A Double-Blind Randomized Placebo Controlled Clinical Trial on the Supplementation of Probiotics in the First Six Months of Life in Asian Infants At Risk of Allergic Diseases – Effects on Development of Allergic Disease and Safety Aspects with a Two Year Follow-up

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LIST OF ABBREVIATIONS

AR	……	Allergic rhinitis
BMI	……	Body Mass Index
CFU	……	Colony-forming unit
CI	……	Confidence interval
CONSORT	……	Consolidated Standards of Reporting Trials
DTPa	……	Diphtheria-Tetanus-Acellular Pertussis vaccine
FAO	……	Food and Agriculture Organization
GALT	……	Gut-associated lymphoid tissue
GRAS	……	Generally Recognized as Safe
HBIG	……	Hepatitis B immune globulin
anti-HBs	……	Hepatitis B virus surface antibody
HBsAg	……	Hepatitis B surface antigen
IFN-γ	……	Interferon-gamma
Ig	……	Immunoglobulin
IL	……	Interleukin
ISAAC	……	International Study of Asthma and Allergies in Childhood
ITT	……	Intention-to-treat
LAB	……	Lactic acid bacteria
LGG	……	*Lactobacillus rhamnosus* GG
LRTI	……	Lower respiratory tract infection
NK	……	Natural killer
OFC	……	Occipitofrontal head circumference
OR	……	Odds ratio
ORadj	……	Adjusted odds ratio
PBMC	……	Peripheral blood mononuclear cells
RR	……	Relative risk
SCORAD	……	SCORing Atopic Dermatitis
SD	……	Standard deviation
SDS	……	Standard deviation scores
TGF-β	……	Transforming growth factor-beta
Th1	……	Type 1 helper T cells
Th2	……	Type 2 helper T cells
TNF-α	……	Tumour necrosis factor-alpha
Tr1	……	T regulatory type 1 cells
T_{reg}	……	Regulatory T cells
URTI	……	Upper respiratory tract infection
WHO	……	World Health Organization

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SUMMARY

Background:

The role of probiotics in allergy prevention remains uncertain but has been shown to have a possible protective effect on allergic diseases. Probiotics can modulate local and systemic immune responses, resulting in decrease in infectious disease and increase efficacy to vaccination.

Objectives:

To assess the effect of probiotic supplementation in the first 6 months of life on

i. allergic diseases at two years of age in Asian infants at risk of allergic disease.
ii. specific antibody response against Hepatitis B as a surrogate marker for infant immune response to vaccination.
iii. protective benefit against infections.
iv. impact on growth and safety.

Methods:

This double-blind, placebo-controlled randomized clinical trial involved 253 infants with a family history of allergic disease. Infants received at least 60ml of milk formula with or without probiotic (*Bifidobacterium longum* [BL999] 1×10^7 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, 12 and 24 months of age, with skin prick tests conducted at the 12 and 24 months. Serum samples were collected from cord blood and at 12 month visit to determine total immunoglobulin E and Hepatitis B virus surface antibody.

Results:

Cumulative incidence of eczema in the probiotic (22%) group was similar to placebo (26%) at 2 years of age (adjusted odds ratio ORadj=0.73; 95% confidence interval CI=0.39 to 1.34). Prevalence of allergen sensitization showed no difference (18.6% vs. 18.9% in placebo, ORadj=0.92; 95% CI= 0.46 to 1.84). No difference in the incidence rate of asthma (probiotic=8.9% vs placebo=9.1%, ORadj=1.15; 95% CI=0.46 to 2.87) and allergic rhinitis (1.61% vs. 2.48% in the placebo, p=0.86) between the two groups was observed.

Improvement in Hepatitis B surface antibody responses in subjects receiving monovalent doses of Hepatitis B vaccine at 0, 1 month and a DTPa-Hepatitis B combination vaccine at 6 months [placebo:187.97 (180.70–195.24), probiotic:345.70 (339.41–351.99) mIU/ml] (p=0.069) was demonstrated, but not in those who received 3 monovalent doses [placebo:302.34 (296.31–308.37), probiotic:302.06 (296.31–307.81) mIU/ml] (p=0.996).

The rates of infections were similar. However, 3.94 times more infants were hospitalized due to infections during the first 6 months in the probiotic group (95% CI=1.21 to 12.75, p=0.022) but this difference was not observed later. Adequate growth was observed with a trend of consistently higher BMI in the probiotic group.

Conclusion:

Early life administration of a cow's milk formula supplemented with probiotics showed no effect on prevention of allergic diseases in the first 2 years of life in Asian infants at risk of allergic disease. However, probiotics may enhance specific antibody responses in infants receiving certain Hepatitis B vaccine schedules. Despite increase hospitalization due to infections, better growth was observed in the probiotic group. Further work is needed to determine whether timing of supplementation, dose and probiotic strain are important considerations. The role and complexities of interaction between the early microbial environment and the developing immune system needs to be unravelled before any recommendations for use in the paediatric population.

Chapter 1: Introduction

The increasing prevalence of allergic diseases worldwide has become a global health and socioeconomic burden including in Singapore [1]. For obvious reasons, effective strategies for the primary prevention of allergic diseases in high-risk infants with family history of atopy would be more attractive compared to treatment of established disease.

Research on immune responses in early life has indicated that early childhood is a critical window of opportunity for intervention. During this period, initial programming of immunologic memory occurs and therefore any stimulus that alters the functional competence of the immune system could result in the susceptibility to allergic sensitization and eventual development of persistent disease into adulthood [2]. This life phase is also a period of intensive growth and remodeling of the organs. Early viral or allergy-mediated inflammatory damage to these rapidly growing tissues can result in long-lasting changes of the allergen responder phenotype [3].

Potential prevention strategies were initially based on allergen avoidance through the control of maternal exposure to allergens and environmental control of allergen levels during infancy [4]. However, these measures are not practicable over a prolonged period of time. A more recently devised strategy involves repeated low dose allergen exposure to induce immune tolerance [5]. The Global Prevention of Asthma in Children (GPAC) Study is double-blind, randomized, placebo-controlled study recruiting children between the ages of 18-30 months at 5 international study sites to receive sublingual drops of either a mixture of allergens or a placebo once a day for a

year to explore the use of sublingual immunotherapy to promote tolerance to common allergens (http://www.globalasthmastudy.org). However, such a regime has the potential for overstimulation of immune responses and could not be employed in early infancy [2].

Enhancement of postnatal maturation of both the innate and adaptive immune functions through early stimulation by the signals of the gut microbiota provides another potential strategy for primary prevention. Approaches such as prebiotics and probiotics, microbial vaccines (in particular mycobacteria) [6] and mixed bacterial extracts have been evaluated. Recent experimental and epidemiological data have suggested that disruption of gut microbiota could drive the development of allergic airway response without any previous systemic priming. The 'microflora hypothesis of allergic diseases' has been postulated to highlight the role of gut microbiota in modulating host immunity [7]. Probiotics which are healthy bacteria of the gut are candidate agents proposed to provide beneficial immunoregulatory signals to potentially prevent the development of sensitization and allergic diseases during early infancy. The primary aim of this study is therefore to assess the effect of administration of probiotics from birth on the prevention of allergic sensitization and allergic diseases. At the initiation of this clinical trial, very few randomized trials had been reported to evaluate the efficacy of this strategy [8]. This study was intended to substantiate or refute these earlier studies as well as to provide data in an Asian population.

Attenuated immune function in atopic infants may also include reduced capacity to respond to vaccines [9-12] and increase susceptibility to infections [13, 14]. The

secondary aims of this study are to assess the effect of probiotic supplementation in the first 6 months of life on protective benefit against illnesses and immune response to vaccination. Safety of the probiotic administration and impact on growth of newborn infants are also documented in this study.

1.1 Atopy and allergic diseases

1.1.1 Definitions

The standardised nomenclature of allergy was revised by the World Allergy Organization as an update of the European Academy of Allergy and Immunology Allergy Position Statement [15]. This nomenclature defines “atopy” as a “personal or familial tendency to become sensitized and produce immunoglobulin E (IgE) antibodies in response to ordinary exposures of allergens, usually proteins, and to develop typical symptoms of asthma, rhinoconjunctivitis, or eczema”. The term atopy cannot be used if IgE sensitization has not been documented by IgE antibodies in serum or by a positive skin prick test.

Allergy is defined as a hypersensitivity reaction initiated by immunologic mechanisms and can be antibody-mediated or cell-mediated which is further classified into IgE-mediated allergy or non-IgE-mediated allergy [15].

Eczema is described by Hanifin and Rajka and modified by Seymour et al. for infants [16] as a pruritic rash over the face and/or extensors with a chronic relapsing course. Similar to the classification of atopy, atopic eczema is based on IgE sensitization and use of the term atopic eczema should be associated with the documentation of a positive skin prick test reactivity or IgE antibodies in serum [15].

The epidemiological definition of clinical asthma involves three episodes of nocturnal cough with sleep disturbances or wheezing, separated by at least seven days, in a setting where asthma is likely and conditions other than allergy have been excluded [17]. Asthma is a complex chronic disorder of the airways and is required to be clinically diagnosed in the presence of variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation [18]. Thus making a diagnosis of asthma in young infants in our study had been difficult due to episodic respiratory symptoms such as wheezing and cough which were symptoms of recurrent respiratory tract infections. Allergic rhinitis will be diagnosed if the child has rhinorrhea, nasal obstruction, nasal itching and sneezing which are reversible spontaneously or with treatment that is not due to a respiratory infection as per recommendations from the World Health Organization (WHO) Allergic Rhinitis and its Impact on Asthma workshop (ARIA) [19]. Despite its high prevalence, allergic rhinitis is often undiagnosed in young children as children lack the ability to verbalize their symptoms and the parents underreported the symptoms as common cold or flu.

1.1.2 Epidemiology of Allergic Diseases in Childhood

The International Study of Asthma and Allergies in Childhood (ISAAC) was conducted in three phases since 1991 to describe the prevalence and severity of asthma, rhinitis and eczema in children living in different countries. In the most recent Phase III study conducted worldwide between 2002 and 2003 in children aged 6-7 years and 13-14 years, the rise in prevalence of symptoms in many centres has been found to be concerning [20]. Wide global variations exist with the prevalence of current wheeze ranging from 0.8% in Tibet, China to 32.6% in Wellington, New Zealand in the 13-14 year olds, and from 2.4% in Jodhpur, India to 37.6% in Costa

Rica in the 6-7 year olds [21]. Similarly the prevalence of current rhinoconjunctivitis symptoms ranged from 1.0% in Davangere, India to 45.1% in Asunción, Paraguay in the 13-14 years old children, and from 4.2% in the Indian Sub-Continent to 12.7% in Latin America in the 6-7 year olds. Co-morbidity with asthma and eczema varied from 1.6% in the Indian sub-continent to 4.7% in North America. [22].

In Singapore, the ISAAC Phase I written questionnaire was administered to 6-7 years old (n=2030) and 12-15 years old (n=4208) schoolchildren in 1994 [23]. The overall prevalence of current wheeze was 12% with prevalence of doctor diagnosed asthma as 20%. In general, current rhinitis was reported by 37.1% and eczema was the least commonly reported with 9.4% having current symptoms. Allergic disorders were found to be common in Singapore and an increasing problem not only in the West but also in an Asian population. By comparing the data from phase I and phase III of the ISAAC surveys conducted in Singapore seven years later in 2001, the prevalence of current wheeze decreased significantly in the 6–7 year age group from 16.6% to 10.2% ($p<0.001$) but increased slightly in the 12–15 year age group from 9.9% in 1994 to 11.9% ($p=0.015$) in 2001. Rhinitis showed increasing severity of symptoms in both age groups and the prevalence of children diagnosed with eczema showed a significant increase from 3.0% to 8.8% ($p<0.001$) in the 6-7 years old group [1]. Furthermore, the prevalence of children who have had more than one atopic disorder increased significantly from 6.0% in 1994 to 10.2% in 2001 ($p < 0.001$) [24].

1.1.3 Immunological basis of atopy and allergic diseases

According to the classic type 1 (Th1) / type 2 helper T (Th2) cells paradigm theory, an individual develops the Th2-dominant immune system when exposed to allergens

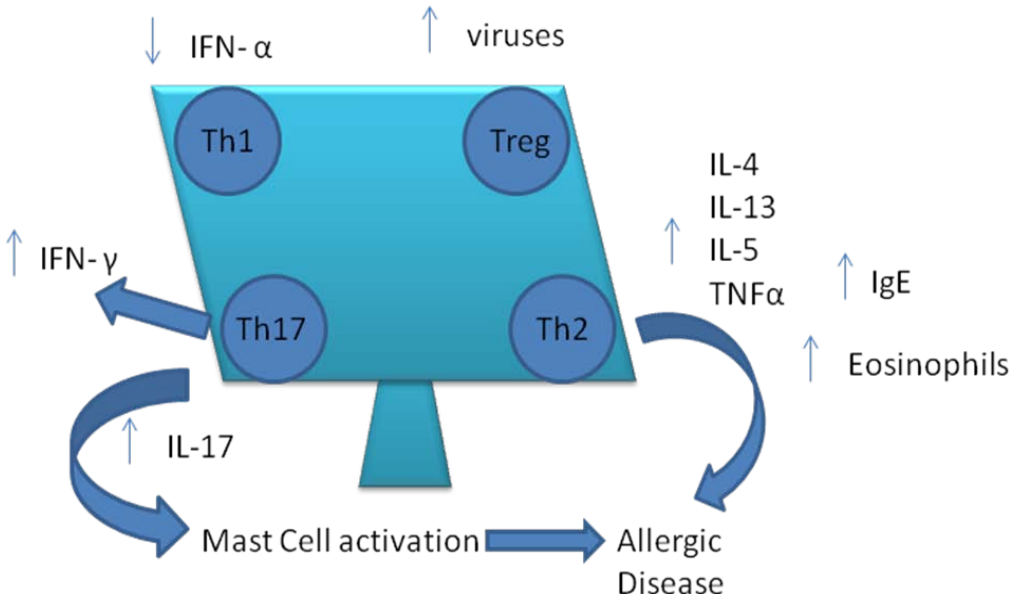
prior to microbial exposure. Generation of the Th2-type cytokines, including interleukin-4 (IL-4), IL-5 and IL-13 promote IgE production and eosinophilia. This hygiene hypothesis suggested by Strachan [25] indicated that a decrease in the microbial load due to clean living environments, antibiotic use and hygienic food standards lead to decreased microbial exposure in early life resulting in an over-expression of the allergic response. There has been much clinical evidence to support this hypothesis. An inverse relationship between infections, including mycobacteria, measles and hepatitis A virus, early in life and atopy have been suggested [26]. Early entry to nurseries [27], greater sib ship numbers [28], living on farms [29] and early gastrointestinal infections [30] are all proposed to be associated with decreased incidence of atopy. These conditions are associated with increased microbial pressure early in life. Endotoxin stimulates antigen-presenting cells to produce IL-12 which triggers the development of antigen-specific Th1 cells and inhibits Th2 cells.

However, this rigid Th1/Th2 paradigm cannot explain the Th1 type inflammation response elicited in chronic atopic eczema and asthma. Furthermore, Th1-mediated autoimmune disease often coexist with Th2-mediated atopic disease [31]. Consequently, an extended version of the hygiene hypothesis of atopic disease has been introduced. Several subsets of $CD4^+$ cells are capable of suppressive mechanisms to control immune responses against both self-antigens and allergens in autoimmune and atopic diseases respectively. These regulatory T (T_{reg}) cells inhibit both Th1 and Th2 cells development *in vitro*. It has further been suggested that the lack of microbial stimulation affects the development of T_{reg} cells, resulting in an atopic phenotype [32]. Allergic patients have been found to have very low IL-10-producing allergen-specific T_{reg} cells as compared to healthy subjects [33]. These IL-

10-secreting T regulatory type 1 (Tr1) cells secrete high levels of IL-10 and transforming growth factor-beta (TGF-β) which can serve to suppress both allergy and autoimmune diseases [34].

There are namely 4 main types of T-cells that regulate one other. The Th1 cells promote cytokine IL-12 to inhibit Th2 cell development, whereas the Th2 cells produce IL-4 to blocks Th1 cell development. The Th1 derived interferon-gamma (IFN-γ) on the other hand, blocks Th17 cell development and prevents IL-17 mediated inflammation in autoimmune murine models [35, 36]. In healthy human individuals, there are less than 1% of Th17 cells in the peripheral blood, but in patients with Crohn's disease, there are slightly higher proportion of Th17 among the $CD4^+$ T cells [37]. IL-17A messenger RNA in sputum has also been found to be significantly higher in asthma patients [38] with the evidence that IL-17 can contributes to the development of allergen-induced airway hyperresponsiveness and airway remodelling [39]. The T_{reg} cells inhibit the development of both Th1 and Th2 cells by direct contact-dependent mechanisms, IL-10 and TGF-β. Onset of allergic diseases may be determined by the ratio of proinflammatory T-cell subsets versus T_{reg} subsets. In chronic allergic diseases, Th17 and tumour necrosis factor-alpha (TNF-α) rich inflammatory Th2 cells can be upregulated while in asymptomatic atopic individuals, IL-10 producing T_{reg} may be upregulated and Th17 cells inactivated [40].

Figure 1-1 Onset of allergic diseases may be determined by the ratio of Th17 and Th2 versus Treg subsets. In patients with chronic allergic diseases, proinflammatory T-cell subsets, namely Th17 cells and Th2 cells, that are capable of producing high levels of TNFα (inducible Th2 cells) are upregulated. (Modified from Orihara et al. [40])



1.1.4 The microflora hypothesis of allergic disease

The role of the indigenous intestinal microbiota has further been proposed to potentially outweigh that of infections in immune maturation. The most common anaerobes within the gastrointestinal microbiota are *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium, Clostridium* and *Lactobacillus*. Other facultative anaerobes such as *Escherichia coli* and *Enterococcus* are also present. Intestinal colonization begins rapidly in the newborn and microbial succession establishes with age in the first year of life until an adult-type highly complex microbiota composition has been achieved. *Bifidobacterium, Clostridium* and *Bacteroides* are among the first anaerobes colonizing the gut [41]. It has been suggested that antibiotic use and dietary changes in affluent countries have disrupted the role of endogenous microbiota in maintaining mucosal immunological tolerance [7]. Differences in intestinal microflora are found in caesarean-delivered infants compared to vaginally delivered infants, and

in babies who are breast fed compared to formula fed babies. Breastfeeding promotes bifidobacteria and lactobacilli colonization that inhibit growth of pathogens [42]. Vaginally delivered babies are colonized with bifidobacteria and lactobacilli earlier than caesarean-delivered babies [43, 44]. Furthermore, children born by means of caesarean section was found to be associated with an increased risk of developing respiratory allergies [45].

A mouse model of antibiotic-induced gastrointestinal microbiota disruption resulted in the development of an allergic airway response to subsequent mould spore (*Aspergillus fumigatus*) exposure in immunocompetent C57BL/6 mice without previous systemic antigen priming. Levels of eosinophils, mast cells, lung leukocyte IL-5, IL-13, IFN-γ, total serum Ig E, and mucus-secreting cells were significantly increased in the microbiota disrupted mice [46]. Similarly in BALB/c mice, antibiotic-induced microbiota disruption promoted the same airway allergic response upon subsequent challenge with mould spores or ovalbumin (OVA) but not in mice with normal microbiota [47].

The same association between altered faecal microbiota and allergic disease has been shown in industrialized and developing countries with a high (Sweden) and a low (Estonia) prevalence of allergy respectively. In both countries, allergic children were colonized with higher levels of aerobic microbes and lower levels of anaerobic microbes, particularly lactobacilli [48]. It is further noted that infants that eventually developed allergies at 2 years of life were colonized with decreased levels of *Enterococcus* species at the age of 1 month and *Bifidobacteria* through the first year of life but increased levels of *Clostridium* species at 3 months of age [49]. These

differences in gut microflora composition between allergic and nonallergic infants can be observed preceding the manifestation of allergies very early in life. Likewise, another prospective epidemiological study demonstrated that infants with atopic sensitization harboured different bacterial cellular fatty acid profile with more clostridia and less bifidobacteria in their stools at 3 weeks of age as compared to non-atopic infants [50].

A case-control study of atopic dermatitis children with age- and sex-matched healthy controls similarly found lower levels of *Bifidobacterium* species in the faecal specimens of patients with eczema. Further, *Bifidobacterium* species were significantly lower in patients with more severe skin symptoms, suggesting a "dose-response" relationship [51]. This finding was further substantiated by another case-control study conducted in Singapore where the eczematous subjects similarly harboured lower counts of *Bifidobacterium*. In this study, higher *Clostridium* and lactic acid bacteria count were also observed [52]. These results are supported by conventional bacterial cultivation and improved culture-independent molecular methods used on targeting different species in the studies. In addition, children with atopic eczema have further been revealed to have predominantly *Bifidobacterium adolescentis* while healthy infants harboured more *Bifidobacterium bifidum* [53]. This difference in microbiota composition might be attributed by reduced adhesive abilities of bifidobacteria to the intestinal mucus in allergic infants [54]. Bifidobacteria from allergic infants induce less IL-10 production but more proinflammatory cytokine *in vitro* eliciting a Th1 type immune response [55]. These data support the microflora hypothesis of allergic disease that the differences in gut microbiota play an influential role in the postnatal maturation of the immune system and development of protective

mechanisms against atopy. This hypothesis paves the way for the use of probiotics intervention as a strategy for the primary prevention of allergy.

1.2 Probiotics

Probiotics in the form of fermented dairy products such as yoghurt and drinks have been consumed by humans for thousands of years and in recent times, freeze-dried bacteria in capsules have become popular dietary supplements. According to Food and Agriculture Organization (FAO) / World Health Organization (WHO) expert panel guidelines, probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [56]. The genus and species of a probiotic can have differential effects thus the strain identity is important to relate the probiotic strain to specific health effects. Strains of *Bifidobacterium* and *Lactobacillus* species, which are the most widely used, are indigenous to the human gut and are resistant to gastric acid digestion to remain viable and adhere to the intestinal epithelium [57, 58]. Majority of the probiotics in food are lactic acid bacteria (LAB) which are generally gram-positive, non spore-forming organisms that are devoid of catalase enzyme and are aerotolerant to produce lactic acid during sugar fermentation [59]. Species from other bacterial genera such as *Streptococcus* and *Enterococcus* and yeasts from the genus *Saccharomyces* have also been considered as probiotics [60]. The common probiotics used in dairy products such as *Lactobacillus acidophilus, Lactobacillus casei* and Bifidobacteria are listed in Table 1-1.

Apart from using probiotics alone, combination of probiotics and prebiotics has been added to milk and nutritional supplements. This combination is known as synbiotics. Prebiotics are nondigestible, fermentable food components that benefit the host by

selectively stimulating the growth or metabolic activity of beneficial intestinal microbiota and reduce the growth of pathogens [61]. Increasing the intake of prebiotics (commonly oligosaccharides) by supplementation to infant feeds has the potential to prevent allergic diseases in infants by modulating the immune system [62, 63].

Table 1-1 Common probiotics associated with dairy products

***Lactobacillus acidophilus* group**	- *L. acidophilus* - *L. amylovorus* - *L. crispatus*	- *L. gasseri* - *L. johnsonni*
***Lactobacillus casei* group**	- *L. casei* - *L. paracasei* - *L. rhamnosus*	
Lactobacillus reuteri		
Lactobacillus plantarum		
***Bifidobacterium* species**	- *B. lactis* - *B. longum* - *B. adolescentis* - *B. animalis*	- *B. bifidum* - *B. breve* - *B. infantis*

1.3 Immunomodulatory effects of probiotics

Studies that demonstrate the efficacy of probiotics is rapidly increasing and one area of particular interest is the effect of administration of probiotics on immune response. Probiotics are promising immunomodulators which enhance both the innate and adaptive immunities in the host [64] as they adhere to epithelial cells and proliferate in the mucosa stimulating the gut immune responses. The gut immune system, which consists of the gut-associated lymphoid tissue (GALT), mucosal lamina propria and the epithelium, protects us against pathogens and also induces tolerance to harmless food and microbial antigens. The intestinal microbiota acts as a microbial stimulation to influence systemic and mucosal immunity and importantly, microbial load acquired in the first days of life primes the immune response [65, 66]. The host-microbe

interaction provides antigenic challenge and aids in the maturation of the mucosal barrier mechanisms and the immune system.

1.3.1 Local effects on gut epithelium

Effect of *Lactobacillus rhamnosus* GG has been observed in several studies. The mitogenic effect of *L. rhamnosus* GG in germ-free rats resulted in increase of cell production contributing to faster mucosal regeneration [67]. This could act as a wash-out mechanism for pathogenic microbes. Furthermore, *L. rhamnosus* GG was observed to stabilize the mucosal barrier and reverse gut permeability disorder when suckling rats were challenged with cow's milk [68]. This reduced systemic antigen load by maintaining the integrity of the intestinal barrier. In addition, Yan et al. reported the increase survival of intestinal cells in the presence of *L. rhamnosus* GG through the prevention of cytokine-induced apoptosis which may be protecting the epithelial cells against inflammation-induced injury [69].

1.3.2 Probiotics and the innate immune system

Both live and heat-killed probiotics and the components of probiotic bacteria have been shown to stimulate the innate immune system. *L. acidophilus* and *L.casei* enhanced the phagocytosis capacity of murine peritoneal macrophages [70]. It is further demonstrated in clinical trials that *L.acidophilus* La1 increased phagocytosis of human leucocytes [71-73]. Other probiotics, namely *Bifidobacterium lactis* Bb12 [72], *B. lactis* HN019 [74] also increased phagocytosis considerably. However, the effect of probiotics in healthy subjects and patients with milk hypersensitivity has been shown to be different. *L. rhamnosus* GG stimulated immunostimulatory

neutrophil activation through upregulation of receptors (CR1. CR3, FcγRIII and FcαR) in healthy individuals but down-regulated immunoinflammatory response by inhibiting phagocytosis in allergic patients [75].

Lactobacilli could enhance antigen presentation of dendritic cells as killed Lactobacillus species upregulated MHC class II and CD86 in murine. *L casei* further induced IL-12, IL-6 and TNF-α while *L. reuteri* inhibited activities of *L.casei* [76]. The differential regulation suggested that the composition of the gut microflora can modify immune response.

