonallergic children.

Polyclonal T-cell responses. Other groups (25–31) have reported that the development of subsequent atopy was preceded by attenuated Th1 IFN- γ responses to polyclonal activation (with PHA) as noted in Fig. 3. This was most evident for children who had developed inhalant sensitization by 2.5 years of age ($P = 0.005$). These children also

had significantly lower production of IL-6 ($P < 0.001$) and IL-10 ($P = 0.001$) at 6 months of age. Furthermore, these differences were still apparent after excluding infants who already had evidence of disease (dermatitis) at 6 months (IL-6 $P < 0.001$; IL-10 $P = 0.005$; IFN- γ $P = 0.012$) and differences in IL-13 responses also became statistically significant ($P = 0.028$).

The same relationships were seen in children with dermatitis at 2.5 years of age, with lower IL-6 ($P = 0.012$) and IL-10 ($P = 0.002$) responses to PHA (Fig. 3).

Allergen-specific responses. At 6 months of age, cytokine responses to food allergen were significantly higher than inhalant allergen responses ($P < 0.001$) (data not shown), which were below detection in many infants. Those who developed a subsequent allergic phenotype at 2.5 years of age had significantly higher Th2 IL-13 responses to allergen (OVA) at 6 months of age. This was seen for both allergic sensitization (positive SPT to any allergen) ($P = 0.032$) and for diagnosed allergic disease ($P = 0.019$) at 2.5 years of age (Fig. 4). There were no significant differences in Th1 allergen-specific responses at 6 months, or in other cytokine responses to either OVA or HDM. As previously reported (23), there were significant but weak positive correlations between allergen-specific Th2 (IL-13) responses and the level of FOXP3 expression after stimulation with the corresponding allergen (OVA: $\tau = 0.19$, $P = 0.008$; HDM: $\tau = 0.13$, $P = 0.048$).

All of the associations between early immune measures and allergic outcomes at the 2.5-year follow-up were independent of probiotic supplementation or other potential confounding factors.

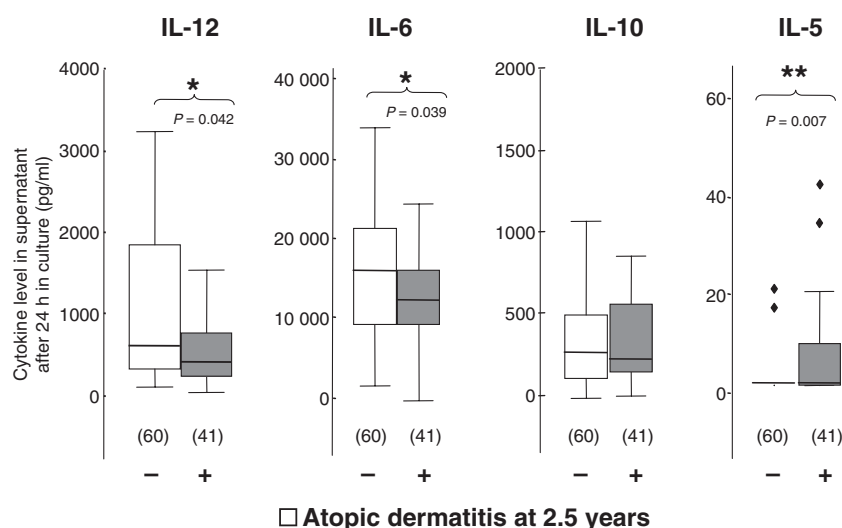


Figure 2. Relationship between early TLR4-mediated responses at 6 months and subsequent allergic disease: the levels of IL-12, IL-6, IL-10, and IL-5 are shown following stimulation with a TLR4 ligand (LPS) and compared in children with (shaded) and without (unshaded) subsequent dermatitis. Levels shown are for the levels above the unstimulated control cultures and denoted, if $*P < 0.05$.

