

ORIGINAL PAPER

Probiotic supplementation in the first 6 months of life in at risk Asian infants – effects on eczema and atopic sensitization at the age of 1 year

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Clinical and Experimental Allergy

Summary

Background The role of probiotics in allergy prevention remains uncertain but has been shown in some studies to have a possible protective effect on eczema.

Objective We aimed to assess the effect of probiotic supplementation in the first 6 months of life on eczema and allergic sensitization at 1 year of age in Asian infants at risk of allergic disease.

Methods A double-blind, placebo-controlled randomized clinical trial involving 253 infants with a family history of allergic disease was carried out. Infants received at least 60 mL of commercially available cow's milk formula with or without probiotic supplementation [*Bifidobacterium longum* (BL999) 1×10^7 colony forming unit (CFU)/g and *Lactobacillus rhamnosus* (LPR) 2×10^7 CFU/g] daily for the first 6 months. Clinical evaluation was performed at 1, 3, 6 and 12 months of age, with serum total IgE measurement and skin prick tests conducted at the 12-month visit. The primary and secondary end-points were eczema and allergen sensitization, respectively.

Results The incidence of eczema in the probiotic (22%) group was similar to that in the placebo group (25%) ($P = 0.53$). The median Scoring Atopic Dermatitis score at 12 months was 17.10 (9.74) in the probiotic group and 11.60 (8.40) in the placebo group ($P = 0.17$). The prevalence of allergen sensitization showed no difference (probiotic = 24% vs. placebo = 19%, $P = 0.26$). The total IgE geometric mean (95% confidence interval) was 18.76 (12.54–24.98) kU/L in the probiotic group and 23.13 (16.01–30.24) kU/L in the placebo group ($P = 0.15$). Atopic eczema (with sensitization) in the probiotic (7.3%) group was comparable to the placebo group (5.8%) ($P = 0.86$).

Conclusion Early life administration of a cow's milk formula supplemented with probiotics showed no effect on prevention of eczema or allergen sensitization in the first year of life in Asian infants at risk of allergic disease. Further work is needed to determine whether timing of supplementation, dose and probiotic strain are important considerations.

Keywords allergen sensitization, allergy prevention, eczema, infants, probiotics

Submitted 15 July 2008; revised 28 August 2008; accepted 29 September 2008

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Introduction

Probiotic supplementation in early life is considered an attractive strategy for the primary prevention of allergic diseases. It has an excellent safety record [1, 2]. Its use is supported by the observation that early life gut microbiota plays an important immunoregulatory role, and an 'imbalance' in its composition may increase susceptibility towards allergies [3]. *In vitro* and animal studies have shown that probiotic bacteria promote immunoregulatory

functions by inducing T cells bearing TGF- β [4] and increasing the production of IL-10 regulatory cytokines [5]. The eventual test of such a strategy lies in intervention studies in the form of clinical trials. Several double-blind, placebo-controlled randomized clinical trials [6–13] have been conducted to examine the effect of probiotic supplementation in primary allergy prevention. However, despite the rigorous testing of this concept on clinical subjects, two recent meta-analyses of the four published clinical trials have concluded that the role of probiotics in

allergy prevention either remains inconclusive due to varied study designs and results [14], or has some protective effect on atopic eczema alone [15]. Additionally, a recently published study that was not included in the meta-analyses failed to show any protective effects on eczema [16].

This study assessed the effect of administration of a probiotic (*Bifidobacteria longum* and *Lactobacillus rhamnosus*) supplemented cow's milk-based infant formula from the first day of life for 6 months on the prevention of eczema and allergic sensitization in the first year of life in Asian infants at risk of allergic disease.

Methods

Study design

A double-blind, placebo-controlled randomized study (ClinicalTrials.gov Identifier: NCT00318695) was conducted to assess the effect of probiotic supplementation in the first 6 months of life on the incidence of eczema and allergen sensitization in the first year in Asian infants at risk of allergic disease. We recruited 253 families with a history of allergic disease from the antenatal clinics at the National University Hospital, Singapore, between May 2004 and June 2006. The sample size was calculated based on published data [8], which reported a reduction in the incidence of eczema from 46% (31/68) in the placebo arm to 23% in the probiotic (15/64) group at 2 years of age. We therefore anticipated the incidence of eczema to be approximately 40%, and that to detect a relative reduction of 50%, with a power of 90% and a two-sided test size of 5%, 110 subjects were required in each group [17]. This sample size was sufficient to evaluate the outcomes at 1 year of age. Efforts were made to ensure a low dropout rate.