Cytokines produced following the interaction of probiotics with the intestinal epithelium plays an important role in the immunomodulatory activity. A significant involvement of toll-like receptors (TLR), including TLR9 [77] and possibly TLR2 and TLR4 expressed on enterocytes contributes to the anti-inflammatory effects of probiotics. In addition, enterocytes produce IL-8 and IL-6 in the presence of probiotic organisms. Adhesion between live *L. plantarum* 299v and HT-29 epithelial cells, which were previously stimulated by TNF-α to induce inflammation, increased the IL-8 mRNA levels in the cells to recruit neutrophils [78]. *B. lactis* Bb12 [79], *L. casei* CRL 431 and *L. helveticus* R389 [80] increased IL-6 secretion in murine models. The data suggested that different species of probiotics would have differential responses with regards to the innate immune system and impact the level of cytokine production.

1.3.3 Probiotics and the adaptive immune system

1.3.3.1 Effect of probiotics on B lymphocytes

Probiotics also influence IgA production. Mice fed with yogurt supplemented with *L. acidophilus* and *Bifidobacterium* species enhanced both mucosal and systemic IgA responses to cholera toxin [81]. *L. rhamnosus* GG enhanced circulating IgA secreting cell response in acute rotavirus-induced diarrhoea patients [82]. In children with Crohn's disease, *L. rhamnosus* GG increased IgA production to cow milk β-lactoglobulin [83]. The effect of probiotics to enhance humoral immune responses to vaccinations has also been evaluated.

1.3.3.1.1 Effects of probiotics on oral vaccination

There is increasing evidence which support potential influences of probiotics on immunological responses to vaccines. Immunological response both to oral and parenteral vaccines have been evaluated with probiotic supplementation. . Gnotobiotic animal models have shown that probiotic has a significant immunostimulating effect on the local and systemic immune responses with increased specific IFNγ in ileum and spleen, IgA and IgG in ileum, and serum IgM, IgA and IgG antibody in oral rotavirus vaccinated pigs with *L. acidophilus* colonization [84]. Another gnotobiotic pigs study suggested that *L. acidophilus* and *L. reuteri* colonization reduced the distribution and frequencies of monocytes/macrophages and dendritic cells in ileum, spleen and blood due to human rotavirus infection [85].

Probiotic have been shown to enhance humoral immune responses to oral immunization such as that of rotavirus [86], Salmonella [87, 88], polio [89, 90] and cholera [91] in double-blind, randomized, controlled studies summarized in Table 1.2. Oral administration of *L. rhamnosus* GG with live oral rotavirus vaccine in 2 to 5 month old infants stimulated a significant increase in rotavirus-specific IgM secreting

cells from 29% in placebo to 79% in probiotic group (p=0.02) indicating an early humoral immune response to rotavirus infection. Furthermore, IgA seroconversion increased from 74% in infants who received placebo to 93% in the probiotic group (p=0.05) [86].

In another study, healthy human volunteers received either *L. rhamnosus* GG, *Lactococcus lactis* or placebo with an attenuated *Salmonella typhi* Ty21a oral vaccine. Although the IgA-, IgG- and IgM-secreting cells were found to be similar but there was a trend towards a higher IgA specific anti-*S. typhi* Ty21a secreting cells among the subjects who received the vaccine with *L. rhamnosus* GG. In addition, subjects who received *L. lactis* showed significantly higher CR3 receptor expression on neutrophils in peripheral blood than those receiving either the placebo or *L. rhamnosus* GG. This suggests that *L. lactis* could influence phagocytosis and affect the non-specific immune response although it did not enhance specific immune responses [87]. The effects of probiotics appear to be strain specific and may be determined by the colonizing properties of the organism. Moreover, administration of probiotics in fermented milk in conjunction with the vaccine could further enhance the immunomodulatory effect of probiotic as milk acts as a carrier to ensure large numbers of viable cells survive the passage through the harsh environment of the gastrointestinal tract. This has been observed in healthy adult subjects who consumed fermented milk containing *L. acidophilus* Lal and bifidobacteria with the administration of an attenuated *Salmonella typhi* Ty21a. The specific serum IgA to *S. typhi* Ty21a in the probiotics group was twice that of the control group (p=0.04). Both specific humoral immune response and systemic immune effect were observed as the total serum IgA was also enhanced at certain time points [88].

Efficacy of oral polio vaccination was also found to be enhanced in 2 studies. In a double-blind, randomized, controlled study, subjects consumed acidified milk products either with *L. rhamnosus* GG or *L. acidophilus* CRL431 or placebo. Subjects were vaccinated orally against polio 1, 2 and 3 in the second week of the study. Both probiotics increased poliovirus neutralizing antibody titres to a maximum of 2 fold and markedly enhanced poliovirus serotype-1-specific IgA. *L. rhamnosus* GG, in particular, increased the IgA titre to 3.9 fold ($p<0.036$). It also increased poliovirus serotype-1-specific IgG by 2.2 fold [89]. These results were substantiated in another study whereby consumption of cow milk-based follow-up formula containing viable *B. lactis* Bb-12 after routine oral polio immunization significantly increased faecal levels of total IgA to a peak level of 2.9-fold ($p<0.05$) with a increasing trend of anti-poliovirus IgA during consumption when compared to prior consumption. The total IgA levels however decreased to the initial levels after cessation of formula intake [90].

The immunomodulatory effect of probiotics was also evaluated in oral cholera vaccination study with 7 strains of *Lactobacillus* or *Bifidobacterium* [91]. Probiotics were supplemented for 21 days and oral cholera vaccination occurred at day 7 and day 14 after the start of supplementation. Specific salivary IgA analysis showed no difference between groups. Serum IgG increased in 2 probiotic groups, namely *B. lactis* Bl-04 and *L. acidophilus* La-14, 7 days after second vaccine administration ($p=0.01$). In contrast, *L. acidophilus* La-14 was found to decrease serum IgA. This change may be due to the concomitant increase of serum IgG in this group. Out of the 7 probiotic strains investigated, 6 showed near significant changes in immunoglobulin

serum concentrations with varying effects compared with controls ($p < 0.1$), although overall vaccination titre was not altered. Strain-specific effects of probiotics were noted as different strains of *L. acidophilus* exhibited different effects and this difference could be due to specific bacterial cell wall protein profiles [92].

Table 1-2 Summary of clinical trials evaluating effects of probiotics on oral vaccination

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Vaccination	Effect of probiotic on Outcome measures
Isolauri et al., 1995 [86]	Probiotic = 30 Placebo = 30	2-5 months (4.1)	*L. rhamnosus* GG	5 x 10^{10} CFU twice daily	5 days	Rotavirus	• Increase specific IgM secreting cells from 29% in placebo to 79% in probiotic (p=0.02) • IgA seroconversion increased 74% to 93% in the probiotic group (p=0.05)
Fang et al., 2000 [87]	Probiotic(1) =10 Probiotic(2) =10 Placebo =10	20-50 years	*(1) L. rhamnosus* GG *(2)* Lactococcus lactis	(1) 4 x 10^{10} CFU daily (2) 3.4 x 10^{10} CFU daily	7 days	*Salmonella typhi* Ty21a	• IgA-, IgG- and IgM-secreting cells similar • Trend towards higher IgA specific anti-*S. typhi* Ty21a secreting cells in *L. rhamnosus* GG group • Higher CR3 receptor expression on neutrophils in *L. lactis* group
Link-Amster et al., 1994 [88]	Probiotic = 16 Placebo = 14	19-59 years (37.3)	*L. acidophilus* La1 and bifidobacteria	1 × 10^{7}-10^{8} CFU/g	3 weeks	*Salmonella typhi* Ty21a	• specific IgA to *S. typhi* Ty21a doubled in probiotic group (p=0.04) • total serum IgA enhanced at certain time points

Table 1.2 Summary of clinical trials evaluating effects of probiotics on oral vaccination (continued)

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Vaccination	Effect of probiotic on Outcome measures
de Vrese et al., 2005 [89]	Probiotic(1) =21 Probiotic(2) =21 Placebo =22	20-30 years	*(1) L. rhamnosus* GG (2) *L. acidophilus* CRL431	10^{10} CFU/100g in yoghurt daily	5 weeks	Polio	• Neutralizing antibody titres increase to a max. of 2 fold • Enhanced serotype-1-specific IgA. *L. rhamnosus* GG, to 3.9 fold ($p<0.036$). • Increased serotype-1-specific IgG by 2.2 fold
Fukushima et al., 1998 [90]	Probiotic = 7 No placebo	15-31 months	*Bifidobacterium lactis* Bb-12	10^{9} CFU in milk daily	21 days	Polio	• Faecal levels of total IgA increase to 2.9-fold ($p<0.05$) with increasing anti-poliovirus IgA
Paineau et al., 2008 [91]	Probiotic= 9 in each group Placebo= 20	18-62 years	(1) *B. lactis* Bi-07 (2) *B. lactis* Bl-04 (3) *L. acidophilus* La-14 (4) *L. acidophilus* NCFM (5) *L.plantarum* Lp-115 (6) *L. paracasei* Lpc-37 (7) *L. salivarius* Ls-33	2×10^{10} CFU	21 days	Cholera	• Specific salivary no change • Serum IgG increased in *B. lactis* Bl-04 (day 0-21) ($p=0.01$) • Decrease serum IgA in *L. acidophilus* La-14 (day 0-21) ($p=0.09$), and day 21-28 ($p=0.05$). • Increased serum IgA in *L. acidophilus* NCFM® (day 21-28) ($p = 0.09$) • Overall vaccination titre not altered.

1.3.3.1.2 Effects of probiotics on parenteral vaccination

Apart from oral vaccinations, the effects of probiotic on antibody responses to diphtheria, tetanus, *Haemophilus influenzae* type b (Hib) and influenza parenteral vaccination [93-97] have also been evaluated and probiotic has been proposed as vaccines adjuvant (Table 1.3).

In a randomized double-blind placebo-controlled study by Kukkonen et al. [93], probiotics supplementation in allergy-prone infants improved immune response to Hib immunization as the geometric mean Hib IgG concentration was higher and there were 2-fold more subjects with protective Hib antibody concentration in the probiotic group than that of control ($p=0.02$). Diptheria and tetanus IgG antibody concentrations however showed no difference between the groups.

Supplementation of *Bifidobacterium breve* strain C50 in milk from birth to 4 months old was also found to increase antipoliovirus IgA titers significantly ($p < 0.02$) as compared to that of subjects in placebo group. This antibody titers correlated with bifidobacteria, especially *B. longum/B. infantis* and *B. breve* levels in the stools ($p < 0.002$) [94]. Furthermore, oral administration of *L. fermentum* CECT5716 increased the immunologic response to an anti-influenza vaccine and lowered the incidence of influenza-like illness 5 months after vaccination by increasing the antigen specific Ig A. The number of natural killer (NK) cells and TNF-α level in serum were higher in the probiotic group compared to the placebo. [95].

Another study performed in infants that received *L. acidophilus* in the first six months after birth reduce the IL-10 response to the vaccine antigen tetanus compared with the

placebo group (p=0.03). Although this effect cannot be directly extrapolated to effects on vaccine responses, the author concluded that probiotics may have immunomodulatory effects on vaccine responses which needs to be determined in further studies [96].

The most recent study conducted by West et al. [97] investigated the impact of *L. paracasei* subspecies *paracasei* strain F19 during weaning on infants who received DTaP (diphtheria and tetanus toxoid and acellular pertussis), polio and Hib-conjugate vaccines. Probiotics supplementation increased the capacity to raise the IgG anti-diphtheria immune response with more marked effects after adjusting for infants who were breastfed for less than 6 months in the 4 week after the second vaccination dose ($p = 0.018$) and prior to the third dose ($p = 0.048$). This similar trend was observed for the specific IgG antibody concentrations to tetanus toxoid after adjusting for breastfeeding duration and probiotic colonization. In contrast, there was no effect of probiotic supplementation on the immune response to the Hib polysaccharide antigen.

In conclusion, there are only a few studies that have looked at the effects of probiotics on different vaccination responses. The efficacy and clinical relevance requires further work to be demonstrated in other studies.

Table 1-3 Summary of clinical trials evaluating effects of probiotics on parenteral vaccination

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Vaccination	Effect of probiotic on Outcome measures
Kukkonen et al., 2006 [93]	Probiotic=47 Placebo=40	At birth	Mixture of 4 strains with prebiotic galacto-oligosaccharides 1. LGG and 2. *L rhamnosus* LC705 3. *Bifidobacterium breve* Bb99 and 4. *Propionibacterium freudenreichii* ssp. *shermanii* JS	Both 5x 10^9 CFU twice daily Both 2 x 10^9 CFU twice daily	Prenatal – 4 weeks Postnatal – 6 months	DTwP (diphtheria, tetanus and whole cell pertussis) at 3,4,5 mo and *Haemophilus influenza* type b (Hib) at 4 mo	• Frequency of Hib antibody conc. (≥1 μg /ml) doubled (p = 0.02) • Hib IgG concentration higher 0.75 (0.15-2.71) μg/ml than in placebo 0.40 (0.15-0.92) μg /ml (p = 0.064). • Diptheria and tetanus IgG no difference
Mullie et al., 2004 [94]	Probiotic= 11 Placebo =9	At birth	*Bifidobacterium breve* strain C50 in milk	Not specified	Birth to 4 months	Combination vaccine against diphtheria and tetanus, poliomyelitis, *Haemophilus influenzae*, and Bordetella pertussis at 2, 3, and 4 mo	• Fecal bifidobacterial level higher at 4 mo (p=0.0498) • *B. longum/B. infantis* carriage higher at 4 months (p=0.0399). • Antipoliovirus IgA titers increased significantly (p <0.02). • Antibody titers correlated with bifidobacteria, especially *B. longum/B. infantis* and *B. breve* levels (p <0.002). • Presence of *B. longum/B. infantis* correlated with higher levels of antipoliovirus IgA (p <0.002)

Table 1.3 Summary of clinical trials evaluating effects of probiotics on parenteral vaccination (continued)

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Vaccination	Effect of probiotic on Outcome measures
Olivares et al., 2007 [95]	Probiotic = 25 Placebo = 25	22-56 years (33)	*L. fermentum* CECT5716	1x 10^{10} CFU daily	28 days	Influenza	• Increase natural killer cells • Significant increase in antigen specific Ig A • Incidence of influenza-like illness during 5 mo after vaccination lower
Taylor et al., 2006 [96]	Probiotic = 58 Placebo = 60	At birth	*L. acidophilus* LAVRI-A1	3 x 10^{9} CFU daily	6 months	Tetanus toxoid	• lower IL-10 responses to tetanus toxoid vaccine antigen compared with the placebo group (p=0.03) • no significant effects of probiotics on either Th1/Th2 cell responses to allergens
West et al., 2008 [97]	Probiotic= 89 Placebo= 90	4 months	*L. paracasei* ssp. *paracasei* strain F19	At least 1 x 10^{8} CFU/serving of cereals	9 months	DTaP (diphtheria and tetanus toxoid and acellular pertussis), polio and Hib-conjugate vaccines	• Increase IgG anti- diphtheria -> adjusting for infants breastfed < 6 months , 4 week after 2nd vaccination (p = 0.018) and prior 3rd dose (p =0.048). • Similar trend for IgG anti-tetanus after adjusting for breastfeeding duration and probiotic colonization. • No effect on immune response to Hib polysaccharide antigen.

1.3.3.2 Effect of probiotics on T lymphocytes

Probiotic supplementation can induce T_{reg} cells which bear TGF-β and production of regulatory cytokines IL-10. *L. reuteri* and *L. casei* influenced monocyte-derived dendritic cells to instruct naïve $CD4^+$ T cells to differentiate into T_{reg} cells which produced increased levels of IL-10 *in vitro*. However, *L. plantarum*, which did not bind to the lectin dendritic cell, was unable to induce T_{reg} cell differentiation [98]. *L. paracasei* NCC2461 was shown in another *in vitro* study to induce the development of a $CD4^+$ T cell subset with immunoregulatory properties that secrete high IL-10 and TGF- β [99] to inhibit the development of bystander T cells and reduces both the Th1 and Th2 cells cytokines, including IFN-γ, IL-4 and IL-5 secretion.

The capability of probiotics to alter the Th1 and Th2 balance has been shown in various studies. Different probiotic strains can have different capacities to drive pro-inflammatory effect towards Th1 development or anti-inflammatory effect towards Th2 development or even stimulate both Th1 and Th2 responses. Skewing of the immune response towards Th1 has been shown by the administration of *L. rhamnosus* GG in children who were allergic to cow's milk resulting in increased production of IFN-γ in peripheral blood mononuclear cells (PBMC) and suppressed secretion of IL-4 [100]. Another study further indicated that *L. rhamnosus* GG degrades cow's milk caseins which down-regulated the IL-4 production to provide protection from dietary antigens [101]. Other probiotic strains such as *L. brevis* subsp. *coagulans* and *B. lactis* HN019 stimulate the production of immunostimulatory cytokines such as IFN-α [74, 102]. On the other hand, reduced production of the pro-inflammatory cytokines Il-12, IFN-γ and TNF- α by splenocytes and Peyer's patches was observed when IL-10 knockout mice, which do not develop colitis until more than 20 weeks old, were fed

with *L. salivarius* and *B. infantis.* This reduction in Th1 cytokines significantly prevented colitis in this murine model. [103]. Subcutaneous injection of *L. salivarius* 118 can also interestingly reduce the production of pro-inflammatory Th1 cytokines in intestinal inflammation murine models, suggesting that the oral route may not be essential for probiotic to demonstrate its anti-inflammatory function [104]. Other probiotics have been found to stimulate both Th1 and Th2 response under different physiological conditions. *L. rhamnosus* HNOO1 in particular raised mixed lymphocyte cytokine production with increased IFN-γ and at the same time enhanced IL-4 and IL-5 production in mice during antigen sensitization [105].

1.4 Clinical benefits of probiotics

1.4.1 Potential benefits from probiotics

To date, potential results have been observed for use of probiotics in the prevention and treatment of gastrointestinal disorders. The evidences for probiotics in the treatment of diarrhoea have been strong. There are more than 10 studies that have investigated the use of probiotics to treat or prevent acute infectious diarrhoea in both children and adult [106-120]. Positive results have been shown for use with *L. rhamnosus* GG [107-109, 111, 117], *L. reuteri* [115, 119], *Saccharomyces boulardii* [118] and other mixtures including *L. acidophilus* [120]. Most of these patients had shorter duration of symptoms and decreased severity with a decreased likelihood of persistent diarrhoea. Meta-analysis further substantiates the efficacy of *L. rhamnosus* GG and *S. boulardii* in the prevention of adverse intestinal effects of antibiotic-associated diarrhoea in children [121]. In other studies, significant lower number of adult patients who received antibiotic treatment experienced nausea and diarrhoea when treated with *L. rhamnosus* GG [122, 123].

In addition, probiotics have shown promising results in the treatment and prevention of relapses of inflammatory bowel disease. Although results have been variable in the small number of studies, VSL#3 has been reported as effective and recommended for the maintenance of remission of pouchitis [124-126]. Beneficial effects of *Bifidobacterium infantis* to relieve symptoms of irritable bowel syndrome have further been reported in large, randomized controlled trials [127, 128].

Limited studies have been performed to propose potential applications of probiotics in other diseases and conditions. The use of probiotics to prevent enterocolitis has been promising in small studies but insufficient information is available to make a concluding recommendation [129]. Similarly, VSL#3 [130, 131] and *L. acidophilus* [132] have been shown to be effective in prevention of radiation enteritis but further studies will be necessary. Evidence is also rapidly accumulating on the use of *L. rhamnosus* GG [133], *L. reuteri* [133, 134] and *L. acidophilus* [135] in the treatment of vaginitis and vaginosis which has produced impressive results in controlled trials.

1.4.2 Probiotics for the treatment of allergic disease

A better understanding of the potential of probiotics as preventive and therapeutic agent has been explored in randomized controlled trials. There have been several studies examining the use of probiotics to treat atopic diseases especially in the treatment of eczema (Table 1.4). Most of these studies classify the severity of eczema based on the SCORing Atopic Dermatitis (SCORAD) index established by the European Task Force on Atopic Dermatitis which combines objective measures such as extent and severity of skin lesions and subjective criteria such as pruritus and sleep

loss [136]. Based on the SCORAD score, the patients can be generally classified as having mild (<25), moderate (25-50) or severe (>50) eczema (Refer to Appendix E).

The first study was conducted in 1997 by Majamaa and Isolauri [137] with 27 infants aged 2.5-15.7 months old fed with 5 x 10^8 colony-forming unit (CFU)/g *L. rhamnnosus* GG fortified extensively hydrolyzed whey formula. The subjects in both the probiotic and placebo group had mild/moderate eczema with baseline SCORAD of 26(17-38) and 21(14-31) respectively. Median SCORAD score improved significantly (p=0.008) from 26 to 15 in the probiotic group but not in the placebo group after one month. Furthermore, faecal α1- antitrypsin and TNF-α concentration which are markers of intestinal inflammation decreased significantly after dietary intervention.

In a second study by the same group, exclusively breastfed infants with mild/moderate eczema were randomized to extensively hydrolysed whey formula, formula with either 3 x 10^8 CFU/g *L. rhamnnosus* GG or formula with 1 x 10^9 CFU/g *B. lactis* Bb-12 [138]. There were 9 subjects in each group. After 2 months supplementation, both the *L. rhamnnosus* GG and *B. lactis* Bb-12 treated group showed significant improvement of the median SCORAD score from 14 to 1 and 12 to 0 respectively, compared to placebo 10 to 13.4 (p=0.002). Significant decrease in serum soluble CD4 and urinary eosinophilic protein X were also observed in both probiotic supplemented group while TGFβ1 was significantly decreased in the *B. lactis* Bb-12 treated group, indicating that the control of inflammation extend beyond the gut.

This study team further investigated the efficacy of 1 x 10^9 CFU/g viable and heat-inactivated *L. rhamnnosus* GG in extensively hydrolyzed whey formula for the management of atopic eczema [139]. However, this study was terminated early due to adverse diarrhoea suffered by infants in the heat-inactivated probiotic group. Although the length of treatment had a great variation from less than a week to more than 10 months, significant decrease in mean SCORAD were noted in all the groups from 13 to 8 in the placebo group, 19 to 5 in the viable probiotic group and 15 to 7 in the heat inactivated probiotic group. This mean decrease in SCORAD was significantly higher in the viable *L. rhamnosus* GG treated group than in the placebo group ($p=0.02$). Presence of some bifidobacteria, lactobacilli, *Bacteroides*, enterococci and clostridia in the faeces were not significantly different before and after treatment in each of the 3 groups when detected with 16S rRNA-specific probes.

Other studies conducted with various strains of lactobacilli further support the favourable effects of probiotics on atopic eczema. In a randomized placebo controlled cross-over trial, 43 moderate/severe eczematous children with a wide age group of 1 to 13 years old were given 1 x 10^{10} CFU *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 each twice daily in water [140]. This cross-over trial was conducted with 6 weeks treatment or placebo and a 6 weeks wash-out period in between. Although no overall significant change in total SCORAD after treatment with probiotics was observed in this cross-over study, a minor improvement of 2.4 SCORAD score was found in the IgE-sensitized group compared to a 3.2 points worsening in the placebo group, however this difference was not clinically significant. The wide age range, method of administration, probiotic species and degree of initial eczema severity

before treatment could have contributed to the reduced efficacy of probiotics in this study.

To date, the largest randomized double blind placebo-controlled trial of the effects of probiotics on treatment of eczema is that of Viljanen et al. [141] on 230 infants with suspected cow's milk allergy. The infants were randomized to either 5 x 10^9 CFU *L. rhamnosus* GG or a mixture of 4 probiotics, namely 5x 10^9 CFU *L. rhamnosus* GG, 5x 10^9 CFU *L rhamnosus* LC705, 2 x 10^8 CFU *B. breve* Bbi99 and 2 x 10^9 CFU *Propionibacterium freudenreichii* ssp. *shermanii* JS, or placebo twice daily. On the whole, no significant difference was noted between the SCORAD scores of probiotic and placebo groups. But in subgroup analysis of allergen-sensitized infants, *L. rhamnosus* GG supplemented group showed a SCORAD improvement of 26.1 points compared to 19.8 points in the placebo group (p=0.036). Similarly, in infants who are not treated with antibiotics, treatment effect was noted in only the *L. rhamnosus* GG supplemented group with a mean SCORAD improvement of 38.4 points versus 28.5 points in the placebo group (p=0.008). Negative effects between the combinations of *L. rhamnosus* GG and other probiotic strains suggested that these strains suppressed the benefits of *L. rhamnosus* GG when used alone. Strain-specific effects and interactions between probiotics need further evaluation.

Other strains such as *L. fermentum* VRI-033 PCC was administered in another randomized study to half of the 56 infants enrolled for 8 weeks [142]. These probiotic-treated infants with moderate or severe eczema showed significant improvement in SCORAD scores (p = 0.03) but not the placebo group. At week 16 follow-up, 92% of probiotic-treated subjects had a better SCORAD than baseline

compared with 63% in the placebo group (p = 0.01). Eventually, 54% of the children in the probiotic group had mild eczema compared to 30% in the placebo group. Probiotic supplementation may accelerate the natural improvement of eczema in young children with apparent effects 2 months after cessation of supplementation.

Interestingly, 3 other subsequent studies conducted using *L. rhamnosus* GG in recent years did not yield favourable results. Brouwer et al. [143] supplemented either *L. rhamnosus* or *Lactobacillus* GG in hydrolysed whey-based formula for 3 months to 17 infants less than 5 months old with eczema in each group. However, this study did not demonstrate any significant effects of probiotics on SCORAD, sensitization, inflammatory parameters or cytokine production. Similarly, Foster-Holst et al. [144] did not demonstrate *L. rhamnosus* GG to be an effective treatment for eczema in a randomized, double-blind, placebo-controlled study with 54 infants randomized to $5x10^9$ CFU of *L. rhamnosus* GG or placebo twice daily for 8 weeks. The most recent study conducted in 2007 by Gruber et al. [145] randomized mild-to-moderate atopic dermatitis infants aged 3-12 months to $5x10^9$ CFU of *L. rhamnosus* GG (n=54) or placebo (n=48) for 12 weeks and showed no therapeutic effect of probiotic even when sub-analysed by age, eczema severity and hydrocortisone treatment.