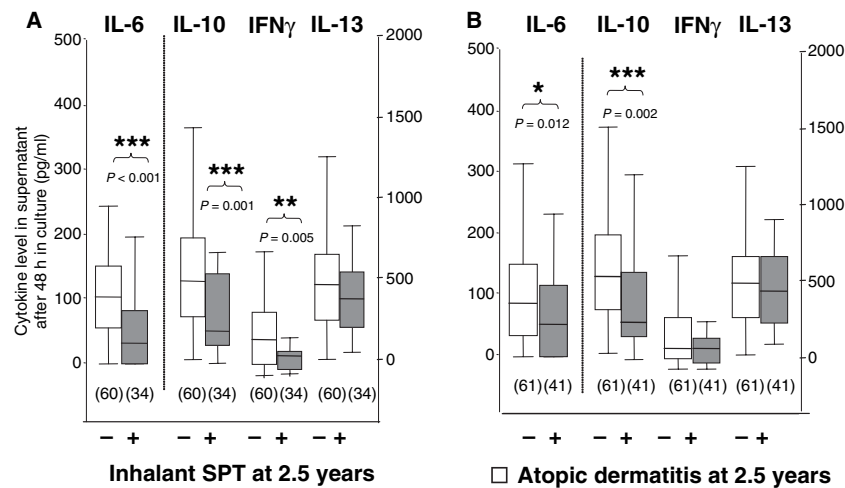


Figure 3. Relationship between mitogen responses at 6 months and subsequent allergic disease: The responses to polyclonal activation with phytohaemagglutinin (PHA) are shown for IL-6, IL-10, IFN- γ , and IL-13. (A) Levels are compared in children with (shaded) and without (unshaded) subsequent inhalant allergen sensitization. (B) Levels are compared in children with (shaded) and without (unshaded) subsequent dermatitis. Levels shown are for the levels above the unstimulated control cultures and denoted, if * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

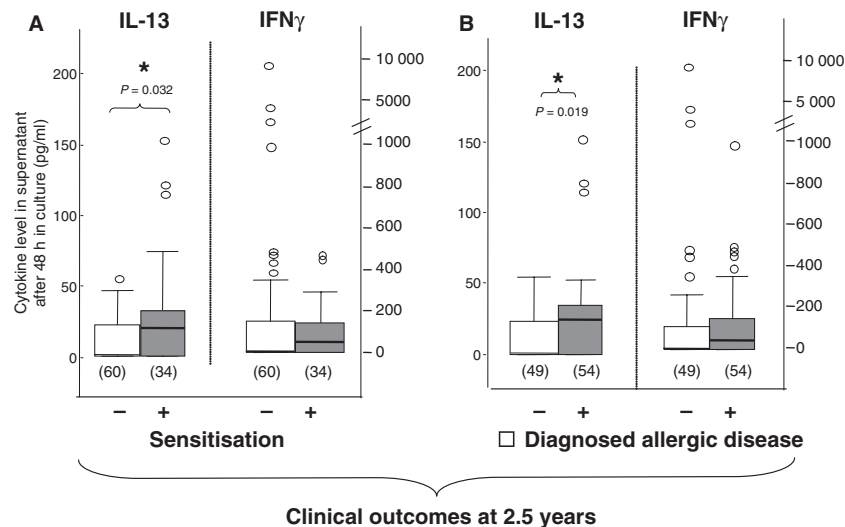


Figure 4. Relationship between allergen responses at 6 months and subsequent allergic disease: the Th1 (IFN- γ) and Th2 (IL-13) responses to allergen stimulation are shown. (A) Levels are compared in children with (shaded) and without (unshaded) subsequent allergen sensitization. (B) Levels are compared in children with (shaded) and without (unshaded) any subsequent allergic disease. Levels shown are for the levels above the unstimulated control cultures and denoted, if * $P < 0.05$.

Discussion

The key findings of this study are that supplementation with the *L. acidophilus* probiotic strain (LAFTI® L10/LAVRI-A1) did not have any long-term effects on allergic outcomes. Specifically, the higher rates of sensitization that were previously seen at 1 year of age in the probiotic group (10) were no longer apparent in the third year of life. Despite documented changes in early colonization patterns (10, 32) and other reports of favorable

effects in humans (11) and in animal models (11–13), this probiotic strain does not appear to have a role in allergy prevention. Administration of this strain to athletes has been shown to increase salivary IFN- γ and whole blood secretion of IFN- γ , although the study did not include a control group who did not receive *L. acidophilus* LAFTI® L10. This study did suggest that using this strain in the first 6 months of life was beneficial in reducing the subsequent rate of gastrointestinal infections. This suggests that early colonization with this strain may have

some beneficial effects in children, and is in keeping with other mucosal effects observed with this *L. acidophilus* in humans (11). However, this was not a primary aim of the study and the limitations of these gastrointestinal data are recognized (and should be interpreted accordingly). Furthermore, we did not previously see any reduction in gastrointestinal infections in these same children at 6 or 12 months of age (i.e. during probiotic treatment and the period immediately after) (10).