The inclusion criteria were: a first-degree relative with a doctor diagnosis of asthma, allergic rhinitis or eczema and a positive skin prick test (SPT) to the dust mites, *Dermatophagoides pteronyssinus* and/or *Blomia tropicalis*, which are the most common causes of inhalant allergen sensitization in our atopic population [18]; gestational age of at least 35 weeks; birth weight above 2 kg; and absence of major congenital malformation or illness. The subject was excluded when the parent was assessed to be mentally or legally incapable of informed consent. Parents who chose to breast-feed exclusively were not considered for the study. Written informed consent was obtained from all families. The study was approved by the National University Hospital's ethics review committee (Ref Code: B/00/322).

Probiotic supplement and infant formula

Subjects received at least 60 mL (9.26 g) a day of commercially available cow's milk-based infant formula

(Nan 1[®]), with either probiotic supplementation [*B. longum* BL999 (ATCC: BAA-999 designation BB536, Morinaga, Japan) 1×10^7 colony forming unit (CFU)/g and *L. rhamnosus* LPR (CGMCC 1.3724) 2×10^7 CFU/g] or without, initiated within 12 h for the first 6 months of life. The infants in the probiotic group, therefore, received at least 2.8×10^8 CFU of probiotic bacteria per day. Mothers were then free to decide whether to make up the remainder of the baby feeds with either the trial formula, or to supplement with breast milk, or another infant formula. Both *B. longum* and *L. rhamnosus* conform to the Food and Agriculture Organization (FAO)/World Health Organization expert panel guidelines for probiotic [19]. Computerized randomization was carried out in blocks of six based on a 1:1 allocation, with the lowest number allocated sequentially. Compliance was monitored by completion of a daily diary chart by parents and biweekly phone calls for the first 6 months. Non-compliance was defined as consumption of <60 mL of trial milk formula daily for a duration of 3 days during the intervention period (birth to 6 months of age).

The milk formula with probiotics was not available commercially and was specially manufactured by Nestle[®], Vevey, Switzerland for this study. Both the probiotic-supplemented and the control formula were not hydrolysed and not supplemented with prebiotics. The formula feeds (test and control) tasted and appeared identical. Quality control testing by the manufacturer showed that the probiotic bacteria in the formulation remained viable in at least the above concentration for 600 days.

Weaning to solids was allowed at between 4 and 6 months of age according to local practices, but parents were advised to avoid potentially allergenic foods including eggs, shellfish, and peanuts until after the first birthday.

Clinical assessment

The primary clinical outcome measure was the incidence of eczema, and the secondary outcome measure was allergen sensitization. Infants were evaluated by a paediatrician at 1, 3, 6, and 12 months of age, which involved a detailed history, recording of anthropometric data and clinical examination, including looking for the presence of eczema. Questionnaires were also administered at these visits to record clinical disease and environmental exposures, including day care, sibship, use of antibiotics, smoking and pets. Biweekly phone calls were performed for the first 6 months, after which monthly phone contacts were carried out to collect data on the health status of the children.

Eczema was defined as a pruritic rash over the face and/or extensors with a chronic relapsing course, as described by Hanifin and Rajka and modified by Seymour for infants [20]. The Scoring Atopic Dermatitis (SCORAD)

index was used to objectively score the severity of atopic dermatitis [21], which were carried out by investigators L. P. C. S., I. G. and Dr Dawn Lim.

Determination of serum total immunoglobulin E and skin prick tests

Serum samples were collected from cord blood and at 1 year of age, and stored at -70°C till assayed. Measurement of total IgE was performed using the fluoroenzyme immunoassay method (UniCAP[®] Phadiatop, Pharmacia Diagnostics, Uppsala, Sweden), with a detection limit of 0.35 kU/L.

The SPT was performed at 12 months of age using a standardized technique with common allergen extracts, including soy (Alyostal, Stallergenes Laboratoires, Antony Cedex, France), milk, egg yolk, egg white, dust mite allergens – *D. pteronyssinus* (Greer Laboratories, Lenoir, NC, USA) and *B. tropicalis* (manufactured in-house [22]). A histamine dihydrochloride solution (10 mg/mL) was used as a positive control and solvent (50% Cocas 50% Gly) as a negative control. A weal > 3 mm in diameter above the negative control was considered positive [23].