Furthermore, lack of beneficial effect of probiotics has been observed in older adolescents and adults with asthma. In the randomized controlled crossover trial, no significant difference in asthma control and inflammation was found between the active group supplemented with 450g of yoghurt with $7.6x10^8$ CFU/g of *L. acidophilus* and the placebo group supplemented with yoghurt containing $3.4x10^8$ CFU/g of *S. thermophilus* and $3.2x10^8$ CFU/g of *L. bulgaricus* for 1 month [146].

Another double-blind, placebo-controlled study was conducted on birch-pollen allergic young adults who were supplemented with *L. rhamnosus* before, during and after the pollen season. But the probiotic supplementation did not alleviate the respiratory and eye symptoms of the patients nor reduce medication use during and after the pollen season [147]. Most recently, 10^8 CFU/ml of *L. casei* in 100ml of fermented milk was administered to 2 to 5 year old preschool children with intermittent to moderate persistent asthma for 1 year and observed longer mean time (4.1 months) free of episodes of asthma as compared to placebo (3.3 months). But this difference was not statistically significant (p=0.23) [148]. A systemic review of these studies concluded that there were no positive effects of probiotic on the treatment of asthma [149]. In this review, the effects of probiotics for the treatment of allergic rhinitis were also assessed. In 9 of the 12 studies evaluated (4 perennial and 8 seasonal allergic rhinitis studies), probiotics improved at least one clinical symptom severity or the amount of medication used or the number of episodes of allergic rhinitis [148, 150-157]. No positive probiotics effect was noted in 3 seasonal allergic rhinitis studies but it is of note that the clinical symptoms did not deteriorate in these subjects [147, 158]. Probiotic supplementation did not show beneficial effects on the total and specific IgE and cytokine and chemokine levels in 9 of these randomized controlled trials which immunological measurements were taken. In conclusion, probiotics may have beneficial effects in allergic rhinitis by reducing symptom severity and medication use.

Due to these conflicting results and difference in study design, meta-analyses and reviews of these studies have proposed that although probiotics are likely to play a role in the management of atopic eczema and allergic rhinitis, the specific treatment

effect is uncertain and cannot be recommended as standard therapy for allergic diseases [159-162]. Probiotics may have more beneficial effects when used early in life as a primary prevention measure while immune responses are still developing and before allergic disease is established.

Table 1-4 Summary of clinical trials evaluating the role of probiotic supplementation in the treatment of atopic dermatitis

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Baseline SCORAD (probiotic/ control)	Effect of probiotic on Outcome measures
Majamaa and Isolauri, 1997 [137]	Probiotic=13 Placebo=14	2.5 -15.7 months	*L. rhamnosus* GG	5 x 10^8 CFU/g	1 month	26 (17-38) / 21(14-31) #	Improvement in SCORAD from median 26 to 15 (p=0.008) but not in placebo
Isolauri et al., 2000[138]	Probiotic (a) = 9 Probiotic (b) = 9 Placebo = 9	4.6 months	(a) *L. rhamnosus* GG (b) *B. lactis* Bb-12	1) 3 x 10^8 2) 1 x 10^9 CFU/g	2 months	13 (6.5-21.0)/ 10 (6.5-26.5) #	Improvement in SCORAD- *B lactis* Bb-12 to 0 (0-3.8), LGG group to 1 (0.1-8.7), vs control 13.4 (4.5-18.2) (p = 0.002)
Kirjavainen et al., 2003[139]	Probiotic (a) = 14 Probiotic (b) = 13 Placebo = 8	3.5-6.8 (5.5) months	(a)Live *L. rhamnosus* GG (b)Heat-inactivated *L. rhamnosus* GG	1 x 10^9 CFU/g	< 1 week to >10 months	(a) 19 (4-47) (b) 15 (0-29) / 13 (4-29) #	More improvement with viable LGG (p=0.02) than for placebo group but diarrhea with heat inactivated LGG
Rosenfeldt et al., 2003 [140]	Cross-over study Probiotic= 22 Placebo= 21	1-13 (5.2) years	*L. rhamnosus* 19070-2 & *L. reuteri* DSM 122460	1 x 10^{10} CFU each twice daily	6 weeks treatment/placebo, washout period, then 6 weeks treatment/placebo	40 (18-66) / 35 (15-66) *	Improvement in extent of eczema from a mean of 18.2% to 13.7% (p =0.02) and SCORAD in IgE sensitized group decreased 2.4 (p=0.04)
Viljanen et al., 2005 [141]	Probiotic (a) = 80 Probiotic (b) = 76 Placebo = 74	1.4 – 11.9 months	(a) *L. rhamnosus* GG (b) LGG, *L rhamnosus* LC705, *B. breve* Bbi99, *Propionibacterium freudenreichii* ssp. *shermanii* JS	(a) 5 x 10^9 (b) 5x 10^9, 5x 10^9, 2 x 10^8, 2 x 10^9 CFU twice daily	4 weeks	34.3(17.2) / 33.3 (15.0) / 29.9 (12.2) †	Mean SCORAD no difference between treatment groups immediately or 4 weeks after treatment. LGG group showed a greater reduction in SCORAD in IgE-sensitized infants (p=0.036) and infants without antibiotic treatment (p=0.008)

Median (IQR)
* Median (range)
† Mean (SD)

Table 1.4 Summary of clinical trials evaluating the role of probiotic supplementation in the treatment of atopic dermatitis (continued)

Study	No. of Subjects	Age Range (mean)	Probiotic	Dose	Supplement period	Baseline SCORAD (probiotic/ control)	Effect of probiotic on outcome measures
Weston et al., 2005 [142]	Probiotic=26 Placebo=27	6-18 months	*L. fermentum* VRI-033 PCC	1 x 10^9 CFU twice daily	8 weeks	40.8 (6.8) / 44.0 (10.4) †	Improvement in SCORAD at 16 weeks (p = 0.03) but not the placebo group. 92% probiotic-treated subjects had better SCORAD than 63% in placebo group (p = 0.01)
Brouwer et al., 2006 [143]	Probiotic (a) = 17 Probiotic (b) = 16 Placebo = 17	1.1 -5.2 months	(a) *L. rhamnosus* (b) *Lactobacillus* GG	(a) 5 x 10^9 (b) 3 x 10^8	3 months	14.2 (3.7-41.1)/ 19.9 (3.5-59.1)/ 22.5 (9.0-39.2)*	No improvement
Folster-Holst et al., 2006 [144]	Probiotic=26 Placebo=27	1-55 months	*L. rhamnosus* GG	5 x 10^9	8 weeks	43.3 / 41.4 ¥	No improvement
Gruber et al., 2007 [145]	Probiotic=54 Placebo=48	3-12 months	*L. rhamnosus* GG	5 x 10^9	12 weeks	24.6 (8.8) / 23.6 (7.8) †	No improvement

Median (IQR)
* Median (range)
† Mean (SD)
¥ Mean

1.4.3 Probiotics for the prevention of allergic disease

Gut microbiota contribute as one of the most abundant sources of microbial antigen exposure to stimulate the early immune system. Thus the potential benefits of probiotics early in life on a developing immune system may provide a window of opportunity for the primary allergy prevention. The first study that addressed the role of probiotics in primary prevention by Kalliomaki et al. [8] reported a reduction by 50% on the incidence of eczema by 2 years of age in the *L. rhamnosus* GG treated group (23%) as compared to the placebo group (46%) (Relative risk RR 0.51; 95% confidence interval CI 0.32-0.84). This double-blind, randomized placebo-controlled trial recruited 132 evaluable pregnant women with a family history of atopic diseases and administered 1 x 10^{10} CFU *L. rhamnosus* GG daily for the last 2-4 weeks of pregnancy. This dose of probiotic was continued postnatally to the breastfeeding mothers or the infants who were on total formula fed directly in water for 6 months. This reduction in eczema persisted at the 4 and 7 year follow-up, where there was a 43% reduction in the risk of developing eczema (RR 0.57; 95% CI 0.33-0.97) at 4 years of age [163]. The cumulative risk of developing eczema during the first 7 years of life was 42.6% in the probiotic-treated group, compared with 66.1% in the placebo group (RR 0.64; 95% CI 0.45–0.92) [164]. However, no differences were observed for total or specific IgE concentration and skin-prick-test reactivity. Although at 4 years of age, there was a reduction in exhaled nitric oxide production in the probiotic-treated group compared to healthy age-matched individuals (from 14.5 to 10.8 parts per billion), effect on respiratory allergic diseases, namely asthma and allergic rhinitis, was not found.

In a second preventive study in another Finnish population by Kukkonen et al., a mixture of 4 probiotic strains, namely *L. rhamnosus* GG, *L rhamnosus* LC705, *B. breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS was administered with prebiotic galacto-oligosaccharides to 925 randomized infants both prenatally to mothers and postnatally for 6 months [165]. This combined supplementation of probiotics and prebiotics reduced eczema (Odds Ratio OR, 0.74; 95% CI, 0.55-0.98; p = 0.035) and atopic eczema (OR, 0.66; 95% CI, 0.46-0.95; p = 0.025) but no effects on sensitization or other allergic diseases at 2 years of age. Kukkonen and colleagues continued to follow up this large study cohort up to 5 years of age [166]. This recent publication revealed that although probiotics did not confer protection against eczema (39.3% vs. 43.3% in placebo), atopic eczema (24.0% vs. 25.1% in placebo), allergic rhinitis (20.7% vs. 19.1% in placebo), or asthma (13.0% vs. 14.1% in placebo) at 5 years of age, subset analysis showed decreased IgE-associated allergic diseases in probiotics-treated caesarean-delivered children (24.3% vs. 40.5% in placebo; OR 0.47, 95% CI 0.23-0.96, p = 0.035).

These initial studies provided positive results but were not substantiated by subsequent studies in other populations. Another double-blind, placebo-controlled trial conducted in an Australian population recruited 178 pregnant mothers with an allergic disease to supplement 3 x 10^8 CFU of *L. acidophilus* to their babies directly after birth for 6 months [167]. At the end of the supplementation period, atopic dermatitis rates were similar in the probiotic (25.8%) and placebo (22.7%) groups (p= 0.629). At 12 months of age, no difference was noted with 43.2% of eczematous toddlers in the probiotic group and 39.1% in the placebo group. Interestingly, the rate of positive skin prick test was significantly higher in the probiotic group (p = 0.03)

with a significantly higher proportion of these children with atopic dermatitis and sensitization (p = 0.04). There was no difference in the rate of food allergic subjects between the groups but children who received probiotic had significantly higher rate of wheezing (OR 2.45; 95% CI 1.11-5.39; p=0.024) in the second 6 months of life. Subsequent follow-up to 2.5 years also did not show a reduction in the risk of eczema with 42% in the probiotic (n=31/74) compared to 34% in the placebo group (n=25/76). No significant reduction in any other allergic disease or allergen sensitization was observed [168].

In a Swedish study, Abrahamsson et al. [169] also could not demonstrate a protective effect of 1x 10^8 CFU of *L. reuteri* on infant eczema when administered daily from gestational week 36 until 12 months of age. However, the probiotic-treated infants had less IgE-associated eczema (8% versus 20% in placebo group, p = 0.02) and less sensitization in a subgroup with allergic mothers (14% versus 31% in placebo group, p= 0.02) at 2 years follow-up.

Several meta-analyses and reviews were performed for these studies conducted before 2008. The Cochrane review by Osborn et al. concluded that there was then insufficient evidence to recommend the use of probiotics in prevention of allergic disease due to the inconsistencies between study designs and probiotic strain [170]. Betsi et al. however recommended that *L. rhamnosus* GG seems to be effective for primary prevention of eczema but more randomized controlled trials needs to be conducted for a more conclusive inference [159]. The meta-analysis by Lee et al. [160] included the data of three follow-up studies from the same study population of Kalliomaki et al. Therefore the conclusion of supporting a preventive potential of

probiotics on paediatric atopic dermatitis might be inappropriate and should be considered with care.

Subsequent randomized double-blind, placebo-controlled trials were published recently. Kopp et al. [171] adopted a similar study design to that of the Kalliomaki et al. study with the hope to elucidate comparable positive results in the German population. The same probiotic strain, *L. rhamnosus* GG was administered at 5×10^9 CFU twice daily to 50 evaluable infants 4 to 6 weeks prenatally and then postnatally for 6 months. This dose was higher than the 1×10^{10} CFU daily of *L. rhamnosus* GG that was administered in the Kalliomaki et al. study. However, no preventive effect of probiotic on the development of eczema was observed in this German population. The cumulative incidence of atopic dermatitis was 38% in the probiotic group and 31.8% in the placebo group (p=0.53). No differences in the total IgE concentration or sensitization to inhalant allergens were noted. Moreover, children with recurrent (≥5 episodes) wheezing bronchitis during the first 2 years were more frequent in the probiotic group (26%) as compared with 9.1% in the placebo group (p=0.03). Eventually, Kopp et al. published a review [172] which argued that although the concept of using probiotics for primary prevention of allergy seems beneficial, further studies need to evaluate specific probiotic strain, the timing, dose and method of administration to determine whether there will be a favourable effect on subgroups.

Differential effects of two probiotic species were further examined by the most recent primary prevention study by Wikens et al. [173] using *L. rhamnosus* HN001 and *B. animalis* subsp *lactis* strain HN019 in another double-blind, randomized placebo-controlled trial conducted in New Zealand. Pregnant women with atopic history (or their husband had atopic history) were recruited to be randomized into one of the

probiotic group or the placebo group to be supplemented with the capsules at 35 weeks gestation. Following which, the breastfeeding mothers were continued to be supplement till 6 months postpartum or the bottle-fed infants were started on the capsules till 2 years of age. This is the longest supplementation period in the primary prevention studies ever conducted. Only the infants who received *L. rhamnosus* but not the *B. animalis* subsp *lactis* had a significantly reduced risk of eczema (14.8%) compared with placebo (26.8%) (p=0.01) by 2 years evaluation. The risk of developing SCORAD≥10 was also reduced in the *L. rhamnosus* group only (24.0% vs. 38.7% in placebo, p=0.009). There was no significant difference of *L. rhamnosus* (21.3%) or *B. animalis* subsp *lactis* (23.5%) on sensitization to any allergens compared to placebo (28.8%) (p=0.42). Other allergic diseases were not evaluated. This suggests a protective effect for only *L. rhamnosus* HN001 but not *B. animalis* subsp. *lactis* strain HN019 and therefore different probiotic species and strains can exert diverse effects on allergic disease.

Despite the disparities between results of different studies, the protective potential for probiotics in the pathogensis of eczema is evident. Responses may be affected by strain-specificity of probiotic effects as closely related strains can show significant different adhesion, competitive exclusion and pathogen displacement properties [174]. In the Taylor et al. study [167], *L. acidophilus* was previously undefined and did not demonstrate a positive reduction of eczema. Probiotics combination needs to be evaluated to ensure that desirable properties are enhanced and not counteracted. Furthermore, the pattern of early allergen exposure including the variations in the timing, dose, interval and regularity may provide the key about how probiotics exert their effects. The target population is also critical as seen in Kopp et al. study in a

German population [171]. Despite using the same concept of the Finnish study published by Kalliomaki et al. [8] that showed a preventive effect of *L. rhamnosus* GG on eczema, the Kopp et al. study failed to demonstrate beneficial effects of probiotics. Host susceptibility to microbial influence and to colonization could be different in various populations. Functional genetic polymorphisms in related microbial recognition pathways may result in varied effects in individuals.

Table 1-5 Summary of clinical trials evaluating the role of probiotic supplementation in the primary prevention of atopic diseases

Study	No. of Subjects	Probiotic	Dose	Supplementation		Age of evaluation (years)	Effect of Probiotics on Clinical Endpoints		
				Prenatal	Postnatal		Eczema	Sensitization	Food Allergy
Kalliomäki et al. , Finland 2001 [8]	n=132	*Lactobacillus rhamnosus* GG (LGG)	1 x 10^{10} CFU daily	Yes	6 months	2	Reduced	No effect	Not evaluated
Kukkonen et al., Finland, 2007 [165]	n=925	Mixture of 4 strains with prebiotic galacto-oligosaccharides 1. LGG and 2. *L rhamnosus* LC705 3. *Bifidobacterium breve* Bb99 and 4. *Propionibacterium freudenreichii* ssp. *shermanii* JS	Both 5x 10^9 CFU twice daily Both 2 x 10^9 CFU twice daily	Yes	6 months	2	Reduced	No effect	No effect
Taylor et al., Australia, 2007 [167]	n=178	*L. acidophilus*	3 x 10^8 CFU daily	No	6 months	1	No effect	Increased rate of sensitization	No effect
Abrahamsson et al., Sweden, 2007 [169]	n=188	*L. reuteri*	1x 10^8 CFU daily	Yes	12 months	2	Reduced only in Ig-E associated atopic eczema	Reduced in children with atopic mothers	No effect

Table 1.5 Summary of clinical trials evaluating the role of probiotic supplementation in the primary prevention of atopic diseases (continued)

Study	No. of Subjects	Probiotic	Dose	Supplementation		Age of evaluation (years)	Effect of Probiotics on Clinical Endpoints		
				Prenatal	Postnatal		Eczema	Sensitization	Food Allergy
Kopp et al., Germany, 2008 [171]	n=94	LGG	5 x 10^9 CFU twice daily	Yes	6 months	2	No effect	No effect	Not evaluated
Wickens et al., New Zealand, 2008 [173]	n=474	(a) *L. rhamnosus* HN001 (b) *B.animalis* subsp *lactis* strain HN019	(a) 6 x 10^9 (b) 9 x 10^9 CFU daily	Yes	2 years	2	Reduced only in *L. rhamnosus* group	No effect	Not evaluated

1.4.4 Impact of probiotics on acute infectious illnesses

Probiotics may reduce the incidence of infections by stimulating humoral and cellular immunity. This immunostimulatory effect of probiotics had previously been shown to improved resistance to respiratory infections in infants attending day care in which *L. rhamnosus* GG supplemented children had fewer days of absence from day care because of illness (age adjusted 5.1 (4.6 to 5.6) vs. 5.7 (5.2 to 6.3) days, p=0.09) suggesting that probiotics may lessen the severity of respiratory infections. There was also a relative reduction of 17% in the number of children who suffered from respiratory infections (otitis media, sinusitis, bronchitis, and pneumonia) (age adjusted OR 0.75, 95% CI 0.52-1.09; p=0.13) and a 19% relative reduction in prescribed antibiotics for respiratory infections (adjusted OR 0.72, 95% CI 0.50-1.03; p=0.08) in the probiotic group [175]. Even though the age adjustment reduced the differences between the groups, the results were near to significance and consistently support the beneficial effects of *L. rhamnosus* GG. However, in another Israeli multicenter trial, *B. lactis* BB12 and *L. reuteri* were not found to protect against respiratory infections among children in day care. Nonetheless, the use of *L. reuteri* but not *B. lactis* BB12 was associated with significantly fewer days of fever, lesser visits to the clinic, lesser absences from the child care and fewer prescribed antibiotics in this study [176].

Data collected from probiotic clinical trials in the primary prevention of eczema also evaluated its effects on infections. In the study by Taylor et al., infants who received *L. acidophilus* postnatally for 6 months did not reduce the risk of atopic dermatitis or respiratory infections and were in fact more likely to be prescribed antibiotics (27.0%) compared to the placebo group (17.0%) [167]. Apart from this study, other studies continue to support the lower frequency of antibiotic use among infants in day care

who received probiotic-supplemented formula such as that of *B. lactis* and *S. thermophilus* [177]. Fewer antibiotic courses throughout the intervention of a mixture of 4 probiotic species by Kukkonen et al. [178] was also observed. Infants received lesser antibiotics in the synbiotic group (23%) than in the placebo group (28%) (OR 0.74, 95% CI 0.55–1.00; p=0.049). During the follow-up period to 2 years of age, respiratory infections occurred less frequently in the synbiotic group (93%) than in the placebo group (97%) (OR 0.49, 95% CI 0.27–0.92; p=0.023).

In this Kukkonen et al. study [178], the synbiotics supplementation failed to prevent episodes of gastroenteritis which was equally common in the synbiotic (74%) and placebo groups (71%) (p=0.736). In the Finnish study among children in day care, *L. rhamnosus* GG supplemented in milk also did not reduce incidence of gastroenteritis (2.9 (2.7 to 3.2) vs. 3.0 (2.7 to 3.3) days in placebo, p= 0.74) [175]. Similarly, the Australian study by Taylor et al. [167] did not observed reduction in gastrointestinal infections in the *L. acidophilus* supplemented group as well. Conversely, in the Rosenfeldt et al. study discussed earlier with regards to the effect of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 in the treatment of children with eczema [140], it was found that probiotics reduced gastrointestinal symptoms (39% during the placebo period vs. 10% during active treatment, p=0.002) and improved small intestinal permeability measured by the lactulose-mannitol test in this cross-over study [179]. Furthermore, stabilization of the intestinal barrier function was positively associated to the reduction in the severity of eczema.

1.5 Safety and adverse effects of probiotics

Probiotics have been regarded as Generally Recognized as Safe (GRAS) by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) expert panel guidelines for probiotic [56, 180]. However, probiotics are strain-specific and based on the U.S. Federal Food, Drug, and Cosmetic Act. [181], probiotics may be regulated as dietary supplements, foods, or drugs depending on the product's intended use. The report of the Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food recommended that it is necessary to know the genus and species of the probiotic strain to evaluate the functionality and safety.

Although there had been only 1 case of significant gastrointestinal effects associated with heat-inactivated *L. rhamnosus* GG [139] in the probiotics treatment and prevention studies evaluated above, there are potential adverse effects due to transmigration on localized and generalized immunologic [182], metabolic and physiologic systems [183]. Antibiotic-resistance transfer within the gastrointestinal tract between probiotics and pathogenic bacteria is also an area of concern [184]. But a few cases reported probiotic bacteremia or fungemia have occurred in immunocompromised patients or patients with underlying chronic disease. There had been 2 reports of *Lactobacillus* bacteremia related to probiotic use in premature infants with short gut syndrome who were fed via gastrostomy or jejunostomy after consumption of *L. rhamnosus* GG supplements [185, 186]. Another case of *L. rhamnosus* GG endocarditis was reported in a 4 month old infant who consumed 10^{10} CFU of *L. rhamnosus* GG daily for antibiotic-related diarrhoea after cardiac surgery [187]. No current reports have described serious adverse effects related to probiotic

use in healthy persons even though the recent widespread use of probiotics especially supplementation in infant formula may increase the prevalence of such problems. [188].

Probiotics may contribute to the host's energy metabolism and enhance the uptake of nutrients to increase nutritional status and improve physical growth. Normal healthy infants who received *L. rhamnosus* GG-supplemented formula for 6 months in a double-blind, randomized study grew to a significantly higher length and weight than the infants who received regular formula [189]. Other studies however observed similar normal growth in both probiotic-treated and placebo study groups and did not find improved growth with probiotics. In a study in the United States, growth was similarly adequate in 3-24 month old infants who received a standard milk-based formula containing *B. lactis* and *Streptococcus thermophilus* or unsupplemented formula.[177]. Another study in France concluded that infants fed a mixture of probiotics or synbiotics showed similar weight gain compared with those fed a control formula [190]. A recent study on extensively and partially hydrolyzed formulas supplemented with *L. rhamnosus* GG also supported normal growth in infants and indicated that probiotic is well tolerated and safe [191]. The study from Kukkonen et al. which evaluated the role of probiotic supplementation in the primary prevention of atopic diseases [165] also found that the anthropometric measures at the ages of 6 months and 2 years showed similar normal growth in the probioic-treated and placebo group [178].

1.6 Gaps in the literature and Aims of the study

Probiotic supplementation in early life is considered an attractive strategy for the primary prevention of allergic diseases. The eventual test of such a strategy lies in intervention studies in the form of clinical trials. Probiotics have the potential to be the ideal interventional strategy as they are safe, can be administered from birth, and can be conveniently added to milk. There were other published trials in the Western countries where the effect of probiotics on allergic diseases were studied. There is a critical need for further contribution of data to support or refute the current body of evidence on the prophylactic effect of probiotics in primary allergy prevention in large studies of other populations. Our study intended to fill this vacuum as well as add new information on the potential effects by examining the effect of probiotics on atopy and immunological responses in an Asian population through a large randomized clinical trial with a combination of two probiotic strains pre-mixed into the formula, as opposed to taking it separately. If proven beneficial, probiotics can be easily added to milk. This would be a major step in reducing the significant morbidity associated with atopic disease and translate into ideas for novel strategies in the primary prevention of allergic diseases in children. Additionally, the prospective nature of this study would enable evaluation of the natural history of individuals at high risk of atopic disease in Singapore. The prospective design of our study excluded recall bias and was the best way to study disease associations as information on exposures and confounders were measured in time. To our knowledge, this was the largest prospective study on Singaporean children at high-risk for atopy. It would thus yield valuable information on the natural history of atopy in Singapore children.

1. This first aim of the study was to assess the effect of administration of probiotics (*Bifidobacterium longum* and *Lactobacillus rhamnosus*) supplemented cow's milk based infant formula from the first day of life for 6 months on the prevention of allergic diseases, namely eczema, asthma, allergic rhinitis, and allergic sensitization in the first 2 years of life in Asian infants at risk of allergic disease. In addition, we correlated atopic outcome at 2 years of age with immune responses at birth and 1 year of age. The incidence of allergic diseases in a high risk cohort (placebo group) and the impact of covariates such as early life infections, family size, presence of pets and passive smoking and other environmental factors were ascertained.