These findings are in contrast to other studies that have found that early supplementation with other probiotic strains can reduce allergic outcomes, particularly dermatitis. Two of these studies found effects using *L. rhamnosus* GG (8, 33), although the later also used prebiotic galacto-oligosaccharides and other probiotic strains (8). A third study using *L. reuteri* (ATCC 55730) found less IgE-associated eczema in the second year and less sensitization in a subgroup with atopic mothers (9). A more recent study has also shown a reduction in dermatitis in a New Zealand cohort supplemented with another *L. rhamnosus* strain (HN001) (34). Two of these studies showed effects on IgE-mediated disease (8, 9). These contrasting findings are likely to reflect differences in the microbiological and immunological effects of these strains, together with a range of other host and environmental differences [as recently outlined in more detail in (1)]. One notable difference is that all of the studies that showed an effect included maternal supplementation in the prenatal period (8, 9, 33), whereas our study did not. The lack of clinical effect in this study is consistent with the lack of immunological effects of *L. acidophilus* (LAFTI® L10) in this population (22–24). In contrast, strains that have been associated with clinical effects on allergic disease have also been shown to have immunological effects in infants (35–37).

To our knowledge, the postnatal development of regulatory T-cell function has not been examined in the context of allergic disease. The study also provided novel data on the relationship between patterns of immune development in the early postnatal period and the development of allergic disease. We noted that a subsequent allergic phenotype (sensitization and/or disease) was associated with increased early expression of regulatory markers. Specifically, there was an increase in the proportion of circulating putative (CD4+ CD25+ CTLA4+) Tregs, which we have previously shown to co-express high levels of the FOXP3 marker (23) in the subsequently allergic population. There was also greater up-regulation of this marker (FOXP3) following allergen stimulation at 6 months of age, in children with subsequent sensitization to inhalant allergens and/or food allergens at 2.5 years of age. Higher FOXP3 expression in children with dermatitis at 2.5 years reinforces the previous association between higher FOXP3 expression in infants and symptoms of dermatitis in the first year of life (23).

It is not clear how this increase in regulatory markers is associated with the other immunological differences seen

in this same group of allergic children. Specifically, at 6 months, these children simultaneously showed higher Th2 responses to allergen but lower innate responses together with lower Th1 responses to mitogens. It is possible that increased regulatory activity may be a secondary compensatory response to inappropriate Th2 responses (as also suggested by positive correlations between FOXP3 and IL-13 responses). In support of this, higher FOXP3 expression at 6 months was more strongly related to pre-existing disease (dermatitis) than subsequent sensitization, suggesting activation of potential counter-regulatory mechanisms. However, it is equally possible that defects in these pathways fail to overcome the developing Th2 diathesis and subsequent allergic sensitization seen in these children. Notably, sensitization developed despite higher proportions of CD4+ CD25+ CTLA4+ Treg at 6 months (which were seen independently of any clinical evidence of allergic disease). Further studies are clearly needed to examine for potential functional differences in the regulatory activity in the first months of life. In this setting, the probiotic strain did not appear to modify the expression of regulatory cell markers (23), but it is noteworthy that colonization (notably with higher proportions of bifidobacteria) patterns in this population have been previously associated with reduced production of most cytokine response to allergens and increased expression of regulatory cell markers ($P = 0.009$) and TGF- β ($P = 0.016$) (32). This supports the notion that microbionics may influence early immune function.

The study also provides further support to the mounting evidence of Th1 immaturity as a presymptomatic event in allergic children (25–31). Furthermore, we also observed attenuated IL-12 responses to innate (TLR mediated) immune activation in the allergic population at 6 months of age. Again, it is not clear if these apparent immaturities are primary defects and to what extent they reflect differences in the early postnatal environment. Other studies have noted that early endotoxin exposure has a protective relationship with allergic disease [including (38, 39)] and that exposure is associated with down-regulated *in vitro* TLR4-mediated mononuclear cell responses to LPS (39). As environmental endotoxin levels were not measured in this cohort, it is not possible to correlate the TLR4 responses with exposure.

The probiotic strain in this study did not appear to influence TLR function directly (22), but we have subsequently shown variations in innate immune function with patterns of colonizing bacteria (32). While these findings suggest that allergy is preceded by attenuated TLR4-mediated responses, there is only preliminary cross-sectional mapping of the normal development of these innate pathways (40) and this has not yet been examined in allergic children. Further studies are needed to more comprehensively map the ontogeny of innate (TLR) responses in allergic and nonallergic children.

In conclusion, this study highlights that there are complex differences in early postnatal immune function in infants that go on to develop allergy and that involved multiple cell populations including innate, effector, and regulatory cell populations. Further studies are needed to understand where the primary defects lie, and how (or if) environmental modification can prevent the continued evolution of these responses into an allergic phenotype. While the probiotic selected in this study did not appear to have a role in allergy prevention, other strains have shown more promise [reviewed in (1)] and these differences are likely to reflect multiple strain, host, and environmental differences in these populations. Further studies are needed to unravel the role and complexities of interaction between the early microbial environment and the developing immune system.

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