Statistical analysis

All statistical analysis was carried out on an intention-to-treat last observation carried forward basis. Atopy was defined as present when the subject had a positive SPT, indicating allergen sensitization. The trial data were collected on printed forms, and subsequently entered into CLINTRIAL [24], a specialized software for managing longitudinal trial data. A comparison of the incidence rates of eczema and atopy in the two treatment groups was made using the χ^2 test, and logistic regression was performed to determine the odds ratio (OR), the associated 95% confidence interval (CI) and adjust for relevant covariates. Because of the non-normality of the data, the Mann–Whitney *U* test was performed to assess the differences between groups. All statistical analyses were performed using SAS v.9.1 and SPSS software (version 15.0 for Windows). A *P*-value of <0.05 was considered statistically significant for all analyses.

Results

Baseline characteristics and participants

At the antenatal clinic, 3703 families were assessed and out of the 865 eligible families, 253 families consented and were recruited into the study. Three subjects in the probiotic group and five subjects in the placebo group withdrew from the study before any follow-up was conducted and were therefore excluded from the analysis (Fig. 1). At the 12-month visit, there were 124 families in

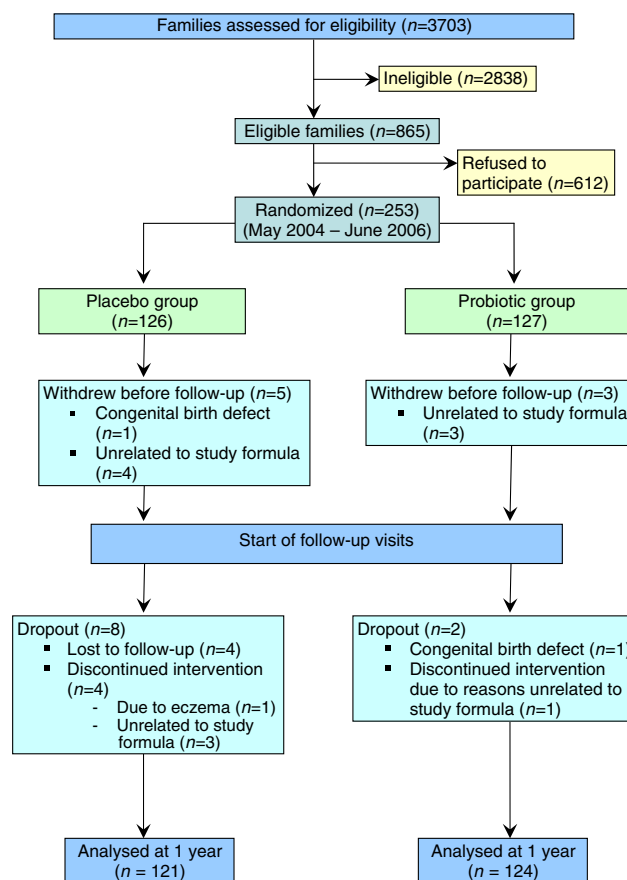


Fig. 1. Flow chart showing progress of participants through the trial.

the probiotic arm and 121 families in the placebo arm. The ethnicity, gestational age, mode of delivery, birth weight, family atopic history, and other baseline characteristics (data not shown) of the two groups were comparable (Table 1). However, imbalances were seen with gender and birth order between the two groups, and these were included as potential confounding factors in subsequent analyses. The compliance level of consuming at least 60 mL of trial formula per day from birth to 6 months was 89% in the probiotic group and 85% in the placebo group. All subjects did not consume any other probiotic preparations or dietary products during the 6-month intervention period.

Effects of probiotic supplementation on eczema and sensitization

The incidence of eczema in the probiotic ($n = 27/124$; 22%) group was similar to that in the placebo group ($n = 30/121$; 25%) ($\text{OR}_{\text{adj}} = 0.82$; 95% CI = 0.44–1.52). In subjects with eczema, the median SCORAD score at 12 months was 17.10 in the probiotic group and 11.60 in the placebo group ($P = 0.17$). The rate of sensitization to common allergens (probiotic = 24% vs. placebo = 19%, $\text{OR}_{\text{adj}} = 1.43$; 95% CI = 0.76–2.70) showed no difference (Table 2). The total IgE geometric mean (95% CI) was 18.76 (12.54–24.98) kU/L

