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Evaluation of the effects of probiotic supplementation from the neonatal period on innate immune development in infancy

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Summary

Background Activation of the innate immune system by microbial stimulation is believed to be critical for normal immune maturation, and there has been speculation that these pathways are important for inhibiting allergic-immune responses.

Objective To assess innate immune function following a 6-month supplementation with probiotic bacteria.

Methods Two hundred and thirty-one allergic, pregnant women were recruited into a randomized, controlled trial. The infants received either a probiotic (*Lactobacillus acidophilus* LAVRI-A1; Probiomix) or placebo (maltodextrin alone) daily for the first 6 months of life. Mononuclear cell samples were available from 118 infants. Functional responses to toll-like receptor (TLR) were assessed using ligands for TLR2 (Pansorbin) and TLR4/CD14 [lipopolysaccharide (LPS)] and measuring cytokine responses in the supernatants. Antigen-presenting cell function, as well as capacity for cytokine production (IL-12p70 and IL-10) was assessed.

Results Infants in the probiotic group did not demonstrate differences in innate immune function compared with those in the control group. No differences were seen when cytokine responses were examined following stimulation with Pansorbin (TLR2) or LPS (TLR4).

Similarly, no differences were seen in the antigen-presenting capacity of these infants. The mean fluorescence intensities of human leucocyte antigen-DR (HLA-DR) on monocytes, B cells and dendritic cells (DC) subsets were not affected, nor were the percentage of circulating DC subsets affected by a 6-month supplementation with *L. acidophilus* LAVRI-A1.

Conclusions Probiotic supplementation with *L. acidophilus* for the first 6 months of life did not alter early innate immune responses in this population at high risk of developing allergic disease.

Keywords allergy, infant, innate, probiotics, TLR

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Introduction

Microbial exposure is a potent source of early immune stimulation and appears essential for development of normal immune function. Bacteria and other microbial agents stimulate a wide range of cells involved in immediate host defence, principally through Toll-like receptors (TLR) and associated pathways. Among these cells, antigen-presenting cells (APC) and regulatory T cells also play a critical role in *programming* and *regulating* subsequent adaptive T cell responses. It has been proposed that TLR-mediated activation of both APC and regulatory T cells plays a central role in reducing the risk of Th2-

mediated allergic responses ([1, 2] and others). This is of most relevance in early life when immune programming is initiated. In support of this, there is emerging evidence that alterations in TLR function, either as a result of differences in early environmental exposures or functional genetic polymorphisms, have an effect on subsequent development of adaptive immune function and disease risk [3–8].

Intestinal flora are arguably the most abundant source of early immune stimulation, and contribute significantly to 'microbial burden' in early life. A number of studies have suggested differences in colonization patterns of infants who go on to develop allergic disease [9–14].

These differences were already apparent at 1 week of age, strongly suggesting that early colonization can influence subsequent patterns of immune development. There is also preliminary evidence that administration of probiotic intestinal flora may reduce the risk of atopic dermatitis in high-risk infants [15, 16]. Although there is evidence in adults that probiotics can enhance production of regulatory cytokine (IL-10) [17] and the activity of APC populations in the human gut [18], the effects on the developing immune system have not been assessed. Animal studies have shown that probiotics enhance the maturation of circulating APC (monocytes) in infant animals [19, 20], providing further support for our hypothesis that supplementation of human infants would have similar effects.

To address this, we assessed the effects of probiotics supplementation with 3×10^9 *Lactobacillus acidophilus* per day (from birth to 6 months of age) on aspects of innate immune function, including markers of APC maturation [human leucocyte antigen-DR (HLA-DR) expression and cytokine production] and the function of TLR pathways. To our knowledge, this is the first study to address this. The samples for this analysis were collected from a cohort of children at high risk of allergic disease who were participating in a randomized control trial to assess the effects of probiotics on allergy prevention.

Methods

Study design

Blood samples were collected at 6 months from children participating in a randomized double-blind, placebo-controlled designed to assess the effect of probiotic supplementation for the first 6 months of life on immune development and allergy outcomes. The aim of this aspect of the study was to compare innate immune function in children who had received probiotics ($n = 58$ with samples available) with those in the placebo group ($n = 60$ with samples available).

Study population

The subjects included in these analyses ($n = 118$ with blood samples available at 6 months of age) were derived from a cohort of 231 infants in a study to assess the role of probiotics in allergy prevention. All infants were at high risk of allergic disease based on maternal allergy. Maternal allergy 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). Infants were ineligible for the study if their mothers smoked or had pregnancy complications including delivery before 37-weeks gestation.