Apart from allergies, prevention of childhood infections has long been recognised as an important target of global health. Infectious disease is the number one cause of mortality in children all over the world. Probiotics are safe and easily available. There is evidence that probiotics can modulate local and systemic immune responses, resulting in decrease in infectious disease, especially diarrhoeal disease [106-120], and increase efficacy to vaccination [86-91, 93-97]. Data is lacking in longitudinal studies with regards to prophylactic use of probiotics on other infections in children, of which viral infections predominate. There is also little information regarding the effect of probiotics on parenterally-administered vaccines especially for important infections in the region, such as Hepatitis B. Viral hepatitis is a common cause of liver disease in Asia. Hepatitis B is the most common form and the Ministry of Health, Singapore reported a prevalence of 4.1% in 1999 and 2.7% in 2005 amongst residents aged 18-69 years. Hepatitis B continues to constitute a major public health concern due to the considerable number of hepatitis B virus carriers in the population. The

national childhood immunisation programme has since been implemented in 1987 for all newborns and we hypothesize that probiotics may have an adjuvant effect resulting in increased immunogenicity with the vaccination, especially in atopic subjects. Delayed immune maturation in atopic infants has been proposed with a deficiency in the ability of T cells to produce Th1-like cytokines on stimulation [192] and exhibit reduced antibody responses to vaccination [193]. An increase in systemic immune response to vaccination would suggest that probiotics may be good vaccine adjuvants and could be exploited in future research, particularly for vaccine development of important infections in Singapore. Increasing the protective antibody response is an attractive strategy to provide long term protection against infection. We proposed that the efficacy of parenteral vaccines can possibly be improved with concomitant or prior use of the probiotics.

2. The second aim of the study was to assess the effect of probiotic supplementation in the first 6 months of life on specific antibody response against Hepatitis B at 1 year of life as a surrogate marker for infant immune response to vaccination.

3. The third aim of the study was to assess the effect of early regular supplementation of probiotics in the infant diet on protective benefit against diarrhoeal and febrile illnesses. The effect on the use of antibiotics and incidence of hospitalization was assessed. We further seek to determine if this effect was short term (6 months, during supplementation) or longer-lasting (2 years follow-up period).

As probiotics consumption are generally considered as safe and no current reports have described serious adverse effects related to probiotic use in healthy persons [188], probiotics are increasingly being used in infancy with more and more products available on the market with high dose of combinations of viable probiotics. Furthermore, probiotics are generally regulated as health supplements and not drugs. Thus the long term use of live probiotics in newborn babies should be continually evaluated with respect to different probiotic strains and combinations. Probiotic may also contribute to the host's energy metabolism and enhance the uptake of nutrients to increase nutritional status and improve physical growth.

4. The fourth aim of the study was to document safety and impact on growth of newborn infants in this study with a 2 year follow-up period.

Chapter 2: Materials and Methods

2.1 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 allergic diseases and effects on safety aspects in Asian infants at risk of allergic disease with a two year follow-up. We recruited 253 families with a history of allergic disease from the antenatal clinics at the National University Hospital, Singapore, between May 2004 to June 2006. Parents were approached and informed of the purpose and design of the trial. The benefits of breastfeeding were emphasized and only those who did not want to totally breastfeed their children were candidates for inclusion in the study.

2.2 Eligibility

2.2.1 Inclusion criteria

2.2.1.1 Pre-delivery evaluation

- Either parent or sibling (first-degree relative) with a history of physician-diagnosed asthma, allergic rhinitis or eczema and a positive skin prick test to the dust mites, *Dermatophagoides pteronyssinus* and/or *Blomia tropicalis*, which are the most common cause of inhalant allergen sensitization in our atopic population [194]
- Parents agreed to the subject's participation in the study as indicated by parent's signature on the informed consent form (refer to Appendix A).
- The parents were willing to comply with procedures and were able to keep to scheduled clinic visits.

2.2.1.2 Post-delivery evaluation

(refer to Screening Form, Appendix B)

- The subject was born at more than 35 weeks gestation and weighed more than 2 kilograms.
- The subject did not have major congenital malformations or major illness as judged by the doctor.
- The subject was in otherwise good, stable health on the basis of medical history, physical examination, and the family appeared to be able to successfully complete this trial on the basis of an interview.

2.2.2 Exclusion criteria

- The subject was excluded when the parent was assessed to be mentally or legally incapable of informed consent.
- The parents were unable or unwilling to comply with procedures.
- Parents who chose to breast-feed exclusively were not considered for the study.

2.3 Randomisation

Computerized randomization was carried out in blocks of 6 (each group having 3 codes) based on a 1:1 allocation, with the lowest number allocated sequentially as per prepared by the milk sponsor, Nestle Research Centre Switzerland. The identical tins of milk formula were labelled with unique trial numbers following the order of the randomisation list by an independent team to ensure concealment of allocation. Six sealed envelopes containing the identity of the milk formula were maintained by the Singapore Clinical Research Institute (previously known as Clinical Trial and Epidemiology Research Unit), Singapore. Investigators, study team and all subjects remained blinded throughout the study period.

2.4 Probiotic Supplement and Infant Formula

Subjects received at least 60ml (9.26 g) a day of commercially available cow's milk based infant formula (Nestle Nan 1®), with either probiotic supplementation (*Bifidobacterium longum* BL999 (ATCC: BAA-999 designation BB536, Morinaga, Japan) 1×10^7 CFU/g and *Lactobacillus rhamnosus* LPR [CGMCC 1.3724] 2×10^7 CFU/g) or without, initiated within 12 hours for the first 6 months of life. The infants in the probiotic group, therefore, received at least $2.8x10^8$ 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 [180].

Compliance was monitored by completion of a daily diary chart (Appendix C) by parents and biweekly phone contacts (Appendix D) with the study nurses for the first 6 months. Non-compliance was defined as consumption of less than 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 probiotic supplemented and control formula were not hydrolyzed 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 to 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 (refer to Weaning Practices Form, Appendix B).

2.5 Ethical Considerations

Written informed consent was obtained from all families pre-delivery. The parent's written informed consent to participate in the trial was obtained after a full explanation had been given of the treatment options, including the conventional and generally accepted methods of treatment and the manner of treatment allocation. After the delivery, subject was evaluated for remaining eligibility criteria and randomized if all were met. The right of the parents to refuse the infant's participation without giving reasons was respected. Similarly, the parents remained free to withdraw at any time from protocol treatment without giving reasons and without prejudicing the subject's further treatment. The study was approved by the National University Hospital's ethics review committee (DSRB Ref Code: B/00/322). This trial was conducted in accordance to the principles of Good Clinical Practice (GCP) and complied with the requirements of the Singapore Guidelines for GCP. The administration of probiotics in standard infant cow's milk formula is discussed in Section 3.4 Discussion, page 95.

Chapter 3: Effects of Probiotic Supplementation on Allergic Diseases and Allergen Sensitization at 2 Years of Age

3.1 Introduction

Probiotic bacteria promote immunoregulatory functions and present as a promising strategy in primary prevention of allergy. Probiotic supplementation has been examined in several double-blind, placebo-controlled randomized clinical trials [8, 163-167, 169, 171, 173] to examine the effect of probiotic supplementation in primary allergy prevention on clinical subjects. However, despite the rigorous testing of this concept on clinical subjects, 4 recent meta-analyses of the published clinical trials have concluded that the role of probiotics in allergy prevention either remains inconclusive due to varied study designs and results [170], or has some protective effect on eczema alone [160]. No beneficial effects on allergen sensitisation and respiratory allergies has been observed in the studies conducted, except for Abrahamsson et al. study [169] which showed an effect of reduced sensitisation in infants with atopic mothers. Other recent studies noted negative effects on allergen sensitisation and wheezing [167, 171]. Generally, the meta-analyses concluded that the concept of using probiotics for primary prevention of allergy is likely, but further studies are needed to evaluate specific probiotic strains, the timing, dose and method of administration to determine the plausible beneficial effects [159, 172].

This study assessed the effect of administration of probiotic (*Bifidobacterium longum* and *Lactobacillus rhamnosus*) supplemented cow's milk based infant formula from

the first day of life for 6 months on the prevention of allergic diseases and sensitization in the first 2 years of life in Asian infants at risk of allergic disease.

3.2 Materials and Methods

3.2.1 Clinical Assessment

The primary clinical outcome measure was the incidence of eczema, and the secondary outcome measures were asthma, allergic rhinitis and allergen sensitization. Infants were evaluated by a paediatrician at 1, 3, 6, 12 and 24 months of age, which involved a detailed history, recording of anthropometric data and clinical examination, including looking for the presence of allergic diseases. Questionnaires (not validated) were also administered by the candidate and research nurses at these visits to record clinical disease and environmental exposures, including day care, sibship, use of antibiotics, smoking and pets (refer to Follow-up Form, Appendix B). Biweekly phone calls were performed for the first 6 months after which monthly phone contacts were done to collect data on the health status of the children (Appendix D).

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 et al. for infants [16]. The SCORAD (SCORing Atopic Dermatitis) index was used to objectively score the severity of atopic dermatitis [136], which were carried out by the paediatricians. Briefly, the doctors applied SCORAD to compare the patient's lesions to standard colour slides and graded each of the six objective intensity items, namely erythema, oedema/papulation, excoriations, lichenification, oozing/crusts and dryness, on a scale from 0-3. The two subjective intensity items, pruritus and insomnia, were graded on a visual analogue scale from 0-10 by the subject's parents or care-givers.

The extent of the disease was determined by grading skin involvement on different parts of the body. The results from the three parameters were then introduced into a weighted mathematical formula to calculate the final results (Refer to Appendix E).

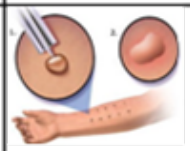
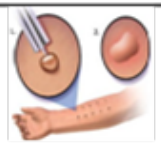
Asthma will be diagnosed if the child had three episodes of nocturnal cough with sleep disturbances or wheezing, separated by at least seven days, in a setting where asthma was likely and conditions other than allergy have been excluded [17]. The subject will be diagnosed with allergic rhinitis if the child had rhinorrhea, nasal obstruction, nasal itching and sneezing which were reversible spontaneously or with treatment that was not due to a respiratory infection as per recommendations from the World Health Organization (WHO) Allergic Rhinitis and its Impact on Asthma workshop (ARIA) [19].

3.2.2 Determination of serum total immunoglobulin E and skin prick tests

Serum samples were collected from cord blood and about 5 ml of blood was drawn (to obtain 2.5 ml of plasma) using EMLA® as an anaesthetic at Month 12. The serum/plasma samples were stored at -70°C till assayed. Measurement of total IgE was performed by the candidate using the fluoroenzymeimmunoassay method (UniCAP® Phadiatop, Pharmacia Diagnostics, Uppsala, Sweden), with a detection limit of 0.35kU/L. Pharmacia Diagnostics indicated that this method is precise and accurate with intra-assay precision (coefficients of variation between 1.4% and 3.3%) and inter-assay precision (coefficients of variation between 3.5% and 11.0%).

Skin prick test was performed by the trained candidate and research nurses at 12 and 24 months of age using standardized technique with common allergen extracts, including soy (Alyostal, Stallergenes Laboratoires, France), milk, egg yolk, egg white, dust mite allergens - *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC) and *Blomia tropicalis* (manufactured in-house [195]). Peanut and shrimp allergens (Greer Laboratories, Lenoir, NC) were added to the panel at 24 months old skin prick test. Histamine dihydrochloride solution (10mg/ml) was used as a positive control and solvent (50% Cocas 50% Gly) as a negative control. A wheal greater than 3mm in diameter above the negative control was considered positive [196]. The study procedures are summarized in Figure 3.1.

Figure 3-1 Study Procedures

Procedures	Birth	Within 72 hrs	1 mth	3 mths	6 mths	12 mths	24 mths
Consumption of milk formula	Within 12 hrs	At least 60ml/day					
Clinical examination + Questionnaire							
Blood collection	 Cord blood						
Skin Prick Test							

3.2.3 Sample size calculation

In this study, the null hypothesis, H_0, represented no difference between the probiotic and placebo group while the alternative hypothesis, H_1, specified a beneficial effect of probiotic supplementation in primary allergy prevention as compared to control. In this hypothesis test, a type I error can occur if the null hypothesis was rejected while it was in fact true and therefore the probability of the type I error was set at a significance level of 0.05. The null hypothesis was to be rejected with the difference happening due to 5% chance. A type II error can occur when the null hypothesis H_0, was not rejected when it was actually false. This type II error was controlled by the power of the study (1 - probability of a type II error). The sample size of this study depended on the size of the difference to be detected between the 2 groups, the power and the level of significance. A two-sided test was carried out rather than a one-sided test which assumed that probiotic intervention will performed clinically better than the control [197-199].

In conclusion, the sample size was calculated based on the study of Kalliomaki et al. in 2001 [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 that the incidence of eczema to be approximately 40%, and that to detect a relative reduction of 50%, with a power of 90% and two-sided test size of 5%, 110 subjects were required in each group [200]. This sample size was sufficient to evaluate the outcomes at 2 years of age. Efforts were made to ensure a low dropout rate.

3.2.4 Statistical Analysis

All statistical analysis was carried out on an intention-to-treat last observation carried forward (ITT-LOCF) basis. Intention to treat strategy included all randomized subjects in the groups according to original treatment assignment, regardless of whether they satisfied the entry criteria, non-adherence with the treatment allocated, and subsequent withdrawal or deviation from the protocol. Noncompliant subjects were included in the ITT analysis, as in clinical practice, some patients are not fully compliant [201-203]. Last observation carried forward approach used the last observation prior to drop-out to impute the outcome values and thus reduced the effects of lost to follow up subjects [204, 205].

The trial data were collected on printed forms (Appendix B), and subsequently entered into CLINTRIAL [197], a specialized software for managing longitudinal trial data. This program facilitates interactive entry and data correction, and maintains consistent and accurate trial data [206]. Atopy was defined as present when the subject had a positive skin prick test, indicating allergen sensitization. Comparison of the incidence rates of allergic diseases and atopy in the two treatment groups was made using Pearson Chi-square test or Fisher's Exact test to test for independence and determine if there were statistically significant relationships between the categorical variables [207]. It tested the null hypothesis that the frequency distribution of the incidence rates of allergic diseases and atopy observed in this sample of subjects was consistent with the expected as according to the chi-square distribution. The Fisher's Exact test was calculated in the case of a 2×2 contingency table with at least one expected cell count less than 5. Using the Fisher's Exact test, the significance of the deviation from the null hypothesis was calculated exactly and does not rely on an

approximation to the chi-square distribution that assumed a sufficiently large sample size [208, 209].

Logistic regression was performed to determine the odds ratio (OR), the associated 95% confidence interval (CI) and adjust for relevant covariates of the categorical dichotomous outcomes of allergic diseases and atopy (occurrence or non-occurrence of outcome event) [210]. Binary logistic regression is a generalized linear model which is used when the dependents (i.e. allergic disease/atopy) are dichotomous and the independents (i.e. covariates) are continuous or categorical variables. The prediction of the probability of occurrence of an event is performed by fitting data to a logistic curve. The odds ratio is defined as the probability of the outcome event occurring divided by the probability of the event not occurring. The 95% confidence interval for the odds ratio is obtained as 1.96 standard errors on either side of the estimate. The change in value of the independent variable is not associated in change in the odds of the dependent variable if the 95% confidence interval around the odds ratio includes the value of 1.0 [211, 212].

Due to the non-normality of the data, Mann Whitney U test was performed to assess the differences between groups. Mann Whitney U test is the nonparametric equivalent of Student's T test and therefore compares medians instead of means. Normal distribution of data is not necessary for use of the Mann Whitney U test [213]. All statistical analyses were performed by using SAS v.9.1 and SPSS software (version 15.0 for Windows). Reporting of this trial was done in accordance to the CONSORT (Consolidated Standards of Reporting Trials) statement [214]. The CONSORT statement is developed by a group of scientists and editors to improve the quality of

reporting randomised clinical trials by providing guidance through checklist and flow diagram [215]. A p value of < 0.05 was considered statistically significant for all analyses.

3.3 Results

3.3.1 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 5 subjects in the placebo group withdrew from the study before any follow-up was conducted and were therefore excluded from the analysis. At the 24 month visit, there were 124 families in the probiotic arm and 121 families in the placebo arm (Figure 3.2). Twin pregnancies were included in the study. The ethnicity, gestational age, mode of delivery, birth weight, family atopic history and other baseline characteristics of the 2 groups were comparable (Table 3.1 and Table 3.2). The median gestational age of all subjects was 39.0 weeks (range: 34.0 to 41.9 weeks). Fifty three percent of them were males and there was a slight imbalance between the groups with more males in the placebo group. The overall racial composition was 44% Chinese, 46% Malays, 10% Indians and 1% Others. There were slightly more first child with 47% in the probiotic group compared to 33% in the placebo group. Imbalances seen with gender and birth order between the 2 groups were included as potential confounding factors in subsequent analyses with adjustment.

Figure 3-2 Flow chart showing progress of participants through the trial.

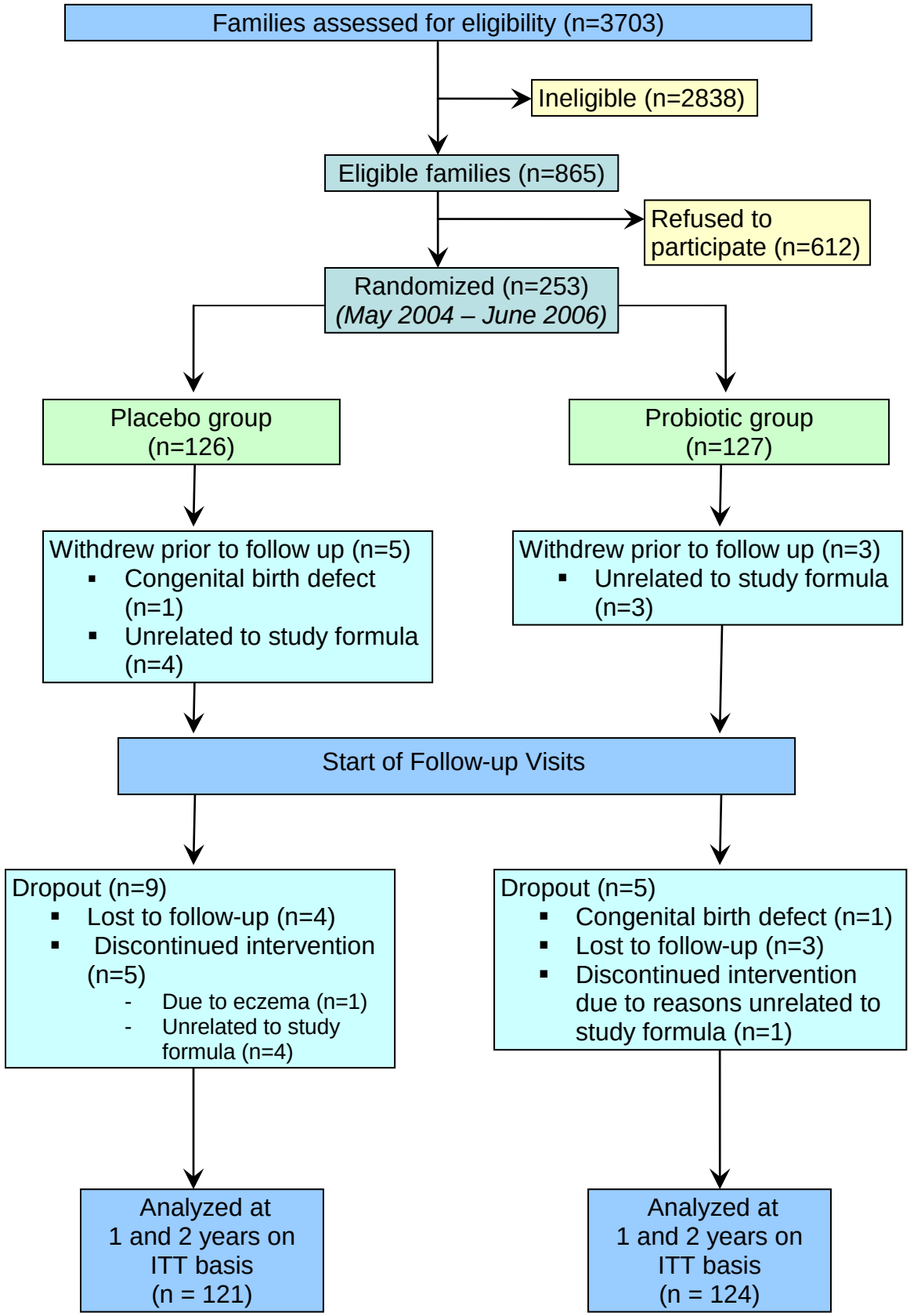


Table 3-1 Characteristics of the Study Population

	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)
Length at Birth (cm)		
Mean (SD)	49.8 (2.8)	49.6 (2.3)
Head circumference at Birth (cm) *		
Mean (SD)	33.4 (2.0)	33.3 (1.4)
Birth Order (%)		
1	41 (33)	59 (47)
2	44 (35)	24 (19)
3	24 (19)	32 (25)
≥4	17 (14)	12 (10)

* Head circumference not measured in one of the subjects in the placebo group

Table 3-2 Family history of allergic diseases

	Placebo n = 126 (%)	**Probiotic n =127 (%)**
Family history of asthma	51 (41)	59 (47)
Father	*19*	*14*
Mother	*22*	*38*
Siblings	*16*	*12*
Family history of allergic rhinitis	87 (69)	92 (72)
Father	*38*	*38*
Mother	*53*	*62*
Siblings	*19*	*9*
Family history of eczema	41 (33)	39 (31)
Father	*13*	*13*
Mother	*20*	*15*
Siblings	*18*	*15*
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

The total monthly family incomes, parents' highest level of education and housing types of the 2 groups were similar (Table 3.3). A total of 151 (60%) families had total monthly income less than the 2nd quartile of $3999 (59% in placebo and 61% in probiotic group). About half of either parent had completed tertiary education (55% in placebo and 57% in probiotic group) and most (87%) of the families are staying in public housing (88% in placebo and 85% in probiotic group).

Table 3-3 Parents' Particulars

	Placebo n = 126 (%)	**Probiotic n =127 (%)**
Total monthly family income		
1st quartile : Below $2000	36 (29)	38 (30)
2nd quartile : $2000-$3999	38 (30)	39 (31)
3rd quartile : $4000-$5999	22 (17)	23 (18)
4th quartile : More than $6000	30 (24)	27 (21)
Father's highest level of education completed		
Primary	8 (6)	8 (6)
Secondary	57 (45)	51(40)
Tertiary	61 (49)	68 (54)
Mother's highest level of education completed		
Primary	11 (9)	5 (4)
Secondary	56 (44)	63 (50)
Tertiary	59 (47)	59 (46)
Type of housing		
Public housing	111 (88)	108 (85)
Private apartments (Condominium)	10 (8)	15 (12)
Landed property	5 (4)	4 (3)

The post-natal histories of the 2 groups were also similar (Table 3.4). Two subjects in the probiotic group were admitted to neonatal intensive care unit (ICU). One of the 3 subjects in the placebo group who was admitted to special care nursery and 3 other subjects (2 placebo and 1 probiotic subjects) who were admitted to the post-natal ward were given antibiotics. Post-natal complications were reported for 16 (6%) subjects (7 in placebo group: mild aspiration, 2 infants of group B streptococcus colonised mothers, pethidine-induced neonatal depression, small for gestational age, suspected sepsis/viral pneumonia/jaundice and swallowed blood syndrome; 9 in probiotic group: Unknown infection, ABO incompatibility, rhesus positive, hypoglycaemic, transitory tachypnea of newborn, insulin-dependent diabetes mellitus, pre-term, respiratory depression and shallow breathing at delivery. As these post-natal complications resolved quickly, these subjects were included into the study except for one who was withdrawn by the investigator as the mother was found to be a drug abuser and deemed unsuitable for the study. A pre-term baby of 34 weeks gestation age was inadvertently included in the study and followed up on an intention to treat basis although the child did not meet eligibility criteria of gestational age above 35 weeks. Most of the abnormalities reported at birth were minor, such as soft systolic murmur, G6PD deficiency and haemangioma over the face, and were not deemed to interfere with the study except for 1 subject diagnosed with Fallot's tetralogy in the placebo group and 1 subject with congenital liver disease in the probiotic group who were excluded from the study (Figure 3.2).

Table 3-4 Subjects' Post-Natal History

	Placebo n = 126 (%)	**Probiotic n =127 (%)**
Admission		
Post-natal ward	123 (98)	119 (94)
Special Care Nursery	3 (2)	6 (5)
Neonatal ICU	0 (0)	2 (1)
Use of antibiotics		
Yes	3 (2)	1 (1)
No	123 (98)	126 (99)
Post-natal complications		
Yes	7 (6)	9 (7)
No	119 (94)	118 (93)
Abnormality		
Heart	4 (3)	4 (3)
Respiratory	0 (0)	0 (0)
Abdominal	1 (1)	1 (1)
Neurologic	1 (1)	0 (0)
Others *	13 (10)	11 (9)

* Others include cephalohaematoma, congenital dislocation of the hips, haemangioma, erythema toxicum neonatorum, supernumerary nipple, acrocyanosis, caput succedaneum, undescended testicles.

3.3.2 Feeding history

The compliance level of consuming at least 60ml 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.

At the end of the 6 months supplementation period, only 2% in the placebo and 3% in the probiotic group had near total breastfeeding with at least 60ml of trial formula. Majority of the subjects had some breastfeeding combined with formula feeding (77% in placebo and 65% in probiotic group). The details of the feeding history are shown in Table 3.5.

All subjects had been weaned by 12 months and the median age of weaning to semi-solids was 6 months for both groups (Table 3.6). All but one or two subjects in each group had egg yolk, egg white, fish and soy products while only about 87% in the placebo group and 83% in the probiotics group took peanuts by 2 years of age.