Table 1. Characteristics of the study population

	Placebo (n = 126)	Probiotic (n = 127)
Gestational age in weeks, mean (SD)	39.0 (1.1)	38.9 (1.4)
Gender (%)		
Male	73 (58)	61 (48)
Ethnicity (%)		
Chinese	53 (42)	57 (45)
Malay	55 (44)	60 (47)
Indian	15 (12)	10 (8)
Others	3 (2)	0 (0)
Mode of delivery (%)		
Lower segment caesarean section	33 (26)	35 (28)
Vaginal delivery	93 (74)	92 (72)
Birth weight (kg)		
Mean (SD)	3.1 (0.4)	3.2 (0.5)
Birth order (%)		
1	41 (33)	59 (47)
2	44 (35)	24 (19)
3	24 (19)	32 (25)
≥4	17 (14)	12 (10)
Family history (%)*		
Eczema	41 (33)	39 (31)
Asthma	51 (41)	59 (47)
Allergic rhinitis	87 (69)	73 (58)
Maternal atopy	77 (61)	87 (69)
Paternal atopy	53 (42)	52 (41)
Atopy in both parents	24 (19)	19 (15)

*Percentages given refer to percentage out of total number of patients.

Table 2. Sensitization characteristics of study subjects at 1 year of age

	Placebo (n = 121)	Probiotic (n = 124)
Allergen sensitization (%)		
Positive skin prick test (any)*	23 (19)	30 (24)
Dietary (any)†	6 (5)	7 (6)
Cow's milk	0	0
Egg white	6	6
Egg yolk	4	4
Soy	0	0
Inhalant allergens (any)	20 (17)	24 (20)
<i>Dermatophagoides pteronyssinus</i>	17	20
<i>Blomia tropicalis</i>	18	19

*Skin prick test not performed for one subject in probiotic group.

†One subject in placebo group not assessed for dietary allergens.

in the probiotic group and 23.13 (16.01–30.24) kU/L in the placebo group ($P = 0.15$). Atopic eczema (with sensitization) in the probiotic (7.3%) group was similar to that in the placebo group (5.8%) ($OR_{adj} = 1.08$; 95% CI = 0.44–2.65).

Assessment of confounding factors

Apart from the imbalances in gender and birth order between the probiotic and the placebo group, differences

Table 3. Prevalence of potential confounding factors

	Placebo (n = 126)	Probiotic (n = 127)
Prenatal smoking exposure (%)		
Prenatal maternal smoking	6 (5)	9 (7)
Passive smoke exposure in household	46 (37)	58 (46)
Smoking exposure by month 6 (%)*	50 (41)	48 (39)
Keep pets (%)	27 (21)	31 (24)
Type of housing (%)		
Public housing	111 (88)	108 (85)
Private housing (Condominium)	10 (8)	15 (12)
Landed property	5 (4)	4 (3)
Feeding history – birth to month 6 (%)*		
Near total breastfeeding with at least 60 mL of formula for 6 months	2 (2)	4 (3)
Any breastfeeding	93 (77)	81 (65)
Total formula	26 (22)	39 (32)
Age at weaning (months)*		
Mean (SD)	5.6 (0.9)	5.7 (1.1)
Use of antibiotics by month 6 (%)*	13 (11)	10 (8)
Day care attendance by month 12 (%)*		
Yes	2 (2)	2 (2)
No	119 (98)	122 (98)

*Five subjects in the placebo group and three subjects in the probiotic group withdrew from study before follow-up and were excluded from analysis.

were also noted for prenatal smoking exposure and feeding history from the list of confounding factors recorded (Table 3). Multivariable logistic regression was performed to account for the possible confounding effects that may influence atopic propensity. Adjustment for imbalance of gender, birth order, prenatal smoking exposure and feeding history between treatment groups did not affect the findings significantly.

Discussion

This randomized-controlled trial, a first in an Asian cohort, did not show a protective effect of probiotic supplementation for the first 6 months of life on eczema or allergen sensitization at 1 year of age. To date, there have been five published clinical trials on the role of early life probiotic supplementation in the primary prevention of eczema. These studies are summarized in Table 4. Our findings are similar to two other clinical trials: one involving an Australian cohort ($n = 178$) [12] and a second involving a German cohort ($n = 94$) [16]. These studies in fact reported negative outcomes. The Australian study showed that allergen sensitization was increased in those who received probiotics, and both studies showed an increased frequency of recurrent wheezy bronchitis. The data from these studies suggest that probiotic supplementation in early life may not be altogether innocuous. Similar to what we observed in our study, this German