Supplementation

Infants were randomized to receive 3×10^9 *L. acidophilus* LAVRI-A1 per day in maltodextrin (Probiomix, Australia) daily or maltodextrin alone. Block randomization was used to ensure that the groups were balanced for (a) the type of maternal allergy (asthma vs. other allergy), (b) parity (first child vs. two or more children) and (c) paternal allergy (allergic vs. non-allergic). Supplements were supplied as stable freeze-dried powder (in sachets) and reconstituted in 1–2 mL sterile water and administered orally via a syringe. This was administered daily for the first 6 months of life, independent of feeding methods. The products were matched in appearance, and the participants and investigators remained blind to the intervention throughout the follow-up period.

Compliance

Compliance was monitored by use of a sachet chart (completed by parents) and sachet counts (returned sachets counted by the pharmacy department).

Cell preparation

Peripheral blood was collected when the infants were 6 months of age and samples were placed into heparinized tubes. Samples were available from 118 of the infants (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. Peripheral blood mononuclear cells (PBMC) were isolated within 2 h using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis. It has been previously shown that cryopreservation and thawing do not distort the pattern of PBMC cellular immune responses [21, 22].

Functional responses to toll-like receptor ligands

To assess functional TLR responses, we measured the pattern and magnitude of cytokine responses following activation with specific microbial ligands for TLR2 (Pansorbin) and TLR4/CD14 [lipopolysaccharide (LPS)]. These are the principal pathways of microbial recognition for Gram-negative (TLR4) and Gram-positive bacteria (TLR2). PBMC (1×10^6 /mL) were cultured in duplicate in 96-well round-bottomed plates in RPMI plus 10% fetal calf serum (not heat inactivated) (Australian Biosearch, Perth, Australia) either alone or with optimized doses of Pansorbin (0.1%; Calbiochem®, 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 ELISA or time-resolved fluorometry (TRF) as outlined

below. The principal cytokines of interest (produced by cells stimulated through these innate pathways) were IL-6, IL-10, tumour necrosis factor- α (TNF- α) and IL-12 (p70); however, other cytokines were also measured.

Antigen-presenting cell surface marker expression and cytokine (interleukin-12p70) responses

We examined surface marker expression and cytokine (IL-12p70) responses as measures of APC function:

Flow cytometry for surface marker expression. For immunophenotyping, cells were examined at time zero, before culture. PBMC were washed in PBS supplemented with 0.1% sodium azide and 1% normal human serum, and incubated for 30 min at 4 °C with one of the following monoclonal antibodies (MAbs) (all obtained from BD Biosciences, Sydney, Australia): fluorescein isothiocyanate (FITC)-conjugated HLA-DR, CD14-allophycocyanin and CD19-PE-Cy5. Cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson). For enumeration of dendritic cells (DC) subsets, cells were stained with a commercial four-colour antibody kit (BD Biosciences) containing lineage-specific antibodies conjugated to FITC (a cocktail of MAbs specific for B cells, T cells, monocytes and NK cells), CD123-phycoerythrin, HLA-DR-PerCp and CD11c-allophycocyanin. Circulating DC were defined as 'lineage-negative' cells expressing HLA-DR and further divided into plasmacytoid and myeloid subsets based on differential expression of CD123 [plasmacytoid DC (pDC)] and CD11c [myeloid DC (mDC)]. At least 150,000 mononuclear cells were acquired on the flow cytometer in order to obtain sufficient numbers of DC subsets for accurate enumeration, and the results are expressed as percentages of mononuclear cells. Data analysis was performed with the FlowJo Software (Tree Star).

Interleukin-12 responses. The capacity of APC populations to produce IL-12 (p70) was assessed in bulk cultures using LPS and IFN- γ priming as published previously [23, 24]. Briefly, PBMC (also at 1×10^6 /mL) were primed with recombinant IFN- γ (10 ng/mL; Pharmingen, Becton Dickinson, San Jose, CA, USA) for 2–3 h before stimulation with 10 ng/mL LPS (*E. coli* LPS, Sigma) for a further 24 h in 10% fetal calf serum and Roswell Park Memorial Institute 1640 (RPMI, Life Technology) culture medium. Supernatants were harvested at 24 h and frozen (at –20 °C) for subsequent IL-12 (p70) measurement by commercial ELISA (OptEIA, Pharmingen, Becton Dickinson) as outlined below.