Table 3-5 Feeding history

	Placebo n = 121 (%)	**Probiotic n =124 (%)**
Feeding history- Birth to Month 6 *		
Near total breastfeeding with at least 60ml of trial formula for 6 months	2 (2)	4 (3)
Any breastfeeding	93 (77)	81 (65)
Total formula	26 (21)	39 (32)
Feeding status †		
Month 1		
Near total breastfeeding with ≥ 60ml of formula	22 (18)	22 (18)
Breastfeeding and formula feeding	70 (58)	60 (48)
Total formula feeding	29 (24)	42 (34)
Month 3		
Near total breastfeeding with ≥ 60ml of formula	11 (9)	15 (12)
Breastfeeding and formula feeding	44 (36)	36 (29)
Total formula feeding	63 (52)	72 (59)
Month 6		
Total breastfeeding with at least 60ml	6 (5)	10 (8)
Breastfeeding and formula feeding	24 (21)	24 (20)
Total formula feeding	85 (74)	88 (72)
Month 12		
Total breast feeding	0 (0)	2 (2)
Breastfeeding and formula feeding	10 (9)	10 (8)
Total formula feeding	103 (91)	110 (90)
Month 24		
Breastfeeding and formula feeding	5 (4)	5 (4)
Total formula feeding	107 (96)	114 (96)

* Five subjects in the placebo group and 3 subjects in the probiotic group withdrew from study before follow-up and were excluded from analysis

† Four subjects (3 placebo, 1 probiotic) not assessed at Month 3 & 6 & 12; 4 subjects (3 placebo, 1 probiotic) not assessed at Month 6 & 12; 2 placebo group subjects not assessed at Month 12 and a further 4 subjects (1 placebo, 3 probiotic) not assessed at Month 24 due to withdrawal of consent/withdrawal by investigator/lost to follow up

Table 3-6 Weaning Practices

	Placebo n = 114	**Probiotic n =122**
Weaned (%) †	114 (100)	122 (100)
< 4 months	*2 (1.8)*	*5 (4.1)*
4 - 6 months	*107 (93.9)*	*105 (86.1)*
> 6 - 12 months	*5 (4.3)*	*12 (9.8)*
Age at weaning (months)		
Mean (SD)	*5.6 (0.9)*	*5.7 (1.2)*
Median (Range)	*6.0 (3.0 to 9.0)*	*6.0 (1.0 to 12)*
Taken egg yolk	113 (99)	120 (98)
Age at taking egg yolk (months)		
Mean (SD)	*9.7 (3.1)*	*9.5 (3.4)*
Median (Range)	*10 (4.0 to 21.0)*	*9.0 (5.0 to 23.0)*
Taken egg white	113 (99)	120 (98)
Age at taking egg white (months)		
Mean (SD)	*10.6 (3.2)*	*10.2 (3.3)*
Median (Range)	*11.0 (4.0 to 21.0)*	*10.0 (6.0 to 23.0)*
Taken fish	112 (98)	120 (98)
Age at taking fish (months)		
Mean (SD)	*7.6 (2.6)*	*7.8 (2.7)*
Median (Range)	*7.0 (4.0 to 22.0)*	*7.0 (4.0 to 18.0)*
Taken soy products	113 (99)	119 (98)
Age at taking soy products (months)		
Mean (SD)	*9.4 (3.6)*	*9.7 (3.4)*
Median (Range)	*9.0 (3.0 to 24.0)*	*9.0 (3.0 to 24.0)*
Taken peanuts	99 (87)	101 (83)
Age at taking peanuts (months)		
Mean (SD)	*15.0 (4.5)*	*15.0 (4.1)*
Median (Range)	*15.0 (6.0 to 24.0)*	*14.0 (6.0 to 24.0)*

† 17 subjects (12 placebo and 5 probiotic) not assessed due to withdrawal of consent/withdrawal by investigator/lost to follow up

3.3.3 Effects of Probiotic Supplementation on Eczema and Allergen Sensitization in Interim Analysis at the Age of 1 Year

At 1 year of age, interim analysis was performed and the incidence of eczema in the probiotic (n = 27/124; 22%) group was similar to that in placebo (n = 30/121; 25%) [adjusted OR (ORadj) = 0.82; 95% CI = 0.44 to 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 (p=0.17).

Rate of sensitization to common allergens (probiotic = 24.2% vs placebo = 19.0%, ORadj = 1.43; 95% CI = 0.76 to 2.70) showed no difference (Table 3.7). Subjects in the probiotic group had slightly higher rate of sensitization to dietary (5.6% vs. 5.0% in the placebo) and inhalant (19.5% vs. 16.5% in the placebo) allergens compared to subjects in the placebo group. The 1 year old serum total IgE geometric mean (95% CI) was 18.76 (12.54 to 24.98) kU/L in the probiotic group and 23.13 (16.01 to 30.24) kU/L in the placebo (p=0.15).

Atopic eczema (with sensitization) in the probiotic (7.3%) group was similar to that in placebo (5.8%) (ORadj = 1.08; 95% CI= 0.44 to 2.65).

3.3.4 Effects of Probiotic Supplementation on Eczema and Allergen Sensitization at 2 Years of Age

At 2 years of age, the cumulative incidence of eczema in the probiotic (n = 27/124; 22%) group was similar to that in placebo (n = 32/121; 26%) (ORadj = 0.73; 95% CI = 0.39 to 1.34). The proportions of children without eczema by the age of 2 years are similar in the 2 groups (p = 0.38 by log-rank test) presented as Kaplan-Meier curves in Figure 3.4. In subjects with eczema, the median most severe SCORAD score by 24 months was 17.70 in the probiotic group and 17.40 in the placebo (p=0.307) (Table 3.8). Atopic eczema (with sensitization) in the probiotic (n=9/118; 7.6%) group was not significantly different from that of placebo group (n=13/111; 11.7%) (ORadj = 0.53; 95% CI= 0.20 to 1.38).

Rate of sensitization to common allergens at 2 years of age was not significantly different between subjects in probiotic (18.6%) and placebo (18.9%) group (ORadj = 0.92; 95% CI = 0.46 to 1.84) (Table 3.7). In contrast to the rate of sensitisation in Year 1, the subjects in the probiotic group had slightly lower rate of sensitization to dietary (3.4% vs. 4.5% in the placebo) and inhalant (16.9% vs. 17.1% in the placebo) allergens compared to those among subjects in the placebo group at 2 years of age. Peanut and shrimp allergens were added to the skin prick test panel at 2 years old. In the probiotic group, 2 subjects had skin prick test reactivity to both peanut and shrimp while 1 subject was sensitized to only shrimp. In the placebo group, only 1 subject was sensitized to peanuts and no shrimp sensitisation was observed.

Longitudinal changes in skin-prick test reactivity over the 1 year period were observed and most distinctively represented by the number of subjects sensitized to

dust mite allergens *Blomia tropicalis* who decreased by about three-fold from 1 year to 2 years of age in both groups (Figure 3.3). Among the 82 subjects (37 in placebo, 45 in probiotic group) who had positive skin prick test at either month 12 or month 24 visit, only 14 subjects (7 in each group) were sensitized to any allergens at both visits. Nineteen subjects (10 in placebo, 9 in probiotic) ever had positive test for dietary allergens and only 3 (1 in placebo, 2 in probiotic) were found to be sensitized to dietary allergens at both visits. Seventy-five subjects (33 placebo and 42 probiotic) ever had positive test for inhalant allergens and only 8 (6 placebo and 2 probiotic) had skin prick test reactivity at both visits. The differences in the rate of sensitization to any allergens between two groups remained insignificant after adjusting for gender, birth order, prenatal smoking exposure and feeding history.

Amongst the 18 subjects (9 in placebo and probiotic groups each) sensitized to dietary allergens at 1 and 2 years of age (Table 3.7), only 2 subjects in the placebo and 1 subject in the probiotic group were observed to manifest symptoms of food allergy. The subject in the probiotic group was found to be sensitized and allergic to egg, peanut, fish and shellfish in the 2 years follow-up period. In the placebo group, 1 subject was allergic to egg white but was outgrown by 2 years old. Another subject in the placebo group was found to be sensitized and allergic to peanuts by 2 years old.

Table 3-7 Sensitization characteristics of study subjects at 1 and 2 years of age

	1 year old		2 year old [# †]	
	Placebo (n = 121)	**Probiotic (n =124)***	**Placebo (n = 112)**	**Probiotic (n =119)**
Allergen Sensitization (%)				
Positive skin prick test (any)	23 (19.0)	30 (24.2)	21 (18.9)	22 (18.6)
Dietary allergens (any)	6 (5.0) **	7 (5.6)	5 (4.5)	4 (3.4)
Cow's milk	*0*	*0*	*0*	*1*
Egg white	*6*	*6*	*3*	*2*
Egg yolk	*4*	*4*	*0*	*1*
Soy	*0*	*0*	*1*	*0*
Peanut	*Not done*	*Not done*	*1*	*2*
Shrimp	*Not done*	*Not done*	*0*	*3*
Inhalant allergens (any)	20 (16.5)	24 (19.5)	19 (17.1)	20 (16.9)
Dermatophagoides pteronyssinus	*17*	*20*	*19*	*18*
Blomia tropicalis	*18*	*19*	*6*	*5*

*Skin prick test not performed for 1 subject in probiotic group

** One subject in placebo group not assessed for dietary allergens

[#] 22 subjects (14 placebo, 7 probiotic) not assessed due to withdrawal of consent / withdrawal by investigator / lost to follow up were excluded

† 1 subject in placebo and 1 subject in probiotic group not assessed due to refusal.

Figure 3-3 Longitudinal changes in skin prick test reactivity at 1 and 2 years old

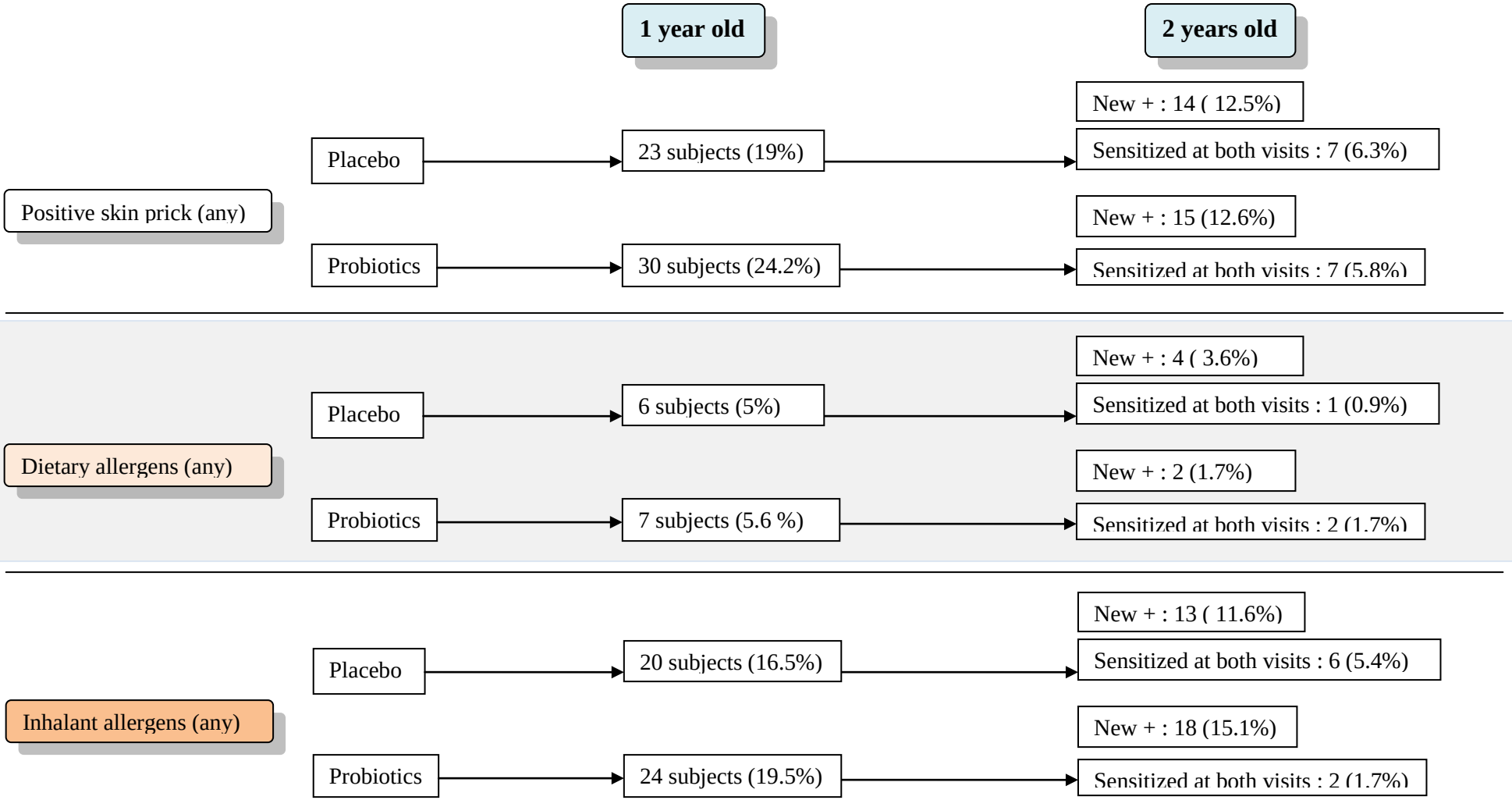


Figure 3-4 Kaplan Meier curves for children without eczema in the probiotic and placebo groups

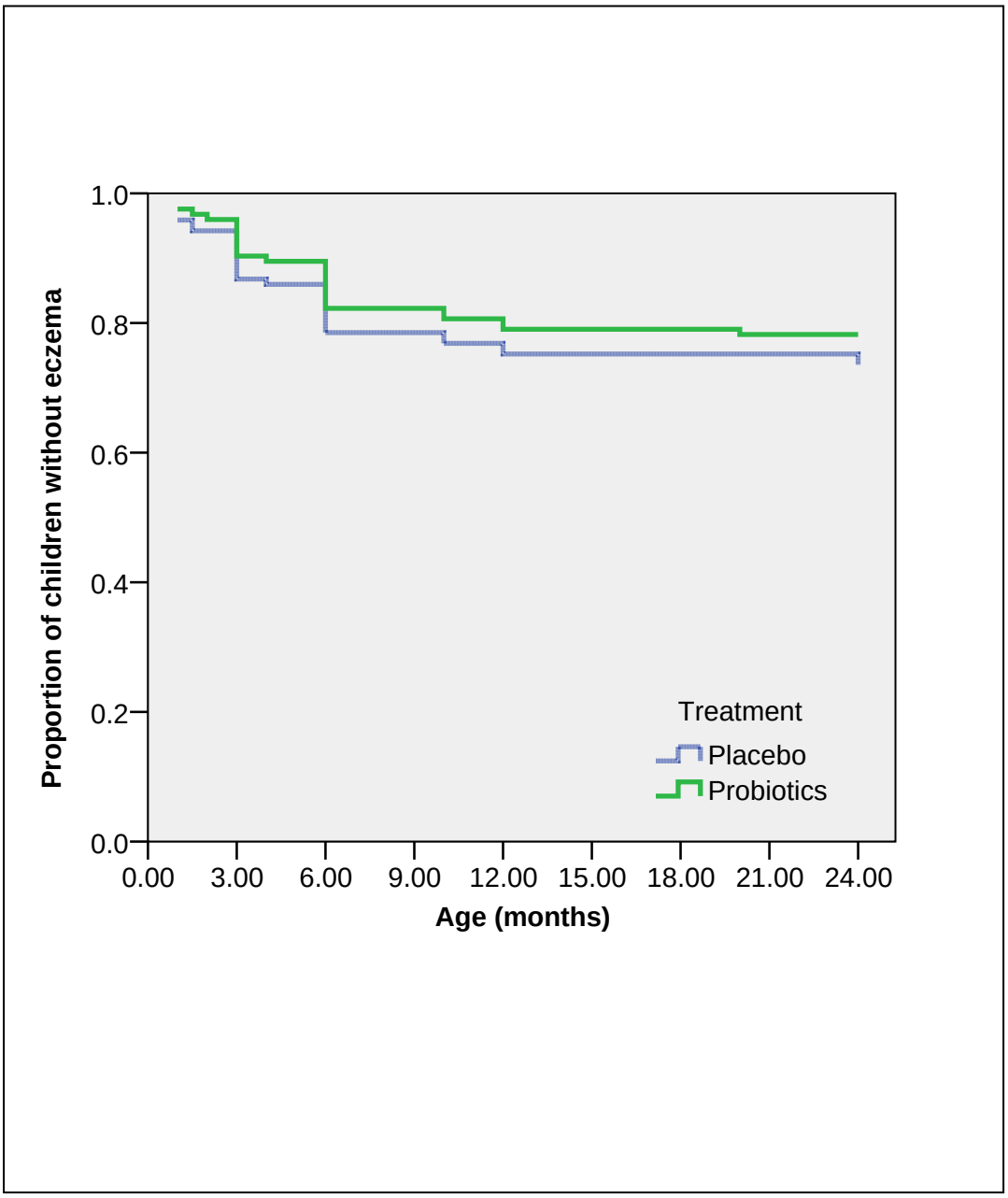


Table 3-8 Details of subjects with eczema by 2 years of age

	Placebo (n = 121)	**Probiotic (n =124)**
Ever eczema by 24 mo (%)*		
Yes	32 (26.45)	27 (21.77)
No	89 (73.55)	97 (78.23)
For those with eczema:		
Age at first diagnosis (months)		
Mean (SD)	5.78 (5.68)	5.54 (4.26)
Median (Range)	3.5 (1 to 24)	6 (1 to 20)
Most severe SCORAD by 24 mo, n †	31	25
Mean (SD)	18.79 (13.50)	20.45 (9.74)
Median (Range)	17.40 (3.9 to 75.0)	17.70 (7.9 to 43.4)

* 8 subjects (5 placebo, 3 probiotic) without any clinical assessment after randomisation due to withdrawal of consent / withdrawal by investigator / lost to follow up were excluded

† 2 subjects (1 placebo, 1 probiotic) were first diagnosed of eczema during external clinic visit and hence no SCORAD was captured. One subject in probiotic group with SCORAD not recorded.

3.3.5 Assessment of confounding factors

Apart from the imbalances in gender and birth order between the probiotic and placebo group, differences were also noted for prenatal smoking exposure and feeding history from the list of confounding factors recorded (Table 3.9). 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.

Table 3-9 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)
Household Passive Smoke exposure by Month 6 (%) †	50 (41)	48 (39)
Keep pets (%)	27 (21)	31 (24)
Dogs/Cats	17 (13.5)	17 (13.4)
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 60ml 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 (%) †	2 (2)	2 (2)
Day care attendance by Month 24 (%) †	21 (17.4)	23 (18.5)

† Five subjects in the placebo group and 3 subjects in the probiotic group withdrew from study before follow-up and were excluded from analysis

3.3.6 Family History and Predictive Capacity of Elevated Cord Blood Total IgE Associated with Eczema and Sensitization at 1 Year of Age

Elevated cord blood total IgE (≥ 0.5 kU/L) was observed in 107 subjects out of 215 (49.8%) infants, whose cord blood was collected. But high cord blood total IgE was not found to be a risk factor for development of eczema (ORadj = 1.05; 95% CI = 0.53 to 2.10) and allergen sensitization (ORadj = 1.47; 95% CI = 0.68 to 3.11) at 1 year of age. Elevated cord blood IgE values were cut off at the level of 0.5 kU/L according to the previous reference cut off value [238, 239].

Maternal atopy and mother with history of eczema were not found to be a risk factor for eczema and allergen sensitization at 1 year of age. Paternal eczema was instead significantly associated. Although paternal atopy was not found to be a risk factor but subjects with paternal history of eczema were 3.02 times more likely to develop eczema and 2.79 times more likely to be sensitized to allergens. Adjusted analyses accounting for gender, ethnicity, birth order, smoking, mode of delivery, housing type, parents' education, pets at birth, treatment and feeding history were made. These risk factors are summarized in Table 3.10.

Table 3-10 Evaluation of risk factors associated with eczema and sensitization at 1 year of age

Risk factor	**Eczema** ORadj (95% CI)	**Allergen Sensitisation** ORadj (95% CI)
Cord blood IgE ≥ 0.5 kU/L † (n=107)	1.05 (0.53 – 2.10)	1.47 (0.68 – 3.11)
Maternal Atopy (Eczema/Asthma/AR) (n=159)	0.77 (0.38 – 1.54)	0.86 (0.41 – 1.80)
Mother with Eczema (n=32)	0.85 (0.33 – 2.19)	0.79 (0.28 – 2.22)
Paternal Atopy (Eczema/Asthma/AR) (n=102)	1.38 (0.71 – 2.66)	0.61 (0.30 – 1.26)
Father with Eczema (n=26)	**3.02 (1.18 – 7.76)**	**2.79 (1.02 – 7.66)**

*Adjusted with gender, race, birth order, smoking, mode of delivery, housing type, parents' education, pets at birth, treatment and feeding history.

† Cord blood not collected from 30 subjects

3.3.7 Subset analysis at 2 years of age

3.3.7.1 Mode of delivery

The incidence of eczema at 2 years of age in caesarean-delivered children supplemented with probiotics was lower in the probiotic (n = 5/34; 14.7%) group as compared to that in placebo group (n = 8/32; 25%) although this difference did not reach statistical significance (ORadj = 0.33; 95% CI = 0.07 to 1.42, p=0.135). The rate of allergen sensitisation was similar in this subset of children with 12.5% (n=4/32) sensitized to any allergens in the probiotics group and 13.8% (n=4/29) sensitized in the placebo group (ORadj = 0.62; 95% CI = 0.10 to 3.68). Atopic eczema (with sensitization) in the probiotic (9.4%, n=3/32) group was also found to be similar to that in placebo group (10.3%, n=3/29) (ORadj = 0.58; 95% CI= 0.07 to 4.48) in these caesarean-delivered children.

In the normal vaginal delivered infants, the incidence of eczema in the probiotics group (n= 22/90; 24.4%) was similar to that in the placebo group (n= 24/89; 27.0%) at 2 years of age (ORadj = 0.80; 95% CI = 0.39 to 1.64). There was no significant difference in the rates of sensitization between the probiotic (n = 18/86; 20.9%) and placebo group (n= 17/82; 20.7%) in this subset of infants (p = 0.804). Vaginally delivered subjects with atopic eczema (with sensitization) in the probiotic (n=6/86; 7.0%) group was less than that in the placebo (n=10/82; 10.2%) although this difference was not found to be significant (ORadj = 0.37; 95% CI= 0.11 to 1.20, p=0.099).

3.3.7.2 Maternal Atopy

Similarly, the incidence of eczema in a subset of children with atopic mothers was not statistically different in the probiotic (n = 19/85; 22.4%) group as compared to that in placebo (n = 17/74; 23.0%) (ORadj = 0.88; 95% CI = 0.41 to 1.92). The rate of allergen sensitisation in this subset of children in the probiotics group (n=11/81; 13.6%) was lower than that in the placebo (n=14/65; 21.5%) group but not significantly different (ORadj = 0.53; 95% CI = 0.21 to 1.33). Atopic eczema (with any positive skin prick test) in the probiotic (9.4%, n=3/32) group was similar to that in placebo (10.3%, n=3/29) (ORadj = 0.58; 95% CI= 0.07 to 4.48) in these children with maternal atopy.

3.3.7.3 Feeding History

To address the variability in probiotic dose resulting from variations in patterns of breast feeding within the intervention group and eczema outcome, analysis of the data according to the amount of probiotic supplemented formula used was made and although a linear increasing trend of 15.4% to 22.2% to 75.0% of subjects with eczema was observed with corresponding decreasing dose of probiotics from fully formula-fed to partially breastfed to near total breast feeding with at least 60ml of trial milk formula for 6 months, the linear trend observed was not found to be significant with p = 0.05266 (Table 3.11). Further comparison of subjects who were on total formula feeding in the probiotic and placebo group also did not show an effect of high dose probiotic supplementation on the development of eczema (p = 0.345).

Table 3-11 Feeding history (%) of subjects with eczema

		Feeding history-Birth to Month 6 (%)					
		Near total breastfeeding with at least 60ml of formula		Partial breastfeeding		Total formula feeding	
		Placebo	***Probiotic***	***Placebo***	***Probiotic***	***Placebo***	***Probiotic***
Ever Eczema by 24 months	Yes	1 (50.0)	3 (75.0)	23 (24.7)	18 (22.2)	8 (30.8)	6 (15.4)
	No	1(50.0)	1 (25.0)	70 (75.3)	63 (77.8)	18 (69.2)	33 (84.6)

3.3.8 Effects of Probiotic Supplementation on Asthma and Allergic Rhinitis at 2 Years of Age

There was no difference in the incidence of asthma (according to definition of ≥ 3 episodes of wheeze) in the probiotic (n=11/124; 8.9%) and that in placebo group (n=11/121; 9.1%) (ORadj = 1.15; 95% CI= 0.46 to 2.87) at 2 years of age. The median age at first diagnosis of asthma was 18 months for subjects in the probiotic group and 15 months for subjects in the placebo group. The incidence of allergic rhinitis was not significantly different between the two groups (n=2/124; 1.61% vs. n=3/121; 2.48% in the placebo, p=0.86). The adjusted OR was 0.84 (95% CI: 0.13 to 5.54). The median age at first diagnosis of allergic rhinitis in the subjects was 12 months for probiotic and 17 months for placebo groups. Adjusted analyses accounting for imbalance of gender, birth order, prenatal smoking exposure and feeding history did not alter the results significantly (Table 3.12).

The coexistence of more than one atopic conditions was observed in 4.1% (n=5/121) of the placebo and 3.2% (n=4/124) of the subjects in the probiotic group (p= 0.747). In both groups, only 1 subject was diagnosed with all 3 atopic diseases, namely eczema, asthma and allergic rhinitis. Figure 3.5 shows that only 1 subject in the placebo group and none in the probiotic group reported having symptoms of both asthma and allergic rhinitis at 2 years of age. Two subjects in both the placebo and probiotic groups had coexistence of eczema and asthma symptoms.