Table 4. Summary of clinical trials evaluating the role of probiotic supplementation in the primary prevention of atopic diseases

Study	No. of subjects	Probiotic	Dose	Supplementation		Effect of probiotics on clinical end-points			
				Prenatal	Postnatal (months)	Age of evaluation (years)	Eczema	Sensitization	Food allergy
Kalliomäki et al., Finland [8]	n = 132	<i>Lactobacillus rhamnosus</i> GG (LGG)	1 × 10 ¹⁰ CFU daily	Yes	6	2	Reduced	No effect	Not evaluated
Kukkonen et al., Finland, [11]	n = 925	Mixture of four strains with prebiotic galacto-oligosaccharides	Both 5 × 10 ⁹ CFU twice daily	Yes	6	2	Reduced	No effect	No effect
		1. LGG and	Both 2 × 10 ⁹ CFU twice daily						
		2. <i>L. rhamnosus</i> LC705							
		3. <i>Bifidobacterium breve</i> Bb99 and							
		4. <i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS							
Abrahamsson et al., Sweden, [6]	n = 188	<i>L. reuteri</i>	1 × 10 ⁸ CFU daily	Yes	12	2	Reduced only in IgE associated atopic eczema	Reduced in children with atopic mothers	No effect
Taylor et al., Australia, [12]	n = 178	<i>L. acidophilus</i>	3 × 10 ⁸ CFU daily	No	6	1	No effect	Increased rate of sensitization	No effect
Kopp et al., Germany, [16]	n = 94	LGG	5 × 10 ⁹ CFU twice	Yes	6	2	No effect	No effect	Not evaluated
Soh et al., Singapore	n = 245	<i>Bifidobacterium longum</i> BL999 and <i>L. rhamnosus</i> LPR	At least 2.8 × 10 ⁸ CFU daily	No	6	1	No effect	No effect	Not evaluated

CFU, colony forming unit.

and three other published Scandinavian trials showed that probiotics did not have an effect on allergen sensitization [6, 8, 11]. The Scandinavian trials, however, demonstrated a reduction in eczema at 2 years, although the Swedish study by Abrahamsson et al. [6] ($n = 188$) showed this benefit only in the subset of IgE-associated eczema. Only one study ($n = 132$) has published long-term follow-up results [9, 10] and has shown a sustained benefit in terms of eczema prevalence, but was disappointing with regard to respiratory allergies.

Besides differences in population characteristics between these clinical trials, variations in study design may have also contributed to the observed differences. A noticeable difference in the study designs of two studies (our study and Taylor et al.) that did not observe a protective effect of probiotics on eczema is the absence of prenatal probiotic supplementation in these protocols. Prenatal supplementation may therefore be an important factor in conferring these benefits. It has been shown that consumption of *L. rhamnosus* GG by the mothers antenatally established specific changes in the transfer and colonization of bifidobacteria in neonates [25]. Furthermore, a recent study found that probiotic supplementation with *Lactobacillus reuteri* during late pregnancy reduced breast milk levels of TGF- β 2 and increased IL-10 [26]. However, prenatal supplementation in the German study did not result in a positive outcome.

Variations in the probiotic strains used and their doses may also contribute to the variability in results. Our study utilized a combination of *B. longum* and *L. rhamnosus*, which are inhabitants of different locations in the gastrointestinal tract. Immunostimulatory oligodeoxynucleotides that suppress IgE and T-helper type 2 cytokine production have been identified in these probiotic strains [27, 28]. Furthermore, *B. longum* BB536 has been reported to have a treatment effect on Japanese cedar pollinosis [29, 30]. It was therefore postulated that the combination of these two probiotic bacteria in our study may exert additive immunomodulatory effects. It has been further shown that the supplementation of these bacterial strains in infant formula is well tolerated [31]. The *L. rhamnosus* LPR strain used in our study has been found to be indistinguishable from *L. rhamnosus* GG using specific molecular probes targeted at 16sRNA (F. Rochat, personal communication, Nestle, Lausanne, Switzerland). Additionally, *L. rhamnosus* LPR was originally derived from a product, Dicoflor[®] (Dicofarm, Rome, Italy), which contains *L. rhamnosus* GG (ATCC 53103) (Certificate of Receipt, China General Microbiological Culture Collection Center), indicating that both these probiotic bacteria are identical. *L. rhamnosus* GG, has shown benefits on eczema most consistently with two studies reporting positive effects [8–11]. However, the inclusion of *L. rhamnosus* in our study, as well as the German study, did not result in the same benefits. The only study (Taylor et al.) that used *Lactobacillus acidophi-*