*Responses to heat-killed *Lactobacillus acidophilus* LAVRI-A1 (HKLA)*

L. acidophilus LAVRI-A1 (Probiomix Ltd, Sydney, NSW, Australia) was cultured in De Man, Rogosa, Sharpe (MRS) broth (Oxoid) and cells were washed twice in PBS before

enumeration by 10-fold serial dilution, and spread plating on MRS agar. Colony-forming units per millilitre of suspension were enumerated after anaerobic incubation at 37 °C for 24 h. Bacteria were heat killed at 75 °C for 45 min and aliquots were stored at –80 °C until use. An aliquot was checked for sterility by plating on MRS agar. To assess MC responses to this, 2×10^6 PBMC/mL were cultured in duplicate in 96-well round-bottomed plates in AIM V (Gibco, Life Technology Ltd, Paisley, Scotland, UK) serum-free medium for 48 h without stimuli (control) or with heat-killed *L. acidophilus* LAVRI-A1 (10^7 CFU/mL). The supernatants were collected and stored at –20 °C for cytokine analysis by ELISA or TRF.

Polymorphonuclear neutrophils preparation and culture

Peripheral blood neutrophils were isolated following PBMC collection and were isolated by dextran sedimentation and red blood cell lysis. Polymorphonuclear neutrophils (PMNs) cell numbers were evaluated by Trypan blue exclusion and purity was analysed microscopically, routinely amounting to >90%. The PMN 2×10^6 cells/mL were cultured in RPMI (Sigma) media supplemented with 10% heat-inactivated fetal calf serum (FCS) (Australian Biosearch), glutamine and 1% (v/v) penicillin/streptomycin. One millilitre of cell suspension was cultured for 24 h at 37 °C with 5% CO₂. Cell suspensions were cultured in medium alone or with LPS (*E. coli*). Supernatants were collected and stored at –20 °C for cytokine analysis.

Cytokine assays

Supernatants from the assays outlined above were analysed for IL-5, IL-6, IL-10, IL-12p70, IL-13, INF- γ and TNF- α with an in-house ELISA using 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, Life Sciences). 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). IL-12p70 and IL-8 (neutrophil cultures) were 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]. Where cytokine responses were defined as a binary outcome (response or no response),

'responders' were defined as children who produced a detectable increase in cytokine response above control levels.

Stool analysis

Stool samples were collected 1 month after commencing the supplement. 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. Lactobacilli, bifidobacteria and coliforms were enumerated in these samples as follows: 10-fold diluted homogenates of the stool samples (w/v) in half-strength Wilkin-Chalgren broth (Oxoid) 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, Prioionic 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).

Data 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 interquartile range and compared using non-parametric tests (Mann-Whitney *U*-test). Correlations were assessed using Spearman's or Kendall's tau (τ) *b* (where a proportion of the variables of interest shared 'zero' values) in order to avoid the problems associated with 'ties' within the data. 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.

Ethical approval

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 informed consent.

Results

Characteristics of the population

Of the 231 participants initially recruited into the study, 178 completed the supplementation period and 118 of these had blood samples collected (60 in the placebo group and 58 in the probiotic group), which were used in this study. There were no significant differences in the characteristics of these groups. Specifically, there were no differences in maternal age, parity, maternal atopic status or paternal atopic status, gestational age, birth weight, birth length, head circumference, Apgar scores, gender, breastfeeding, use of formula milk, age of solid introduction, antibiotic usage or day care attendance between the probiotic and control groups.

Compliance with the supplementation (monitored by both sachet counts and charted parental reporting) was good, with approximately 80% of doses administered and no difference between the groups (*P* = 0.607).

Effects of probiotic supplementation on toll-like receptor-2- and toll-like receptor-4-mediated immune responses

The purpose of these functional experiments is to compare the pattern and magnitude of cytokine responses with various microbial elements that are inherently recognized by cells of the innate immune system, principally via different TLR pathways. We used specific ligands to assess key pathways, including endotoxin (LPS) from Gram-negative bacteria to assess TLR4 function (in association with CD14) and Pansorbin (Gram-positive bacteria) to assess TLR2 function.