Table 3-12 Prevalence of asthma and allergic rhinitis at 2 years of age

	Placebo (n = 121)	**Probiotic (n =124)**
Ever asthma[†] (%)*		
Yes	11 (9.09)	11 (8.87)
No	110 (90.91)	113 (91.13)
For those with asthma:		
Age at first diagnosis (months)		
Mean (SD)	14.18 (6.18)	16.09 (5.96)
Median (Range)	15 (3 to 24)	18 (6 to 24)
Ever allergic rhinitis[#] (%)*		
Yes	3 (2.48)	2 (1.61)
No	118 (97.52)	122 (98.39)
For those with rhinitis:		
Age at first diagnosis (months)		
Mean (SD)	14.33 (10.26)	12 (8.48)
Median (Range)	17 (3 to 23)	12 (6 to 18)

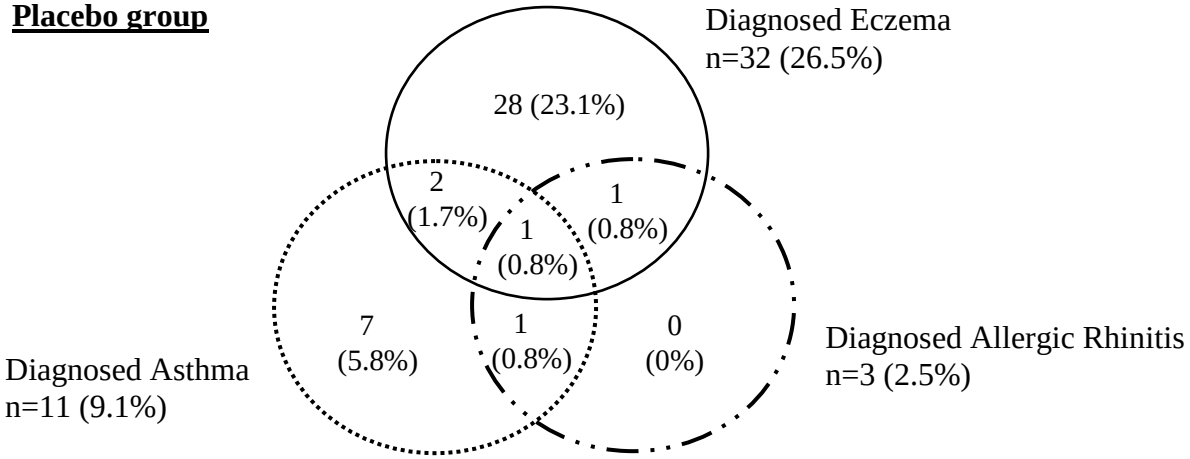
* 8 subjects (5 placebo, 3 probiotic) without any clinical assessment after randomisation due to withdrawal of consent / withdrawal by investigator / lost to follow up were excluded

[†]Asthma - 3 episodes of nocturnal cough with sleep disturbances or wheezing, separated by at least seven days, in a setting where asthma was likely and conditions other than allergy have been excluded

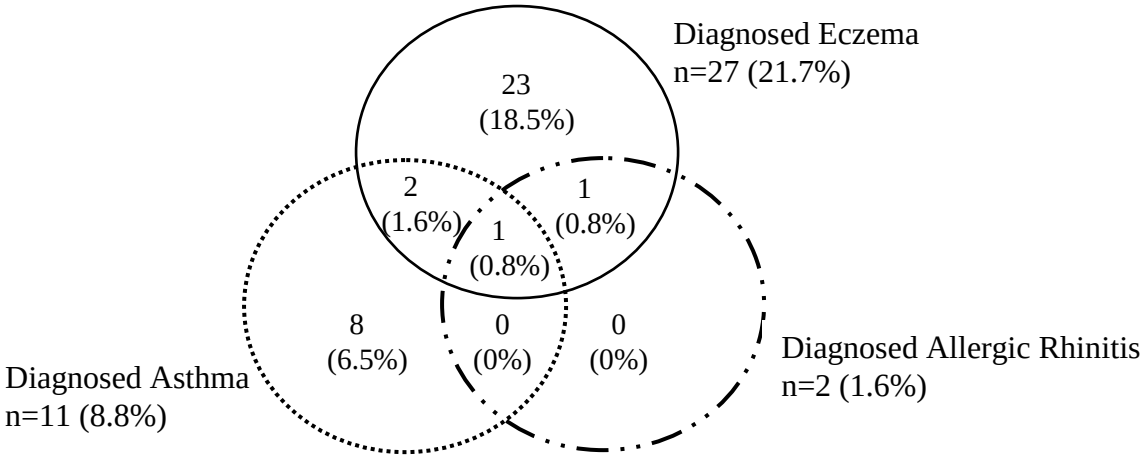
[#]Allergic rhinitis - rhinorrhea, nasal obstruction, nasal itching and sneezing which were reversible spontaneously or with treatment that was not due to a respiratory infection.

Figure 3-5 Incidence of multiple atopic conditions in the placebo and probiotic groups at 2 years of age

Placebo group



Probiotic group



3.4 Discussion

This randomized controlled trial, a first in an Asian at risk cohort, did not show a protective effect of probiotic supplementation for the first 6 months of life on eczema, asthma, allergic rhinitis or allergen sensitization at 1 and 2 years of age. To date there have been 6 published clinical trials on the role of early life probiotic supplementation on the primary prevention of eczema. These studies are summarized in Table 1.5. Our findings are similar to 2 other clinical trials, one involving an Australian cohort (n=178) [167, 168] and a second German cohort (n=94) [171]. 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 study [171], a New Zealand study [173] and 3 other published Scandinavian trials [8, 165, 169] showed that probiotics did not have an effect on allergen sensitization. The Scandinavian trials, however, demonstrated a reduction in eczema at 2 years, although the Swedish study by Abrahamsson et al. (n=188) showed this benefit only in the subset of IgE-associated eczema [169]. Only one study (n=132) has published 7 years long-term follow up results [163, 164] and has shown a sustained benefit in terms of eczema prevalence, but similar to our study at 2 years, the results were disappointing with regards to respiratory allergies.

In subset analysis of interactions between mode of delivery and probiotic intervention, caesarean-delivered babies supplemented with probiotics did not show a significant difference in incidence of eczema, allergen sensitization and atopic eczema in our

study although the incidence of eczema at 2 years of age in caesarean-delivered children supplemented with probiotics was about 40% lower in the probiotic (14.7%) group as compared to that in placebo (25%). An increase in sample size could increase the power to possibly detect a statistical difference between the 2 groups. The follow-up study of one of the Scandinavian trial (Kukkonen et al. [165]) to 5 years of age demonstrated that probiotics prevented cumulative IgE associated allergic diseases, in particular IgE associated eczema and positive food skin prick test response and/or food-specific IgE > 0.7 kU/L, in caesarean-delivered children but not in the total cohort [166]. It has been shown that vaginally delivered babies are colonized with bifidobacteria and lactobacilli earlier than caesarean-delivered babies [43, 44]. Furthermore, children born by means of caesarean section was found to be associated with an increased risk of developing respiratory allergies [45]. The deprivation of the massive microbial load during vaginal delivery might be substituted by probiotic supplementation.

Besides differences in population characteristics between these clinical trials, variations in study design may have also contributed to the observed differences. A common difference in the study designs of 2 studies (our study and Taylor et al.) that failed to observe a protective effect of probiotics on eczema was the absence of prenatal probiotic supplementation in these protocols. Prenatal supplementation may therefore be an important factor in conferring these benefits. Supplementation of *L. rhamnosus* GG to the mothers antenatally enhanced specific changes in the transfer and colonisation of bifidobacteria in neonates [216]. Furthermore, in the Swedish study by Abrahamsson et al. [169], prenatal supplementation resulted in a more pronounced effect of reduction of IgE associated eczema and sensitization in infants

with atopic mothers. This study found that probiotic supplementation with *L. reuteri* during late pregnancy reduced breast milk levels of TGF-ß2 and increased IL-10 [217]. Different strains of probiotics can have varying immunomodulatory effects as Prescott et al. observed on the contrary that *L. rhamnosus* HN001 or *B. lactis* HN019 prenatal supplementation increased breast milk levels of TGF-ß1 and IgA [218]. This observation was in consistent with Rautava et al. study which showed higher TGF-ß2 isoform with no difference in TGF-ß1 in the breast milk of women who received *L. rhamnosus* GG [219]. Animal studies suggested that TGF-ß in breast milk may have anti-inflammatory effects [220] and induces allergen-specific tolerance [221]. The advantage of prenatal supplementation of probiotics has been further highlighted by the presence of small quantities of viable bacteria with a range of bacterial DNA signatures in breast milk and greater biodiversity of maternal peripheral blood mononuclear cells which could program the neonatal immune system [222]. Prenatal supplementation of *L. rhamnosus* GG to mothers was also found to promote newborn colonization with *L. rhamnosus* GG for as long as 6 months and may even persist to 24 months [223]. Consumption of L. rhamnosus GG by pregnant mothers was further showed to increase the bifidobacterial diversity in infants with more *B. breve* and lesser *B. adolescentis* than the placebo group [216]. However, prenatal supplementation in the German study did not result in a positive outcome. This could further suggest that probiotics supplementation to mothers in late pregnancy is of crucial importance.

The different probiotic strains studied and their doses may also contribute to the inconsistency in results between studies. Our study utilized a combination of *B. longum* and *L. rhamnosus* which are inhabitants of different locations in the

gastrointestinal tract. Immunostimulatory oligodeoxynucleotides which suppress IgE and Th2 cytokines production have been identified in these probiotic strains [224, 225]. Furthermore, *B. longum* BB536 has been reported to have a treatment effect on Japanese cedar pollinosis [151, 152]. It was therefore postulated that the combination of these 2 probiotic bacteria in our study may provide additive immunomodulatory effects. It has been further shown that the supplementation of these bacterial strains in infant formula is well tolerated [190]. 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 (personal communication, F Rochat, Nestle, Lausanne, Switzerland). Additionally, *L. rhamnosus* LPR was originally derived from a product, Dicoflor® which contains *L rhamnosus* GG (ATCC 53103) (Certificate of Receipt, China General Microbiological Culture Collection Center), indicating that both these probiotic bacteria are identical. *Lactobacillus rhamnosus* GG has shown benefits on eczema most consistently with 2 studies reporting positive effects [8, 163-165]. However, the inclusion of *L. rhamnosus* in our study, as well as the German study did not result in the same benefits. The New Zealand study that used *L. rhamnosus* HN001 and *B. animalis* subsp *lactis* strain HN019 demonstrated that *L. rhamnosus* HN001 supplementation but not *B. animalis* subsp *lactis* could reduce the prevalence of eczema with no effect on allergen sensitization [173]. The only study (Taylor et al.) that used *L. acidophilus* for supplementation resulted in increased allergen sensitization in the supplemented subjects. Another study (Kukkonen et al.) used a mixture of 4 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 [226] reported a reduction in eczema as this study had

a lost to follow up rate of 20%. Another study [227], which did not select infants at risk of allergy, reported no significant difference in eczema in infants up to four months of age. Meta-analysis of these 2 studies could not determine the role of prebiotics in the prevention of eczema due to the heterogeneity of the study design [228].

Our study involved a relatively lower dose of probiotics (approximately 2 logs) compared to the other primary prevention trials which reported protective effects on eczema [8, 163-165]. However at these lower doses, we did document consistent transit of the supplemented probiotic in the stools of infants in the probiotic group compared to placebo [229, 230]. Significantly more *L. rhamnosus* (OR= 111.93; 95% CI = 23.18 to 540.45, $p < 0.001$) and *B. longum* (OR= 3.75; 95% CI = 1.27 to 11.07, $p= 0.017$) were detected by polymerase chain reaction method in the probiotic group over the first 3 months of supplementation [229]. 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 breast feeding with infant formula [231] 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 breast feeding within the intervention group and eczema outcome, analysis of the data according to the amount of probiotic supplemented formula used (ie. exclusive formula vs minimum of 60ml)

was made and a near to significance increasing trend of subjects with eczema was observed with corresponding decreasing dose of probiotics (p = 0.05266). The difference might have been detected with a larger sample size to increase the statistical power of the study. In this trial 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 [232]. It is also not a practice to use hydrolyzed formula in at risk infants in Asian communities including ours. Of great interest was the absence of cow's milk sensitization (except 1 in the probiotic group at 2 years old) 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 (26%) was lower than the 39% in the placebo group at 1 year [167] and 46% at 2 years [8] in the other studies. Despite this lower observed prevalence of eczema (26%), 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 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 originate [233].

Allergic airway diseases usually manifest later in life and in our study the low prevalence of 9% asthma and 2.5% allergic rhinitis at 2 years of age did not yet allow for their comparison. An additional follow-up period will be critical for the evaluation of respiratory allergies in the form of clinical asthma, allergic rhinitis and sensitization to inhalant allergens. These tend to develop later in life after the age of 2 years and this step-wise, temporal development of respiratory allergies has been described as the "Atopic March" [234-236]. Thus only a small proportion of subjects with atopic symptoms had symptoms for more than one atopic condition.

Atopic diseases are multifactorial diseases influenced by various familial and environmental factors. Thus identifying useful predictive markers for effective screening remains a challenge. Family history and elevated cord blood serum IgE [237-239] have been proposed as markers to screen newborns for atopy risk. However, the predictive capacity of cord blood IgE has been questioned [240-242] while family history of atopy has generally been regarded as a useful predictor. In our study, positive family history and elevated cord blood serum IgE were not found to be associated to eczema, allergen sensitization, atopic eczema at 1 year of age. Combining parental atopy with elevated cord IgE also failed to identify babies at risk of eczema and allergen sensitization although these have been demonstrated in other studies to be predictors of atopy in newborn babies [238, 239]. In contrast to published literature [243-245], maternal atopy and mothers with eczema were not found to be a risk factor for eczema. Paternal eczema was instead significantly associated. The associations between parents' atopic disease and the risk of eczema in the subjects may vary according to the type of atopic disease with parental eczema being a better marker than parental asthma or parental allergic rhinitis and not

according to parental gender [246]. It is also possible that questionnaires were usually completed by mothers resulting in a misclassification of paternal symptoms. But this possibility could be eliminated as this should have a greater regression dilution bias observation as opposed to results observed in our study.

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. Further work is needed to determine whether prenatal supplementation, probiotic dose and probiotic strain are important considerations. A larger study will have to be performed to increase the power and determine the difference observed between the placebo and probiotic supplemented groups.

Chapter 4: Effect of Probiotic Supplementation on Specific Antibody Responses to Infant Hepatitis B Vaccination

4.1 Introduction

Probiotics are promising immunomodulators which enhance innate and adaptive immunity in the host [64]. Gnotobiotic animal models have shown that probiotics have significant immunomodulatory effects on local and systemic immune responses. Furthermore, its safety record in humans has made probiotic supplementation an attractive strategy to modulate and enhance the immune system. Probiotics have been conferred GRAS (generally regarded as safe) status by the Food and Agriculture Organization(FAO)/World Health Organization expert panel [56], and are considered safe in the neonate [84, 85]. Probiotic supplementation in young children has been shown to protect against gastrointestinal infections such as rotavirus gastroenteritis [117]. Probiotics have also been shown to enhance specific immune responses to vaccination in young children and adults. It increased the immunogenicity of orally administered vaccines such as that of rotavirus [86], Salmonella [87], polio [89] and cholera [91]; as well as enhanced antibody responses to parenterally administered vaccines, namely diphtheria, tetanus, and Haemophilus influenzae type b [93-97]. Probiotics therefore have an adjuvant effect by enhancing immunogenicity of vaccines. This study assessed the effect of probiotic supplementation in the first 6 months of life on specific IgG antibody responses to Hepatitis B vaccination. To our knowledge, there have been no previous reports on the effect of probiotics on Hepatitis B vaccination in infants.

4.2 Materials and Methods

4.2.1 Vaccination

Depending on the attending vaccination centre, majority of the infants received either 1 of 2 schedules of Hepatitis B vaccination with intramuscular injection in the anterolateral aspect of the thigh at ages 0, 1 and 6 months respectively, following the Singapore national immunization program. Schedule A consisted of monovalent Hepatitis B vaccination, (HBVax, MSD, Whitehouse Station, NJ, USA) at Dose 1 and 2 (2.5μg each) and a hexavalent diphtheria-tetanus-acellular pertussis (DTPa) combination vaccination containing a Hepatitis B component (10μg) (Infanrix HEXA, GSK Biologicals, Rixensart, Belgium) at Dose 3. Schedule B consisted of monovalent Hepatitis B (HBVax) 2.5μg/dose for all three doses. Infants born to Hepatitis B surface antigen (HBsAg)-positive mothers received the Hepatitis B vaccine with Hepatitis B immune globulin (HBIG) at birth to prevent transmission of perinatal Hepatitis B viral infection.

4.2.2 Antibody analysis

Venous blood was collected at 12 months of age and analyzed for Hepatitis B serology with measurement of the Hepatitis B virus surface antibody (anti-HBs) immunoglobulin G using ADVIA Centaur Anti-HBs (Bayer Health Care, Tarry town, NY, USA).

4.2.3 Statistical analysis

SPSS software (version 15.0 for Windows) was used to perform independent two-sample t-test to compare anti-HBs IgG geometric mean titres between placebo and probiotic groups. The geometric mean titres were obtained by computing the exponentiated values of the arithmetic mean of the logarithm transformed values of the anti-HBs IgG titres. The logarithmic transformation gave the data a good fit to the normal distribution to enable the use of parametric Student's t-test [209, 247]. A p value of < 0.05 was considered statistically significant for all analyses.

4.3 Results

4.3.1 Baseline characteristics and participants

Families were assessed at the antenatal clinic and 253 newborns were recruited into the study. During the follow-up period, 3 subjects from the probiotic group and 6 subjects from the placebo group did not complete the study and blood samples were not collected. At the 12-month visit, 11 subjects refused blood taking. There were 20 subjects who received alternative vaccine schedules (other than schedule A or B) involving combination vaccines and these subjects were excluded from analysis. As the primary clinical outcome of the study was eczema, vaccine schedule was determined by the vaccination centre the subject attended.

The demographic and social characteristics, gender ratio, gestational age, birth weight, number of siblings, daycare attendance, smoking at home and breastfeeding rate of the 2 vaccine groups were comparable (Table 4.1). There were 11 subjects (4.72%) including one born to a Hepatitis B carrier (HBeAg positive) mother, who failed to seroconvert after 3 doses of vaccination (Table 4.2). Of these, 3 received vaccine

schedule A (probiotic = 2) while 8 received vaccine schedule B (probiotic = 3) (Figure 4.1). Seven of the 11 subjects seroconverted after an additional booster dose of vaccine (HBVax, 5 μg), and the eighth subject after 2 doses. The ninth subject was born to Hepatitis B carrier mother failed to respond and was found to be HBsAg positive. The remaining 2 subjects (10^{th} and 11^{th}) refused further blood evaluation.

There were therefore 202 evaluable subjects. Fifty-seven infants received vaccine schedule A (probiotic=29, placebo=28) and 145 infants received vaccine schedule B (probiotic= 77, placebo= 68).

Figure 4-1 Flow chart showing progress of participants through the study

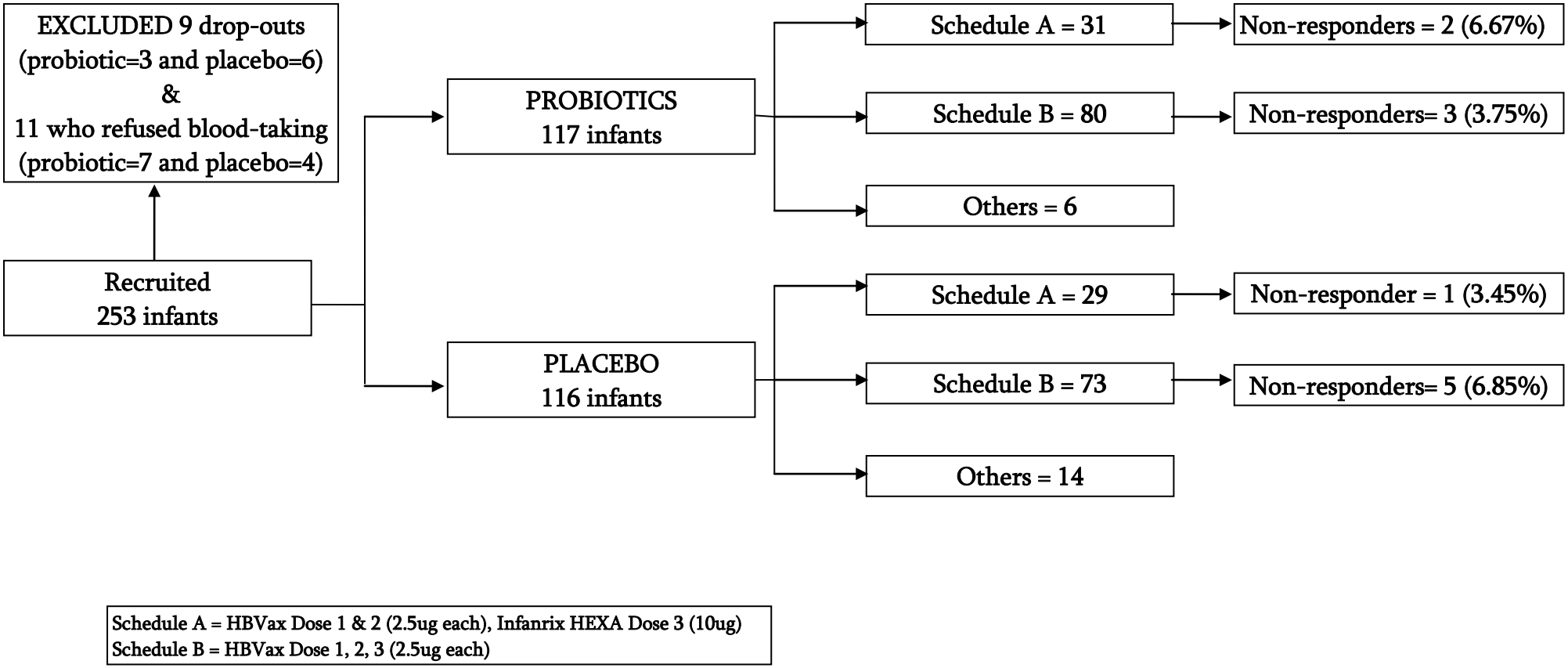


Table 4-1 Characteristics of the Study Population

	Vaccine Schedule A		Vaccine Schedule B	
	Probiotic n=29 (%)	Placebo n=28 (%)	Probiotic n=77 (%)	Placebo n=68 (%)
Gestational age in weeks, Mean (SD)	38.3 (1.6)	38.8 (1.1)	38.5 (1.4)	38.4 (1.1)
Gender				
Male	12 (41.4)	15 (53.6)	41 (53.2)	40 (58.8)
Birth Weight (kg)				
Mean (SD)	3.1 (0.5)	3.2 (0.4)	3.2 (0.5)	3.1 (0.4)
Birth Order				
1	17 (58.6)	10 (35.7)	33 (42.9)	21 (30.9)
2	2 (6.9)	12 (42.9)	16 (20.8)	23 (33.8)
3	10 (34.5)	4 (14.3)	20 (26.0)	14 (20.6)
≥4	0 (0)	2 (7.2)	8 (10.4)	10 (14.7)
Smoking exposure	10 (34.5)	8 (28.6)	43 (51.2)	29 (39.7)
Day care attendance by Month 12	0 (0)	0 (0)	0 (0)	2 (2.7)
Feeding history-Birth to Month 6				
Near total breastfeeding with at least 60ml of formula for 6 months	2 (6.9)	1 (3.6)	1 (1.2)	0 (0)
Any breastfeeding	19 (65.5)	24 (85.7)	54 (64.3)	54 (74.0)
Total formula	8 (27.6)	3 (10.7)	29 (34.5)	19 (26.0)

4.3.2 Effects of probiotic supplementation on Hepatitis B surface antibody response

The seroconversion rates were almost similar between the 2 schedules. However, of 5 infants who were born to Hepatitis B carrier mothers, vaccine failure occurred in one infant who was randomized to the probiotic group and received Schedule A vaccinations. This child was the only subject to develop the Hepatitis B carrier state.

Within the placebo group, the anti-HBs geometric mean titre (95% CI) of subjects in Schedule A [187.97 (180.70 – 195.24) mIU/ml] were lower than that in subjects with Schedule B [302.34 (296.31 – 308.37) mIU/ml], although this difference did not reach statistical significance (p=0.076). In contrast, within the probiotic group, anti-HBs geometric mean titres of those receiving schedule A [345.70 (339.41 – 351.99) mIU/ml] and B [302.06 (296.31 – 307.81) mIU/ml] were comparable (p=0.575). In other words, probiotic supplementation could potentially increase anti-HBs responses in those receiving Schedule A to levels more comparable with those in Schedule B. Hence, for infants who received Schedule A, the anti-HBs geometric mean (95% CI) titres were 345.70 (339.41 – 351.99) mIU/ml in the probiotic group and 187.97 (180.70 –195.24) mIU/ml in the placebo (p = 0.069). This difference was not observed for infants receiving vaccine Schedule B where anti-HBs geometric mean (95% CI) titres were very similar [probiotic: 302.06 (296.31 – 307.81) mIU/ml, placebo: 302.34 (296.31 – 308.37) mIU/ml] (p = 0.996). The data is summarized in Table 4.2.

Table 4-2 Hepatitis B surface antibody response in vaccine schedule A and B

Vaccine group	**Treatment group**	**Sample size (n)**	anti-HBs **IgG geometric mean titre (mIU/ml)**	**95% C.I.**	**p value**	**Seroconversion rate (%)**	**p value**
A	placebo	28	187.97*	180.70 – 195.24	0.069	96.6	1.000
	probiotics	29	345.70	339.41 – 351.99		93.5	
B	placebo	68	302.34*	296.31 – 308.37	0.996	93.2	0.259
	probiotics	77	302.06	296.31 – 307.81		97.5	

Schedule A: Monovalent HepB vaccines at 0, 1 month and DTPa-IPV-HiB- HepB combination vaccine at 6 months

Schedule B: Monovalent HepB vaccines at 0, 1, 6 month**s**

*p= 0.076 (Placebo group: Schedule A vs Schedule B)

4.4 Discussion

Prevention of childhood infections through vaccination is an important target of global healthcare. Hepatitis B vaccination is part of the WHO expanded program of immunization. Many countries have adopted universal Hepatitis B vaccination. In countries where Hepatitis B is endemic, vertical transmission of the infection is still a concern. Strategies that may improve immunogenicity of the vaccine are welcomed, especially in infants born to Hepatitis B carrier mothers.