lus for supplementation resulted in increased allergen sensitization in the supplemented subjects. Another study (Kukkonen et al.) used a mixture of four probiotics with prebiotic galacto-oligosaccharides and showed a reduction in eczema and atopic eczema. There is, however, still insufficient evidence to determine the role of prebiotics in allergy prevention, even though a study [32] reported a reduction in eczema as this study had a lost to follow-up rate of 20%. Another study [33], which did not select infants at risk of allergy, reported no significant difference in eczema in infants up to 4 months of age. Meta-analysis of these two studies could not determine the role of prebiotics in the prevention of eczema due to the heterogeneity of the study design [34]. Our study involved a relatively lower dose of probiotics (approximately 2 logs) compared with the other primary prevention trials that reported protective effects on eczema [8–11]. At these doses, we did document a consistent transit of the supplemented probiotic in the stools of infants in the probiotic group compared with the placebo group [35, 36]. Significantly more *L. rhamnosus* (OR = 111.93; 95% CI = 23.18–540.45, $P < 0.001$) and *B. longum* (OR = 3.75; 95% CI = 1.27–11.07, $P = 0.017$) were detected by the polymerase chain reaction method in the probiotic group over the first 3 months of supplementation [35]. Furthermore, the viability of this strain combination in the milk formula was monitored at the end of the study period to ensure preservation of bacterial viability at the required dose.

A unique feature of our protocol was the supplementation of probiotics in infant formula. We felt that this was consistent with the situation in 'real life' as a large proportion of our mothers and within and outside Asia, either supplement breastfeeding with infant formula [37] or use infant formula only. Furthermore, supplementation in formula would improve compliance. To address the variability in probiotic dose resulting from variations in patterns of breastfeeding within the intervention group and eczema outcome, analysis of the data according to the amount of probiotic supplemented formula used (i.e. minimum of 60 mL vs. exclusive formula) was performed and no differences were found (data not shown). In this study, we resolved to use standard infant cow's milk formula rather than a hypoallergenic formula so as to avoid an added parameter that might confound atopy development [38]. It is also not a practice to use hydrolysed formula in at-risk infants in Asian communities including ours. Of great interest was the absence of cow's milk sensitization or clinical cases of cow's milk allergy despite exposure in our cohort.

Another interesting point was that the prevalence of eczema in our cohort (25%) was lower than the 39% in the placebo group at 1 year [12] and 46% at 2 years [8] in the other studies. Despite this lower observed prevalence of eczema (25%), our study was not underpowered as we could still detect a relative reduction of eczema of 50%,

with a power of 80%. Interestingly, the German study also reported relatively low rates of eczema (28%). It may be possible that probiotics are ineffective in a population with lower rates of eczema. The reasons for the lower prevalence of eczema are almost certainly multifactorial and would include the different genetic make-up and its interaction with the lifestyle and the environment. These findings are nonetheless consistent with the ISAAC Phase 3 studies, where the cumulative prevalence of eczema in schoolchildren in the Singapore cohort (8.2%) was significantly lower than those reported in Australia (32.3%) and in Scandinavia (38.6%), where the other studies were conducted [39].

In conclusion, our study does not support the role of early life probiotic supplementation as a modality for primary eczema prevention. An extended period of follow-up of this cohort is intended to determine longer term outcomes and effect on other manifestations of allergy in this population.

Acknowledgements

We thank the PROMPT (PRObiotic in Milk for the Prevention of aTopy trial) team – Dr Dawn Lim, Ms Hor Chuen Yee, Ms Corinne Kwek Poh Lian and Ms Judy Anthony, who assisted with the follow-up of the subjects; Ms Siti Dahlia Mohd Dali, Ms Mavis Yeow Bee Ling and Mr Jerome Rex Cruz, who facilitated the supplement allocation; the obstetricians and midwives who helped during collection of cord blood; and Dr Stefan Ma for his statistical advice. The voluntary participation of all subjects in the study is sincerely appreciated.

This study was funded by the National Medical Research Council, Singapore (R178-000-085-213). S. E. Soh is supported by National University of Singapore Research Scholarship. The study milk formula was kindly sponsored by Nestle®, Vevey, Switzerland.

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