Effects on toll-like receptor-2 signalling pathways. As anticipated, these responses were dominated by IL-6, IL-10, TNF- α and IFN- γ . All children showed detectable responses (with production of one or more of these cytokines). Supplementation with probiotics was not associated with any significant effects on TLR2-mediated immune responses (to Pansorbin) as shown on Fig. 1a. There were no differences in the frequency or magnitude of these or other cytokine responses Fig. 1a. Children in the probiotic group showed a trend for higher TNF- α (*P* = 0.47). Those with detectable IFN- γ responses (*n* = 55; 94%) also showed a trend for higher responses compared with the placebo group (*P* = 0.28). No other trends were observed.

Effects on toll-like receptor-4 signalling pathways. TLR4 activation (by LPS) was dominated by strong IL-6 and TNF- α responses, which were seen in 98% of the placebo group and 100% of the probiotic group. There were no differences in the magnitude of these or any other cytokine responses between the groups (Fig. 1b).

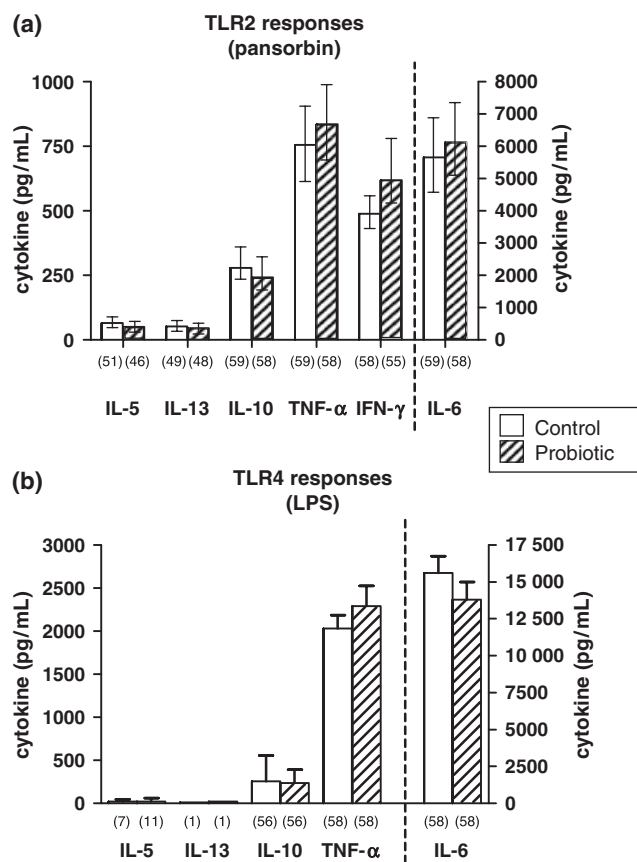


Fig. 1. Comparison of functional responses to toll-like receptor (TLR) activation in the probiotic and placebo groups. Peripheral blood mononuclear cells cytokine responses in children with detectable responses in the probiotic group (shaded) compared with the placebo group (unshaded). The number of children who responded to stimuli are indicated (in brackets): (a) Responses to pansorbin (TLR2) and (b) Responses to lipopolysaccharide (TLR4).

Effects of probiotic supplementation on responses to heat-killed *Lactobacillus acidophilus* LAVRI-A1. We also assessed the responses to the *L. acidophilus* (LAVRI-A1) that was used as the probiotic supplement. This heat-killed extract is likely to activate the innate immune system through a number of potential pathways (including TLR). However, although there was a trend for higher TNF- α ($P=0.415$) and IFN- γ ($P=0.36$) responses in the children who had received probiotics, there were no significant differences in any of the cytokine responses measured (Fig. 2).

Effects of probiotic supplementation on specific aspects of antigen-presenting cells function

Effect of probiotics on interleukin-12 responses. To measure APC IL-12 (p70) production, MC cultures were primed with recombinant IFN- γ before LPS stimulation [24]. This potent method of stimulation allows assessment of APC