Probiotic supplementation has been shown to enhance the immunogenicity of various vaccines [86, 87, 89, 91, 93-97]. This study evaluated the effects of probiotics on Hepatitis B vaccine responses in infants vaccinated from birth. Two vaccine schedules were compared as the majority of subjects received these schedules. Our results show that the schedule with 3 monovalent doses of Hepatitis B vaccine resulted in better anti-HBs responses compared to the schedule consisting of 2 monovalent doses followed by a third dose as a DTPa combination vaccine, although this difference was not statistically different ($p=0.076$). These results differ from two recent studies conducted in Singapore, where anti-HBs responses involving monovalent vaccine and combination vaccine were similar [248, 249]. This observed difference may be related to the dose of the monovalent vaccines (2.5μg, half-dose of HBVax, MSD in our study vs 10μg, full-dose Engerix B, GSK Biologicals, Rixensart, Belgium in previous studies [248, 249]) used between studies, and the combination of Hepatitis B vaccines from different manufacturers in schedule A (MSD for monovalent and GSK Biologicals for combination vaccine) in our study. These schedules represent real life schedules used by vaccine centres in Singapore.

There was only one documented vaccine failure in our cohort. This subject was born to Hepatitis B (HBeAg +) mother and despite hyperimmune globulin at birth and 3 doses of Hepatitis B vaccines, developed the carrier state. This failure to respond may be genetically determined as suggested in a recent report which revealed risk haplotypes in the genetic variants of the HLA-DP locus [250]. The remaining 8 subjects who seroconverted after one or two additional booster doses of vaccination should not be considered non-responders as is defined by a failure to seroconvert after completion of two full 3-dose series of the Hepatitis B vaccine and for whom an acute or chronic Hepatitis B infection has been ruled out [251].

Interestingly, probiotic supplementation in the first 6 months of life could improve anti-HBs responses in subjects receiving schedule A (2 doses monovalent + 1 dose combination) but not in those receiving schedule B where higher antibody responses were observed. The immune response ceiling has possibly been reached in those subjects that received Schedule B. This difference, however, did not reach statistical significance. This is likely to have arisen from the smaller sample size of subjects in schedule A, since the vaccine schedules were not randomized for this study but were dependent on the vaccine centres attended. This restriction on sample size could have compromised the statistical power of the study, in particular for schedule A. This study may be statistically underpowered as the number of subjects receiving vaccine schedule A were less than those in vaccine schedule B. Nonetheless, the data suggests that probiotics can potentially be used as an adjuvant for immune responses in schedules with less than optimal responses.

Breastfeeding is unlikely to have influenced the observed differences between groups, since only a very small proportion of subjects were totally breastfed apart from the study formula (Table 4.1). There were also little differences in breast feeding practices between groups. Breastfeeding has shown contrasting results in some studies. In a study to evaluate Hib conjugate vaccine response, breastfed infants produced higher antibody concentrations than formula-fed infants [252]. In contrast, in another Australian study, breastfed infants had lower anti-Hib capsular polysaccharide antibody concentrations both before and after immunization with Hib conjugate vaccine as compared with formula-fed infants [253].

In conclusion, our data suggests that probiotic supplementation from birth could enhance Hepatitis B antibody response in infants receiving certain vaccine schedules. These findings however, would require larger studies to confirm these observations.

Chapter 5: Effects of Probiotic Supplementation on Acute Infectious Illnesses

5.1 Introduction

Supplementation of certain strains of probiotics may enhance resistance against infections. The potential effects have been studied in day care centres where infants are more prone to develop gastrointestinal and respiratory tract infections than children at home. These infants supplemented with *L. rhamnosus* GG for 7 months resulted in a lower rate of use of antibiotics for respiratory tract infections and a 0.6 day shorter absence period due to illness compared to the placebo group, when adjusted for age. Another study in infants attending day care centres studied the effects of two different probiotics, *B. lactis* (BB-12) and *L. reuteri* on prevention of infections. Both probiotics reduced the number of days and number of episodes with diarrhea and fever (>38 ˚C). Furthermore, *L. reuteri* but not *B. lactis* was associated with fewer visits to the doctor, antibiotics prescriptions and reduced absence of day care.

The significant effects were modest with difference in the number of days of illnesses calculated as less then 1 day in both studies and hence the clinical relevance is unclear and cannot be extrapolated from these studies performed over a short period of time.

This study therefore aims to assess the effect of early regular supplementation of probiotics in the infant diet on protective benefit against diarrhoeal and febrile illnesses in this longitudinal study to determine if this effect is short term (6 months, during supplementation) or longer-lasting in a 2 years follow-up period.

5.2 Materials and Methods

5.2.1 Ascertainment of infections

Infants were reviewed by a paediatrician at 1, 3, 6, 12 and 24 months of age, which involved a detailed history, recording of anthropometric data and clinical examination. Questionnaires were also administered at these visits to record clinical illnesses. In addition, questionnaire phone surveys (Appendix D) were performed biweekly for the first 6 months and after which conducted at monthly intervals to document the incidence of infectious episodes, defined as fever more than 38.5˚C, diarrhoea lasting more than 3 day, upper respiratory tract infection (URTI) lasting more than 14 days, lower respiratory tract infection (LRTI) and wheezing, with particular emphasis on those that require antibiotic use of more than 3 days period, doctor visits or hospitalizations (Appendix B, Adverse Events Form and Serious Adverse Events Form). Diary charts (Appendix C) were also used by the parents to record details of infections to lessen recall bias.

5.2.2 Statistical analysis

All statistical analysis was carried out on an intention-to-treat approach. Incidence of infectious episodes were calculated and expressed as percentages. SPSS software (version 15.0 for Windows) was used to perform Chi-square test to compare differences in incidence of infectious episodes, antibiotic usage and hospitalization between placebo and probiotic groups. The effects of confounding factors such as sibling number, attendance at child care and feeding history were assessed using multiple logistic regression analysis and expressed as odds ratio (OR) with 95% confidence interval (CI). The number of antibiotic courses was logarithmically transformed and Student's t-test for independent samples was used for group

comparision of geometric mean. A p value of <0.05 was considered statistically significant for all analyses.

5.3 Results

5.3.1 Effect on Infections and Antibiotics Usage during Intervention period

In the first 6 months of life, incidence of febrile episodes more than 38.5^{o}C occurred in 16.5% of the infants in the placebo group. Only 5.8% of the subjects suffered from gastroenteritis more than 3 days in the placebo group. The incidences of URTI and LRTI were about equal at 6.6% and 5.8% respectively in the placebo group. During the probiotic supplementation period (0–6 months), we observed no significant difference between the probiotic and placebo groups in the occurrence (at least once) of febrile episodes more than 38.5^{o}C (18.5% vs. 16.5%; p=0.677), gastroenteritis lasting more than 3 days (7.3% vs. 5.8%; p=0.640), URTI more than 14 days (4.0% vs 6.6%; p=0.367), LRTI (5.6% vs. 5.8%; p=0.962) and wheezing (6.5% vs. 4.1%; p=0.418). No difference was also found between infants who received antibiotics in the probiotic group (n=10/124; 8.1%) as compared to that in the placebo group (n=13/121; 10.7%) (p=0.472). The geometric mean number of antibiotics courses taken was 1.07 in the probiotic and 1.05 in the placebo group (ratio: 1.01; 95% CI: 0.84 – 1.21; p=0.854). However interestingly, more infants were hospitalization due to infections in the probiotic group (n=14/124; 11.3%) than that in the placebo group (n=4/121; 3.3%) during the first 6 months intervention period (p=0.016) (Table 5.1). After adjustment with possible confounding factors such as sibling number, attendance at child care and feeding, the infants in the probiotic group is 3.94 times (95% CI = 1.21 to 12.75, p=0.022) more likely to be hospitalized due to infections by

6 months than subjects in the placebo group. Further analysis showed that the ratio of hospitalization per episode of acute infections was higher in the probiotic group (12.0%; n=15/125) compared to only 3.6% (n=5/137) in the placebo group (p=0.018). The 15 subjects in the probiotic group were hospitalized due to diarrhoea more than 3 days (n=1), fever higher than 38.5˚C (n=1), URTI (n=1), LRTI (n=3) and other febrile illnesses (n=9). The 5 subjects in the placebo group were hospitalized due to fever higher than 38.5˚C (n=1), LRTI (n=3) and urinary tract infection (n=1) summarised in Table 5.2.

Table 5-1 Occurrence (at least once) of infectious episodes, antibiotics use and hospitalization per subject between treatment groups during intervention (0-6 months) period

	Placebo n = 121 (%)	**Probiotics n = 124 (%)**	**Significance *P***
Incidence of infectious episodes by 6 months			
Febrile episode more than 38.5^{o}C	20 (16.5)	23 (18.5)	0.677
Gastroenteritis lasting more than 3 days	7 (5.8)	9 (7.3)	0.640
URTI lasting more than 14 days	8 (6.6)	5 (4.0)	0.367
LRTI	7 (5.8)	7 (5.6)	0.962
Wheezing	5 (4.1)	8 (6.5)	0.418
Incidence of antibiotics use by 6 months			
Used antibiotics	13 (10.7)	10 (8.1)	0.472
Incidence of hospitalization due to infections by 6 months	4 (3.3)	14 (11.3)	0.016*

- 8 subjects (5 placebo, 3 probiotic) without any clinical assessment after randomisation due to withdrawal of consent / withdrawal by investigator / lost to follow up were excluded

- URTI - upper respiratory tract infection
- LRTI - lower respiratory tract infection

Table 5-2 Episodes of hospitalization due to infections by 6 months in the placebo and probiotic groups

Episodes of hospitalization due to infections by 6 months	***Placebo***	***Probiotic***
Fever > 38.5˚C	1	4
Diarrhoea > 3 days	0	1
URTI	0	1
LRTI	3	3
Urinary Tract Infection	1	3
Viral Infection	0	2
Erysipelas	0	1
Total	5	15

- URTI - upper respiratory tract infection
- LRTI - lower respiratory tract infection

5.3.2 Effect on Infections and Antibiotics Usage during Follow-up (6-24 months)

The subjects were continued to be follow-up for infectious episodes from 6 to 24 months of age (Table 5.3). Febrile episodes more than 38.5^{o}C occurred in about half of the subjects (46.3%) in the placebo group. The incidence of gastroenteritis more than 3 days increased to 25.6% in the follow-up period. Incidence of URTI and LRTI also doubled to 12.4% and 10.7% in the placebo group as compared to the first 6 months of life. The incidence of acute infectious illnesses in the probiotic and placebo group, including febrile episodes more than 38.5^{o}C (55.6% vs. 46.3%; p=0.142), gastroenteritis lasting more than 3 days (19.4% vs. 25.6%; p=0.239), URTI more than 14 days (13.7% vs 12.4%; p=0.760), LRTI (7.3% vs. 10.7%; p=0.350) and wheezing (12.9% vs. 13.2%; p=0.940) were observed to be similar in the probiotic and placebo groups from 6 to 24 months of life. Recurrent wheezing episodes ($\geq$ 2) occurred in 9.5% of the children in the probiotic group and 10.2% in the placebo group from 6 to 24 months (p=0.849). By 2 years of age, about half of the subjects received antibiotics with no significant difference between the probiotic group (46%) and the placebo (53.7%) group (p=0.225). The geometric mean number of antibiotics courses taken was 1.51 in the probiotic and 1.58 in the placebo group (ratio: 0.95; 95% CI: 0.79 – 1.14; p=0.606). The rate of hospitalization due to infections between the probiotic group (9.9%) and placebo group (12.1%) were also similar (p=0.496).

Table 5-3 Occurrence (at least once) of infectious episodes, antibiotics use and hospitalization per subject between treatment groups during follow-up (>6-24 months) period

	Placebo n = 121 (%)	**Probiotics n = 124 (%)**	**Significance *P***
Incidence of infectious episodes, 6-24 months			
Febrile episode more than 38.5^{o}C	56 (46.3)	69 (55.6)	0.142
Gastroenteritis lasting more than 3 days	31 (25.6)	24 (19.4)	0.239
URTI lasting more than 14 days	15 (12.4)	17 (13.7)	0.760
LRTI	13 (10.7)	9 (7.3)	0.350
Wheezing	16 (13.2)	16 (12.9)	0.940
Recurrent wheeze ≥ 2	12 (9.5)	13 (10.2)	0.849
Incidence of antibiotics use, 6-24 months			
Used antibiotics	65 (53.7)	57 (46.0)	0.225
Incidence of hospitalization due to infections, 6-24 months	15 (12.1)	12 (9.9)	0.496

- 8 subjects (5 placebo, 3 probiotic) without any clinical assessment after randomisation due to withdrawal of consent / withdrawal by investigator / lost to follow up were excluded

5.4 Discussion

Probiotics may influence the incidence of infections by enhancing humoral and cellular immunity [254]. Several studies have been published on the preventive effect of probiotics on infectious illnesses in infants and children [168, 175, 176, 178, 255-257]. We aimed to evaluate the effects of probiotics on the type, frequency and severity of acute infectious illnesses in infants. Short term (intervention period, 0-6 months) and long term effects in a 2 years follow-up period were investigated. This study did not to demonstrate any protective effect of probiotic supplementation on infection. Rates of febrile episodes, gastroenteritis and respiratory infections were similar in both groups during the intervention and follow-up period. The rate of antibiotic usage and number of courses were also similar between the two groups. Interestingly, more infants were hospitalization for infections by 6 months in the probiotic group than in the placebo group (ORadj: 3.94; 95% CI = 1.21 to 12.75, p=0.022). This difference was not observed later during the follow-up period.

These findings contrast with many previous studies demonstrating that probiotic agents are able to prevent or treat gastrointestinal infections, particularly those of viral etiology [258-260]. The study in Israeli infants in child care centre demonstrated decreased febrile episodes and diarrhoea episodes in infants supplemented with *L. reuteri* or *B. lactis.* In particular, *L. reuteri* supplementation decreased antibiotic prescriptions. But similar to our study, probiotic supplementation had no effect on respiratory illness [176]. The difference in outcomes may be explained by the higher incidence of gastrointestinal infections in Israel. The Swedish study by Abrahamsson et al. [169] with the primary aim to evalutate the prevention of eczema with supplementation of probiotics, similarly used the *L. reuteri* strain as the study in Israel,

but found no effect on gastroenteritis with 30% in the placebo group and 29% in the probiotic group by 1 year of age. In comparision, our study showed a lower incidence of 15.7% of gastroenteritis lasting more than 3 days in the first 12 months of life in the placebo group. This lack of protection in communities with low prevalence of diarrhoea illness has also been demonstrated in other studies. These include 2 Finnish studies among children in day care, one using *L. rhamnosus* GG [175], and another by Kukkonen et al. [178] where supplementation of synbiotics were used. On the other hand, the Australian eczema prevention study study (Taylor et al.) which used *L. acidophilus* supplementation for the first 6 months of life found significantly fewer gastrointestinal infections from 1-2.5 years of age (12%) compared with the placebo (27%) group ($p = 0.023$) [168]. This difference was observed only after the 1st year of life at the 2.5 years follow-up analysis [167]. Taylor et al. also demonstrated no protective effect of *L. acidophilus* supplementation on respiratory tract infections and paradoxically showed a greater frequency of wheeze in the first 6 months of life [167]. This finding corresponded with the data from another German study on the prevention of eczema where a significantly higher proportion of *L. rhamnosus* GG supplemented children with recurrent (≥5 episodes) wheezing bronchitis was observed compared to placebo in the first 2 years of life [171]. Infants who are genetically at risk for atopy have been proposed to have compromised resistance to respiratory infections [261].

Our results did not demonstrate a reduction in the rate of antibiotic use with probiotic supplementation. Moreover, a significantly higher proportion of children in the probiotic group were hospitalized due to infections during the first 6 months of life suggesting more severe infections. Adjustment with sibling number, attendance at child care and feeding did not alter the significance of the result. This finding is in

line with Taylor et al.'s study in which a higher rate of antibiotic use in the probiotic group, particularly in the first 6 months during *L. acidophilus* supplementation [167]. This trend is similarly shown by Abrahamsson et al. as antibiotics were more frequently prescribed in the *L. reuteri* group during the first year of life [169]. Rate of hospitalization was not assessed in these studies but the higher rate of antibiotics used similarly suggest more severe infections in the probiotic group.

There is currently insufficient evidence to advocate the use of probiotics for the prevention of common acute childhood infections. Although accumulating data suggest that these organisms may help prevent both respiratory and diarrhoeal diseases in children at increased risk of such infections, such as those in day care facilities or living in developing countries, it is probable that in our study, the cohort of infants being examined is generally healthy and this study was conducted in a developed community where infant nutrition is optimal with diligent hygiene practise, and hence the effect of probiotics on preventing acute infections was not discernable.

However, the concerning increase in the rate of hospitalization during probiotic supplementation period could be of importance in view of the wide availability of probiotics in infant formula. It is inappropriate to recommend probiotics for prevention of childhood acute infections in Singapore until more studies in communities unravel the role and complexities of interaction between the early microbial environment and the developing immune system.

Chapter 6:
Effects of Probiotic Supplementation on Growth

6.1 Introduction

Nutrition is the main determinant of childhood growth during the first few years of life. Infants receiving formulas with probiotics can have an impact on their growth during the supplementation and also in the long term. Probiotics may alter the gastrointestinal flora and contribute to the host's energy metabolism which enhances the uptake of nutrients to increase nutritional status and improve physical growth. In a double-blind, randomized study, healthy term infants who received *L. rhamnosus* GG-supplemented formula for 6 months grew to a significantly higher length and weight than the infants who received regular formula [189]. Other studies observed similar normal growth in both probiotic-treated and placebo study groups [177, 178, 190, 191, 262]. Safety and tolerance of infant formulas supplemented with probiotics needs to be further evaluated to assess the possible influence of these microorganisms on growth in early infancy. We therefore aim to document safety and impact on growth of newborn infants in this study during the 6 months intervention and 2 year follow-up period.

6.2 Materials and Methods

6.2.1 Growth measurements

Infants were reviewed by a neonatologist at birth and a paediatrician at 1, 3, 6, 12 and 24 months of age, which involved a detailed history, recording of anthropometric data and clinical examination. Weight and length measurements were made according to standardized techniques by using an infant stadiometer (length board) and calibrated infant electronic scale. The Body Mass Index (BMI) was calculated as the ratio of weight (in kg)/recumbent length or standing height (in m^2). The occipitofrontal head circumference (OFC) of subjects was measured to the nearest 2mm with standard measuring tapes.

6.2.2 Statistical analysis

Data analysis was conducted by using SPSS software (version 15.0 for Windows). Means plus/minus (±) standard deviation of the anthropometric measures of infants in the placebo and probiotic groups, who were followed up to 1, 3, 6, 12 and 24 months were calculated and analyzed by using the Student's t-test for independent samples. Changes in weight (weight gain), recumbent length / height, Body Mass Index (BMI) (weight/height^2), and OFC from birth to 24 months were analyzed by a mixed model correcting for gender and feeding history. The mixed model describes the development of growth parameters over time by a quadratic curve, taking into account each subject's intercept and slope (random effects) and is robust against dropouts [263]. Weight-for-age, length-for-age, head-circumference-for-age and BMI-for-age z-scores (also called standard deviation scores - SDS) were calculated based on the WHO Child Growth Standards which can be applied to all children from birth to 5

years old in any country, regardless of ethnicity [264]. A p value of <0.05 was considered statistically significant for all analyses.

6.3 Results

The anthropometric measures of weight (kg), length (cm), BMI (kg/m^2) and OFC (cm) at birth and 1, 3, 6, 12 and 24 months old showed similar normal growth in the probiotic and placebo groups (Table 6.1 and Table 6.2). No differences in birth weight (3.15 ± 0.45 and 3.14 ± 0.42 kg; p=0.775) and length (49.62 ± 2.32 and 49.76 ± 2.78 cm; p=0.656) were observed between the probiotic and placebo group. A comparison of weight, length and BMI z-scores with the WHO Child Growth Standards showed that the mean z-scores of infants in both the probiotic and placebo group were close to 0 at all times during the study (Figure 6.1, 6.2 and 6.3).

The infants in the probiotic group had a higher weight for age z-scores (Means ± SD) from 1 to 24 months of age (Figure 6.1), particularly at one time point of 6 months old (0.18 ± 0.89) compared to the placebo group (-0.02 ± 1.05), this difference was not significant (p = 0.089) and the z-scores were comparable at other time points (Table 6.2). Weight gain (z-scores) during the treatment and follow-up period were similar among infants in the different formula groups. Growth in weight, expressed in z-scores, was particularly higher in the probiotic group (0.00 ± 0.67) than the placebo group (-0.15 ± 0.68) between 1 to 3 months and this difference was near to significant (p = 0.072) (Table 6.3). There were no significant differences in subjects' weight changes associated with treatment between probiotic and placebo groups from birth to 24 months of age (F = 2.474, p= 0.117) with adjustment for gender and feeding history (during the first 6 months intervention period) using mixed model analysis. No

differences were also found for change in mean length (probiotics vs. placebo; F = 0.044, p= 0.835) and OFC (probiotics vs. placebo; F = 0.271, p= 0.603). Mean changes in length, OFC, and BMI z-scores during the treatment period were not different between the probiotic and placebo group (Table 6.3 and 6.4).

The curve of the probiotic group showed consistently higher BMI than that among the subjects in the placebo group from birth to 24 months of age (Figure 6.4). Mean differences in BMI between probiotic and placebo groups (F=3.359, p=0.068) with adjustment for gender and feeding history (during the first 6 months intervention period) using mixed model repeated measures analysis showed a near to significance difference in trend between the two groups.

6.4 Discussion

In this longitudinal study we evaluated the safety of infant formula supplemented with *B. longum* and *L. rhamnosus* LPR in normal healthy term infants. The outcomes of weight gain, changes in length, head circumference and BMI after 6 months of intervention were similar in the probiotic and placebo groups. In addition, the long term follow-up to 2 years of age found no significant difference between the weight, length/height, head circumference and BMI of the two treatment groups. During both periods infants in both groups grew normally as the weight-for-age, length-for-age, BMI-for-age and head circumference z-scores indicated growth rates comparable to the WHO Child Growth Standards. These are good indications of the nutritional sufficiency and adequate growth of both the probiotic-supplemented and normal cow's milk based formulas fed subjects in our study as these standards are based on data from healthy, exclusively breastfed infants.

Although not statistically significant, the BMI between the placebo and probiotics supplemented groups revealed that the probiotics group demonstrated higher BMI compared to the placebo group consistently to 24 months of age even though the BMI at birth were similar (Figure 6.4). The BMI for age z-score in the probiotic group was closer to zero (the expected value for the reference distribution) from 3 to 12 months of age, indicating better growth status as WHO proposed the Child Growth Standards to be a standard for normal growth in infancy applicable throughout the world [264]. This is consistent with the study by Vendt et al. [189] which reported more weight gain, expressed in age-adjusted SDS (z-scores), at 3 months and better growth in length and weight at 6 months of age in the probiotic group compared with the placebo. BMI was not analysed in this study. Therefore the difference in BMI in our study suggests that there might be a difference in the effect of the probiotic-supplemented and placebo formula. Our results might not have been sufficiently powered to detect the difference as this was not a primary aim of the study and the limitations of these data are recognized.

Other studies observed similar normal growth in both probiotic-treated and placebo study groups. In a study in the United States, growth was similar in infants who received a standard milk-based formula containing *B. lactis* and *Streptococcus thermophilus* or unsupplemented formula [177]. Although, the change in z scores of weight, height and weight/length during the study period (210 ± 127 days) were not significantly different between the groups, the growth trend over the supplementation period was not analysed as only 2 data sets at entry and at discharge were reported.

Another study in France concluded that infants fed a mixture of probiotics or synbiotics showed similar weight gain and changes in length, head circumference, and

BMI measurements compared with those fed a control formula [190]. In spite of this, the higher z-scores for length at 12 months in the *B. longum* and *L. rhamnosus* LPR group suggested a possible effect of the probiotics compared with the control. However, the study was not designed with sufficient sample size to detect this difference. Furthermore, all of the comparisons in weight gain between treatment and control showed a trend toward better weight gain in the probiotics and synbiotics group. Similarly, the authors suggested a bigger sample size in future studies to see a difference in weight-for-age z-scores.

Probiotics may alter the gastrointestinal flora where different composition of gut microbiota may have direct action on the villous epithelium and determine differences in the efficiency of caloric extraction from food for energy storage [265]. Ley et al. [266] reported that obese human subjects have relatively less Bacteroides and more Firmicutes in the stools compared to lean human subjects. This was also confirmed in the study of gut microbiota of lean and obese mice where genetically obese mice had half the abundance of Bacteroidetes and higher proportion of intestinal Firmicutes compared to their lean siblings [267]. In another animal study, microbiota of obese and lean mice was transferred to lean germ-free recipient mice, and over a two week period, mice colonized with the microbiota from obese mice had significantly greater increase in total body fat than that of mice colonized by microbiota from lean mice [268]. Kalliomaki et al. study, with the primary aim to evaluate prevention of allergy with probiotic supplementation from birth, reported lower bifidobacteria and higher *Staphylococcus aureus* in the stools at 6 and 12 months of age being associated with increased likelihood of becoming overweight at 7 years of age [269].

Serial stool samples at 3 days, 1 month, 3 months, and 1 year were also collected from the subjects in our study. Stool microbiota analysis of 37 consecutive subjects with (n= 20) or without (n= 17) probiotic administration were reported recently [229]. The probiotics *B. longum* (p=0.005) and *L. rhamnosus* (p <0.001) were detected more frequently in probiotic subjects during supplementation, but no difference were found after the probiotic-supplemented formula intervention had stopped. More colony-forming units of lactic acid bacteria were also cultured in the stools of probiotic-supplemented babies at month 3 during treatment period (p=0.035). Transient alteration of gut flora in early life through probiotic supplementation can possibly results in programming and alteration of subsequent growth trajectory and adiposity gain.

Breastfed infants are generally healthier than formula-fed infants as breast milk is an optimal source of nutrition with complex oligosaccharides to selectively stimulate the growth of beneficial bacteria and inhibit the growth of pathogens [270]. In our study, feeding history did not differ between the study groups. At the end of the 6 months supplementation period, only 2% in the placebo and 3% in the probiotic group had near total breastfeeding with at least 60ml of trial formula. Majority of the subjects had some breastfeeding combined with formula feeding (77% in placebo and 65% in probiotic group) (Refer to Table 3.5 in Chapter 3). Introduction of solid food did not differ between the two groups. All subjects had been weaned to semi-solids by 12 months and the median age of weaning was 6 months for both groups (Refer to Table 3.6 in Chapter 3). All but one or two subjects in each group had egg yolk, egg white, fish and soy products and about 87% in the placebo group and 83% in the probiotics group took peanuts by 2 years of age. The feeding history in the two groups were

therefore comparable and should not have influenced the difference in growth significantly

This study confirms the adequate growth and safety in healthy probiotic supplemented infants. It also raises the possibility that probiotic supplementation leads to better growth which is closer to the WHO standards. This finding needs to be confirmed on longer follow-up as it can have significant impact on clinical practice and recommendations and also provides strong evidence for the influence of gut microbiota on early life programming.