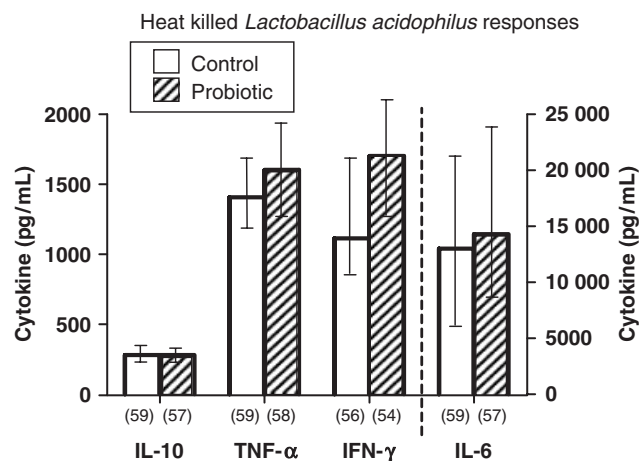


Fig. 2. Comparison of immune responses to the *Lactobacillus acidophilus* probiotic by children in the supplement and placebo groups. Peripheral blood mononuclear cells cytokine responses to heat-killed *L. acidophilus* in the probiotic group (shaded) were compared with the placebo group (unshaded).

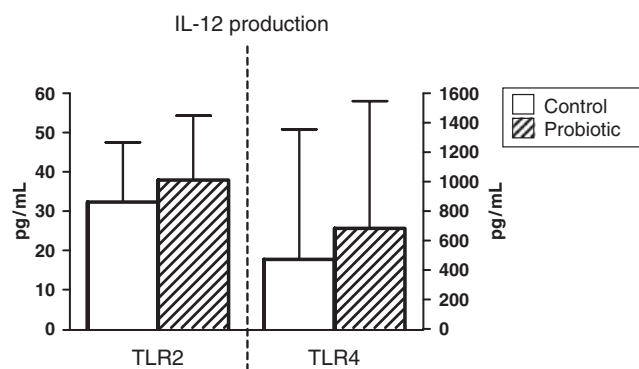


Fig. 3. Comparison of interleukin (IL)-12 responses in the probiotic and placebo groups. The levels of IL-12 response to pansorbin (TLR2) and lipopolysaccharide (TLR4) were assessed in children with detectable responses, making comparisons between the probiotic group (shaded) and the placebo group (unshaded). Cytokines are expressed as median, IQR, 95% confidence intervals (pg/mL). The groups were compared by the Mann-Whitney *U*-test. No significant differences were seen between the groups.

capacity to respond under intense pro-inflammatory conditions, and is a good measure of 'capacity' to respond. We also assessed responses to less potent methods of stimulation (i.e. with Pansorbin), which resulted in 10-fold lower responses (Fig. 3).

Children who received probiotics did not show any significant differences compared with children in the placebo group, although there was a trend for higher IL-12 responses to both Pansorbin ($P=0.912$) and LPS ($P=0.322$) in the probiotic group.

Effects of probiotic supplementation on human leucocyte antigen-DR expression on B cells and monocytes. Based

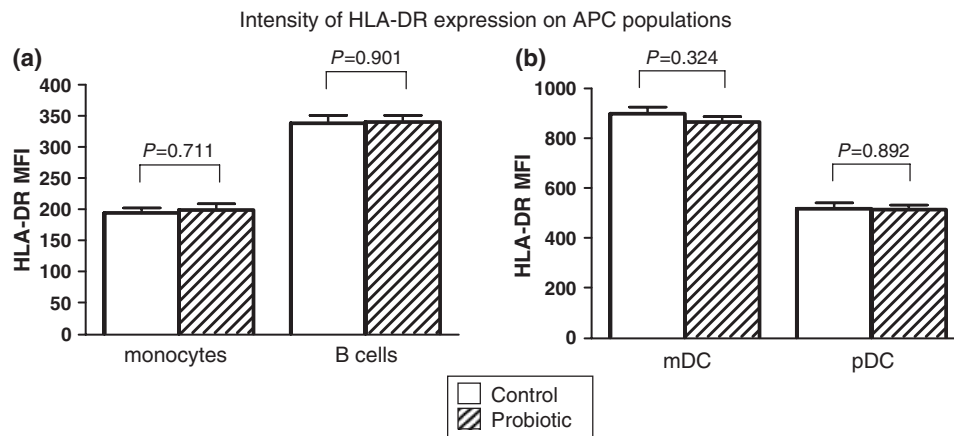


Fig. 4. Comparison of major histocompatibility complex class II expression in the probiotic and placebo groups. Human leucocyte antigen-DR expression on antigen-presenting cell populations of 6-month-old children comparing the probiotic group (shaded) and the placebo group (unshaded). (a) monocyte and B cell human leucocyte antigen-DR (HLA)-DR expression. (b) Myeloid DC (mDC) and plasmacytoid DC (pDC) HLA-DR expression. MFI, mean fluorescence intensity.

on reports that probiotics may influence function and maturation of APC [18–20], we assessed for any differences in a key marker of antigen-presenting capacity (HLA-DR expression).

Supplementation with *L. acidophilus* for the first 6 months of life did not affect the number of cells expressing HLA-DR or the intensity of expression on infant CD14⁺ monocytes ($P=0.711$) or CD19⁺ B cells ($P=0.901$) at 6 months of age (Fig. 4a).

Effects of probiotic supplementation on human leucocyte antigen-DR expression on dendritic cell subsets. Children supplemented with probiotics tended to have higher numbers of both mDC and pDC (as a percentage of all PBMC) compared with the placebo group, although this was not statistically significant (Fig. 5).

We also examined the intensity of expression of HLA-DR on mDC and pDC at 6 months of age. There were no differences in the intensity of expression on mDC ($P=0.324$) or pDC ($P=0.892$) subsets when the treatment and control groups were compared (Fig. 4b).

Effects of probiotic supplementation on neutrophil function

We assessed neutrophil responses following stimulation with LPS. The probiotic group demonstrated a trend for higher IL-8 production, however, this did not reach statistical significance ($P=0.414$) (Fig. 6).

Relationship between colonization patterns and innate immune function

There were no relationships between innate immune outcomes (outlined above) and the detection of cultureable

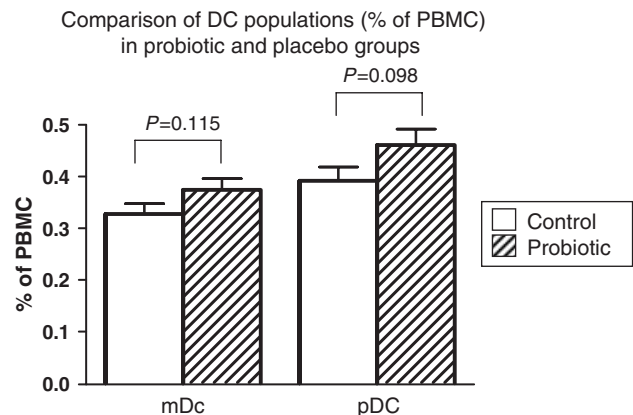


Fig. 5. Enumeration of DC subsets in 6-month-old children. Using flow cytometry, myeloid dendritic cell (mDC) and plasmacytoid DC (pDC) were enumerated and expressed as a percentage of peripheral blood mononuclear cells. Error bars indicate standard error of mean. The groups were compared by Student's *t*-test.

lactobacilli, bifidobacteria or coliforms in the stools during the first month of life.

Discussion

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 [25]. It has been proposed that a similar, but more subtle process may be occurring in humans, with progressively cleaner environments contributing to the recent increase in allergic disease (reviewed in [9]). Because intestinal flora are arguably the most abundant source of early immune stimulation, there has been logical interest in the role of probiotic supplementation in promoting immune

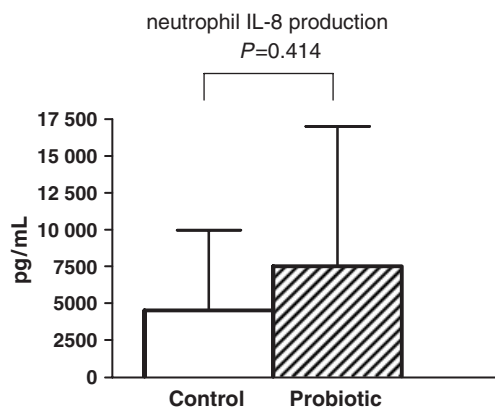


Fig. 6. Comparison of neutrophil IL-8 responses in the probiotic and the placebo groups. The level of the IL-8 cytokine response after 24 h of culture with lipopolysaccharide was measured by ELISA. Levels are expressed as median, IQR, 95% confidence intervals (pg/mL) as compared by the Mann-Whitney *U*-test.

tolerance and reducing the risk of allergic disease. One study has already provided support for this concept by showing that probiotic supplementation for the first 6 months of life (and in the weeks before delivery) was associated with a 50% reduction in the risk of atopic dermatitis [15, 16]. The fact that they observed no effects on allergen-specific IgE suggested that the effects were mediated by other pathways.

Our interest in innate immune pathways is based on evidence that (microbial) TLR activation in early life can modify innate immune function. Intrauterine infections have been associated with increased TLR4 expression in the placenta [26] and in animals, antenatal (maternal) exposure to bacterial LPS enhances neonatal Th1 IFN- γ responses and inhibits allergen sensitization in the offspring, presumably also via TLR4 pathways [27]. Tulic et al. have shown similar effects of this TLR4 ligand (LPS) in the postnatal period [28], but notably, the inhibition of allergic responses was observed only when LPS was given before responses were established. There is only preliminary evidence that the anti-inflammatory effects of probiotics are mediated through TLR-receptor signalling in experimental models of colitis [29]. Intestinal epithelial cells express functional TLR (TLR2 and TLR4) that can mediate the effects of bacterial flora, again in animal models [30]. In humans, there are reports that changes in colonization patterns are associated with increased expression of TLR (TLR2) [31]. DC, that express a range of TLR play a pivotal role in the gut where they penetrate the epithelium without disrupting barrier function and directly sample gut-associated bacteria [32]. *Lactobacillus* strains have also been shown to up-regulate DC major histocompatibility complex (MHC)-class II expression [33] and modify DC cytokine responses [18] *in vitro*. There is growing evidence that aspects of TLR pathways and DC function are less mature in the neonatal period, and

postnatal maturation is driven by microbial exposure. For all of these reasons, we hypothesized that administration of microbial products would be associated with enhanced maturation of these pathways in infancy.

However, despite this background evidence, we did not see any evidence that probiotic supplementation for the first 6 months of life had any significant effects on either TLR2 or TLR4 function, or on markers of APC (monocytes, B cells and DC) maturation. The only trends that are worth noting are that probiotics were associated with a higher proportion of mDC ($P=0.115$) and pDC ($P=0.098$) populations (Fig. 5), although, if anything, there was a trend for lower HLA-DR expression on these cells (Fig. 4b). There were also trends for higher IFN- γ and TNF- α responses following TLR2 ligation in the probiotic group, and higher IL-12 and TNF- α responses following TLR4 ligation. It is possible that these trends, which support our hypothesis, do not reach statistical significance because of the sample size. However, our numbers (around 60 in each group) are similar to those in the study by Kalliomaki et al. [15], which noted a significant reduction in allergic disease in children receiving *Lactobacillus* GG ($n=64$) compared with a placebo group ($n=68$). Thus, we would anticipate that we should be able to detect any relevant effects on innate pathways in our population sample's size.

There are a number of reasons why we did not observe significant effects. Firstly, it is possible that the strain we used did not have significant immunomodulatory effects, as differences between probiotic strains have been recognized (although largely *in vitro*) [18]. Secondly, it is possible that compliance was not as good as we measured, although this seems unlikely. Thirdly, it is possible that 6 months is too young an age to observe this effect; however, other studies suggest that infants prone to allergic disease already show immunological differences at this age or younger [34–38]. Fourthly, it has been demonstrated that in order for some probiotic species (*Lactobacillus reuteri*) to induce inhibitory (IL-8) responses *in vitro*, they required pre-incubation with human epithelial cells and had to be adherent and alive (not heat killed or γ -irradiated) [39]. The species that we used was previously shown to have good survival and adherence properties, but this is difficult to confirm in the current setting. Despite this, however, there is other evidence that suggests live bacteria are not mandatory and that the anti-inflammatory effects of probiotics (in a murine model of colitis) can be mediated by bacterial DNA on TLR9 in the absence of live microorganisms [29]. Finally, it is possible that genetic differences in this population (compared with other studies [15]) may alter the effects of probiotics on immune development. Functional polymorphisms in TLR signalling pathways are known to confer variability in functional responses of these pathways to microbial stimulation. Specifically, TLR4 polymorphisms are associated with endotoxin hyporesponsiveness [40]. Other

genetic polymorphisms have also been associated with allergic disease risk including polymorphisms in TLR2 [5], and CD14 (involved in TLR4 signalling) [3]. The protective effects of some genetic polymorphisms appear dependent on environmental microbial exposure. Similarly, the effects of microbial exposure will depend on genetic polymorphism. It is likely that this also has implications for therapeutic strategies that use microbial products.

In summary, despite *L. acidophilus* LAVRI-A1 conforming 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) [41, 42], supplementation with this probiotic strain for the first 6 months of life did not have any significant effects on key aspects of innate immune function in high-risk infants. We intend to follow this population further to assess the effects of this probiotic on the development of allergic disease and sensitization, and to examine how this relates to early innate immune function.

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