Table 6-1 The growth characteristics (mean ± SD) of the study population (from birth to 3 months) with two-sample t-test for comparison between placebo and probiotic group

Measured Parameters (mean ± SD)	At Birth		Significance *P* values	1 month		Significance *P* values	3 months		Significance *P* values
	Placebo (n=126)	**Probiotic (n=127)**		**Placebo (n=121)**	**Probiotic (n=124)**		**Placebo (n=118)**	**Probiotic (n=123)**	
Length(cm)	49.76±2.78	49.62±2.32	0.656	54.55±2.55	54.22±2.60	0.316	61.40±2.85	61.36±2.62	0.908
Length z-scores	0.16±1.24	0.06±1.23	0.515	0.16±1.23	0.04±1.26	0.456	0.33±1.27	0.38±1.24	0.752
Weight (kg)	3.14±0.42	3.15±0.45	0.775	4.45±0.55	4.43±0.55	0.694	6.19±0.83	6.26±0.69	0.520
Weight z-scores	-0.37±0.92	-0.31±0.99	0.641	0.16±0.86	0.16±0.90	0.984	-0.21±1.11	-0.03±1.16	0.157
BMI (kg/m^2)	12.70±1.72	12.79±1.31	0.651	14.97±1.28	15.05±1.33	0.644	16.39±1.54	16.63±1.51	0.234
BMI z-scores	-0.69±1.00	-0.53±1.12	0.231	0.13±0.92	0.20±0.98	0.540	-0.23±1.03	-0.03±1.02	0.139
OFC (cm)	33.42±1.97	33.34±1.41	0.715	37.23±1.34	37.18±1.36	0.795	40.09±1.48	40.08±1.29	0.950
OFC z-scores	-0.75±1.00	-0.66±1.16	0.481	0.25±1.07	0.27±1.09	0.871	0.00±1.11	0.07±1.00	0.603

Table 6-2 The growth characteristics (mean ± SD) of the study population (from 6 to 24 months) with two-sample t-test for comparison between placebo and probiotic group

Measured Parameters (mean ± SD)	6 months		Significance P values	12 month		Significance P values	24 months		Significance P values
	Placebo (n=115)	**Probiotic (n=122)**		**Placebo (n=110)**	**Probiotic (n=122)**		**Placebo (n=108)**	**Probiotic (n=117)**	
Length(cm)	67.50±2.90	67.88±2.82	0.317	76.25±3.84	76.31±3.15	0.890	87.34±3.98	87.07±3.90	0.611
Length z-scores	0.31±1.20	0.48±1.13	0.276	0.51±1.47	0.53±1.15	0.923	0.25±1.23	0.20±1.29	0.752
Weight (kg)	7.69±1.01	7.82±0.877	0.299	9.41±1.21	9.56±1.16	0.347	12.14±1.76	12.38±2.11	0.358
Weight z-scores	-0.02±1.05	0.18±0.89	0.089	0.00±1.06	0.18±1.00	0.178	0.10±1.15	0.18±1.05	0.572
BMI (kg/m^2)	16.83±1.57	16.94±1.32	0.533	16.16±1.44	16.39±1.44	0.226	15.89±1.80	16.32±2.30	0.123
BMI z-scores	-0.27±1.05	-0.15±0.88	0.342	-0.34±1.01	-0.19±1.03	0.259	-0.09±1.33	0.11±1.36	0.239
OFC (cm)	42.64±1.58	42.73±1.47	0.668	45.78±1.54	45.96±1.50	0.358	48.40±1.59	48.26±1.37	0.495
OFC z-scores	-0.15±1.10	-0.02±1.06	0.347	0.16±1.06	0.32±0.99	0.264	0.44±0.99	0.41±0.94	0.800

Figure 6-1 Weight for age z-scores (Means ± SD), relative to WHO standards, during intervention period to 6 months and follow-up period up to 24 months of age

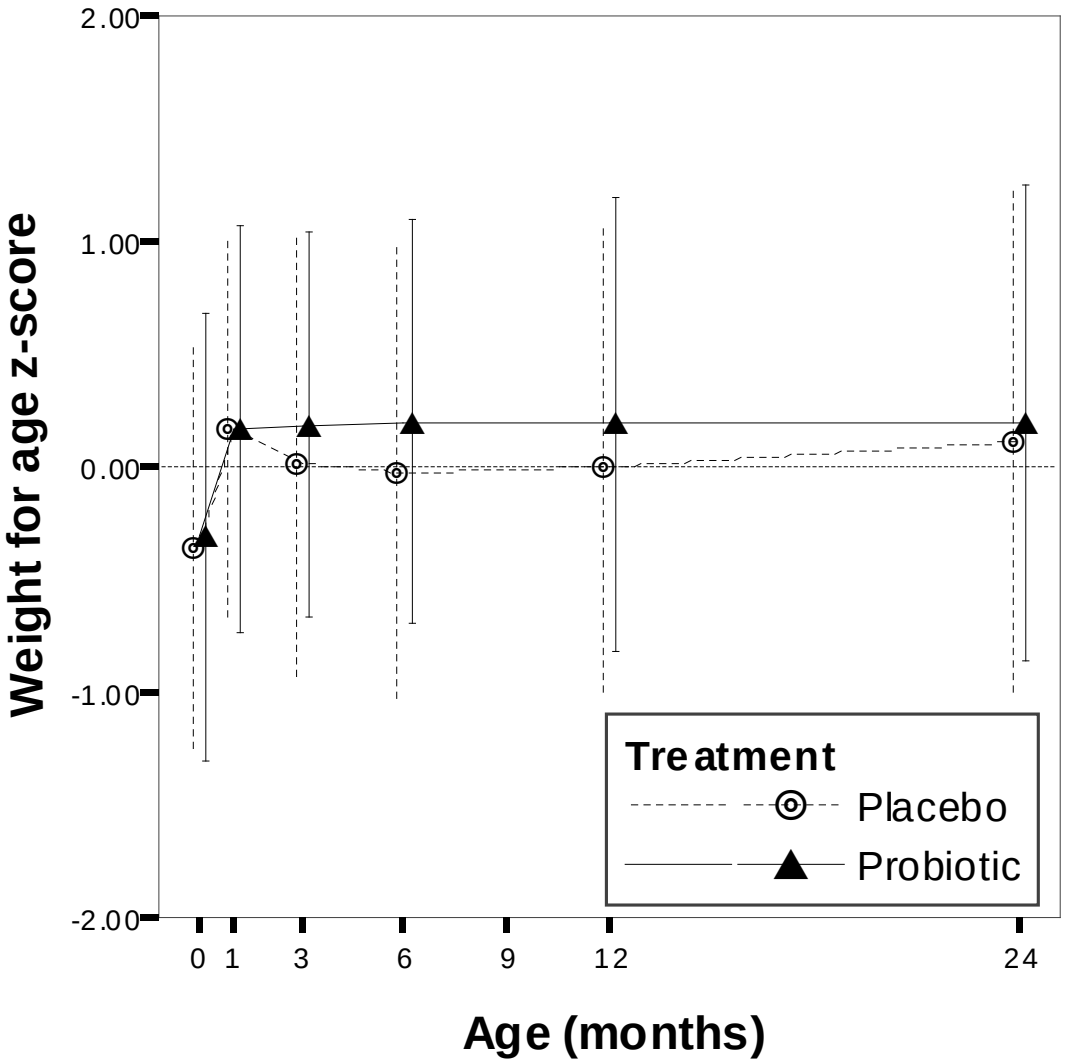


Figure 6-2 Length / Height for age z-scores (Means ± SD), relative to WHO standards, during intervention period to 6 months and follow-up period up to 24 months of age

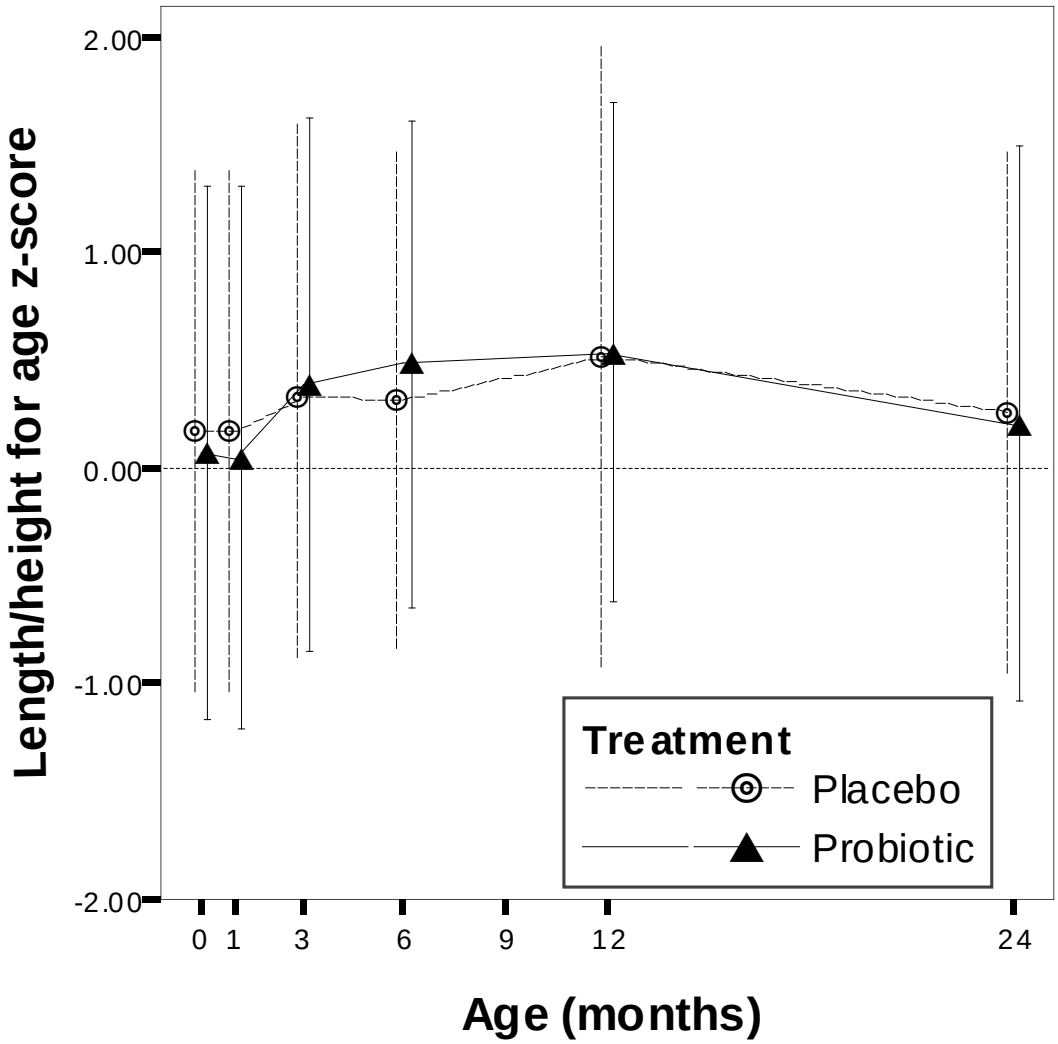


Figure 6-3 BMI (kg/m^2) for age z-scores (Means ± SD), relative to WHO standards, during intervention period to 6 months and follow-up period up to 24 months of age

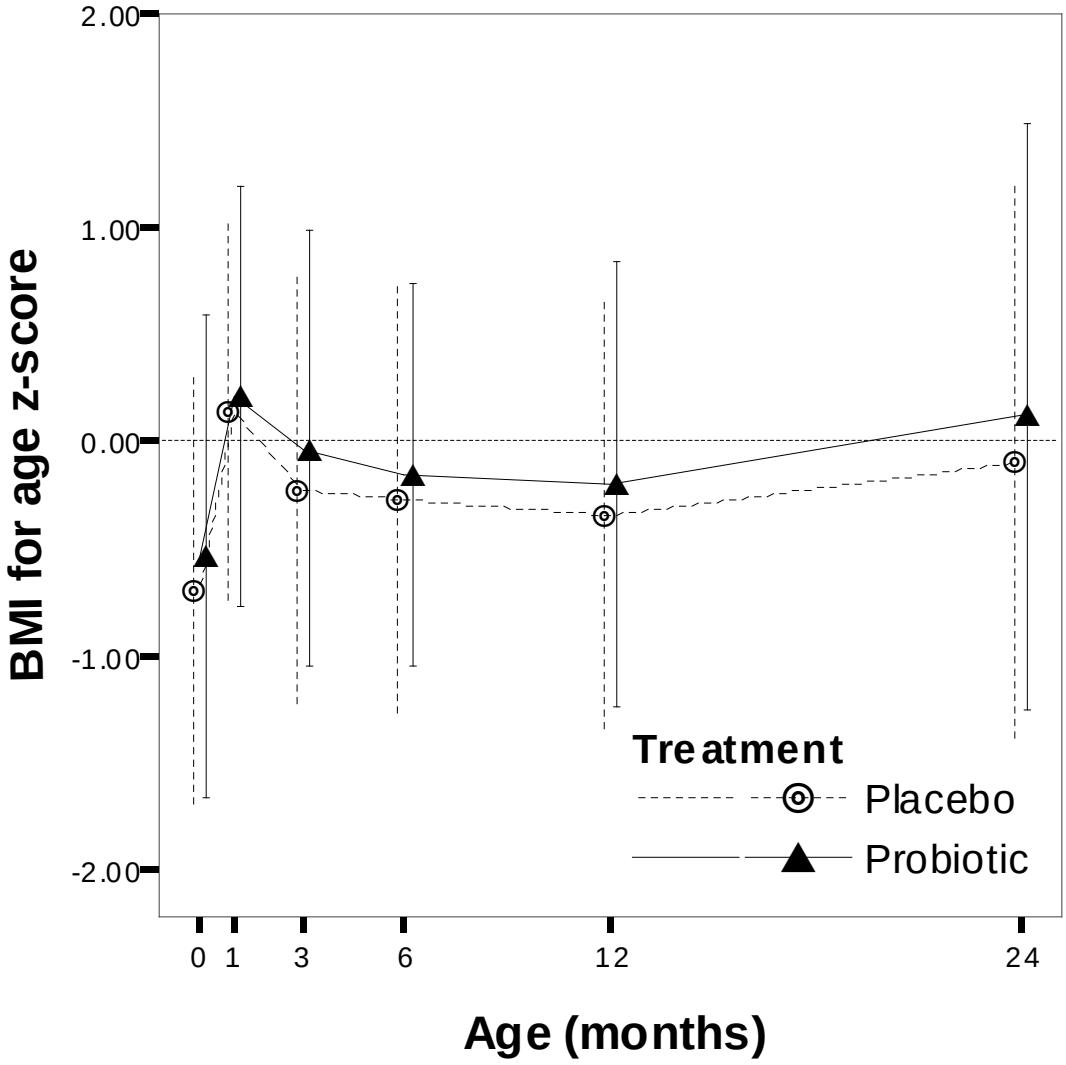
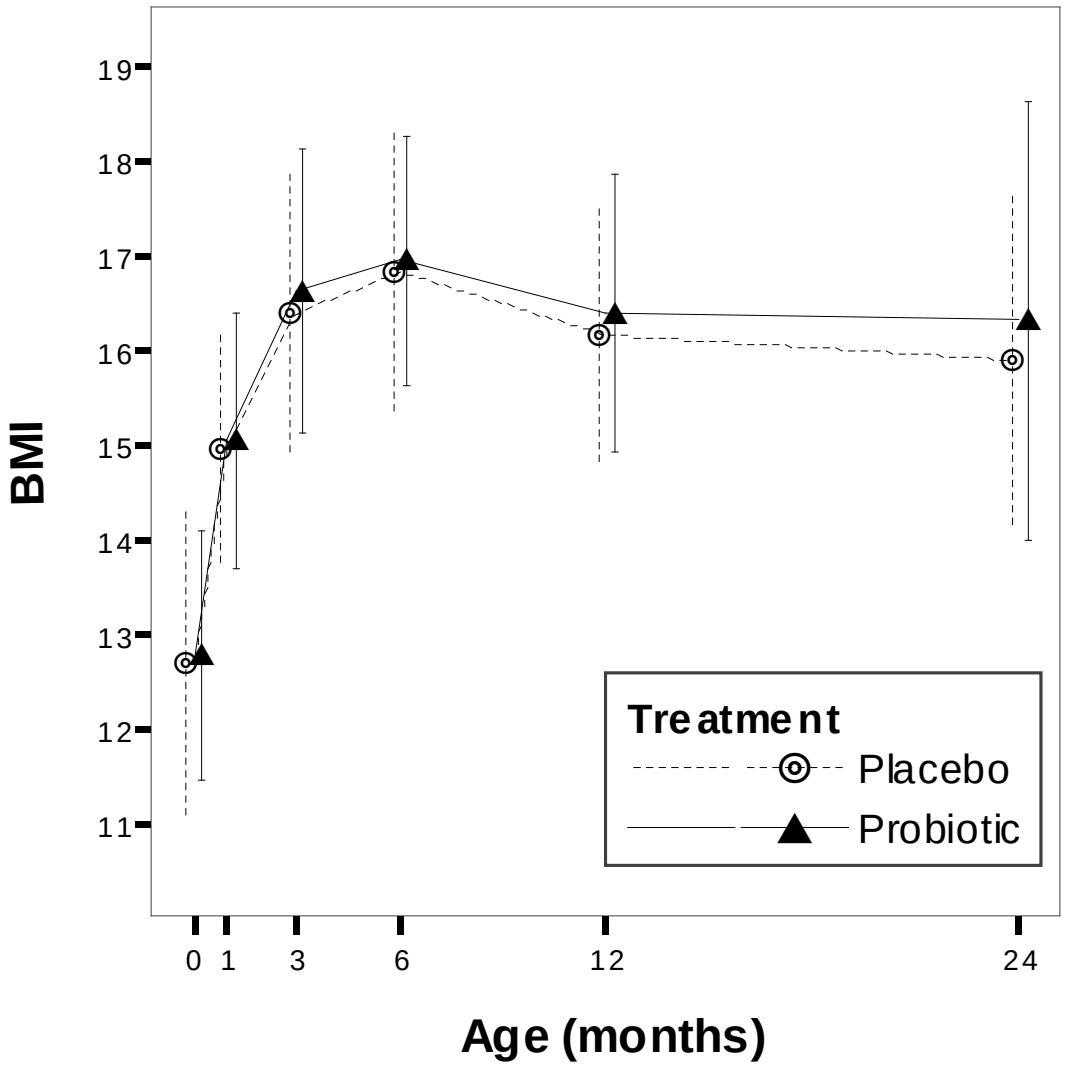


Figure 6-4 BMI (kg/m^2), Means ± SD, during intervention period to 6 months and follow-up period up to 24 months of age



* Mean differences in BMI between treatment groups from birth to 24 months of age (F=3.359, *p*=0.068) with adjustment for gender and feeding history (first 6 months) using Mixed model repeated measures analysis

Table 6-3 Mean (±SD) weight gain and changes in length, head circumference, and body mass index (BMI) for age and gender z-scores from birth to 6 months during intervention period with two-sample t-test for comparison between placebo and probiotic group

Measured Parameters (mean ± SD)	Birth to 1 month		*Significance P values*	1 to 3 months		*Significance P values*	3 to 6 months		*Significance P values*
	Placebo (n=119)	**Probiotic (n=123)**		**Placebo (n=117)**	**Probiotic (n=122)**		**Placebo (n=114)**	**Probiotic (n=121)**	
Length (cm) z-scores	0.01 ± 1.01	-0.42 ± 1.07	0.670	0.14 ± 1.02	0.31 ± 1.27	0.244	-0.11 ± 0.87	0.14 ± 1.30	0.300
Weight (kg) z-scores	0.51 ± 0.65	0.46 ± 0.59	0.498	-0.15 ± 0.68	0.00 ± 0.67	0.072	-0.05 ± 0.46	0.01 ± 0.57	0.292
BMI (kg/m^2) z-scores	0.79 ± 1.02	0.72 ± 1.13	0.606	-0.34 ± 1.04	-0.24 ± 1.04	0.475	-0.07 ± 0.72	-0.11 ± 0.95	0.724
OFC(cm) z-scores	1.00 ± 0.99	0.92 ± 1.07	0.549	-0.24 ± 0.91	-0.19 ± 0.95	0.685	-0.19 ± 0.79	-0.07 ± 0.83	0.273

Table 6-4 Mean (±SD) weight gain and changes in length, head circumference, and body mass index (BMI) for age and gender z-scores from 6 to 24 months during follow-up period with two-sample t-test for comparison between placebo and probiotic group

Measured Parameters (mean ± SD)	6 to 12 months		*Significance P values*	12 to 24 months		*Significance P values*
	Placebo (n=108)	**Probiotic (n=121)**		**Placebo (n=105)**	**Probiotic (n=115)**	
Length (cm) z-scores	0.16 ± 1.25	0.04 ± 1.07	0.443	-0.23 ± 1.11	-0.33 ± 1.18	0.509
Weight (kg) z-scores	0.00 ± 0.71	-0.005 ± 0.56	0.944	0.09 ± 0.81	0.06 ± 0.66	0.727
BMI (kg/m^2) z-scores	-0.08 ± 1.12	-0.05 ± 0.96	0.834	0.21 ± 1.28	0.35 ± 1.26	0.432
OFC(cm) z-scores	0.28 ± 0.87	0.34 ± 0.88	0.587	0.28 ± 0.84	0.08 ± 0.89	0.091

Chapter 7: Conclusions and Future Directions

Several specific aims were achieved in this dissertation: 1) to assess the effect of administration of *Bifidobacterium longum* and *Lactobacillus rhamnosus* supplemented cow's milk based infant formula from birth to 6 months on the prevention of allergic diseases, namely eczema, asthma, allergic rhinitis, and allergic sensitization in the first and second year of life among Asian infants at risk of allergic disease; 2) to investigate the effect of probiotic supplementation in the first 6 months of life on specific IgG antibody responses to Hepatitis B vaccination; 3) to determine the short and long term effect of early regular supplementation of probiotics in the infant diet on protective benefit against diarrhoeal and febrile illnesses; and 4) to document safety and impact on growth of newborn infants in this study with a 2 years follow-up period.

This double-blind, randomized, placebo controlled clinical trial on the supplementation of probiotics in the first six months of life in Asian infants at risk of allergic diseases did not show a protective effect of probiotic supplementation on eczema, asthma, allergic rhinitis or allergen sensitization at 1 and 2 years of age. Subset analysis of interactions between mode of delivery and probiotic intervention did not show a significant difference in prevalence of eczema, allergen sensitization and atopic eczema in caesarean-delivered babies supplemented with probiotics. The prevalence of eczema in our cohort (26%) was lower than the 39% in the placebo group at 1 year and 46% at 2 years in the other studies in Australia and Finland. An additional follow-up period will be critical for the evaluation of respiratory allergies as the low prevalence of 9% asthma and 2.5% allergic rhinitis at 2 years of age did not yet allow for their comparison. Furthermore, as different timing of supplementations,

dose and probiotic strain are used in various studies, additional studies need to be conducted to ascertain how probiotics exert their effects on allergic diseases.

Family history and elevated cord blood serum IgE were not found to influence the development of eczema, allergen sensitization, atopic eczema at 1 year of age. Parental atopy in combination with elevated cord IgE also fails to identify babies at risk of eczema and allergen sensitization. Paternal eczema was instead significantly associated while maternal atopy and mothers with eczema were not found to be a risk factor for eczema.

In the determination of the effects of probiotic supplementation to enhance the immunogenicity of Hepatitis B vaccine responses, our results show that the schedule with 3 monovalent doses of Hepatitis B vaccine resulted in better anti-HBs responses compared to the schedule consisting of 2 monovalent doses followed by a third dose as a DTPa combination vaccine, although this difference was not statistically different. Probiotics can potentially be used as an adjuvant to enhance immune responses in schedules with less than optimal responses, but these findings need to be explored in studies with larger sample size.

The effects of probiotics supplementation on protective benefit against acute infectious illnesses in infants were not demonstated in our study. Rates of febrile episodes, gastroenteritis and respiratory infections were similar in the probiotic and placebo groups during the intervention and follow-up period. Antibiotic usage and courses were also similar between the two groups. To note, more infants were hospitalized due to infections during the intervention period in the probiotic group

than in the placebo group but this difference was not observed later during the follow-up period. Although probiotics did not prevent common childhood infections, no adverse events related to the study formula was observed. However, it raises the possibility of increased hospitalization during probiotic supplementation period which needs to be further determined in more controlled clinical studies to confirm the safety of the administration of *Bifidobacterium longum* and *Lactobacillus rhamnosus* from birth.

In order to evaluate the safety of the probiotic formula in early infancy, we assessed the possible influence of these probiotics on growth. The outcomes of weight gain, changes in length, head circumference and BMI were similar in the probiotic and placebo groups during the intervention and long term follow-up period to 2 years of age. Adequate growth was observed during both periods in the 2 groups as the weight-for-age, length-for-age, BMI-for-age and head circumference z-scores indicated normal growth rates. Notably, the BMI between the placebo and probiotics supplemented groups revealed that the probiotics group had higher BMI compared to the placebo group consistently to 24 months of age even though the BMI at birth were similar. This difference in trend was near to significance. This study confirms that the supplementation of probiotics from birth yielded adequate growth similar to the infants in the placebo group. Despite appearing to be safe for newborn infants, probiotic supplementation can possibly leads to increase growth which needs to be confirmed on longer follow up.

In conclusion, the findings can have significant impact on clinical practice and recommendations, but further studies are needed to determine the role and complex

interaction of specific strains of probiotics and the developing immune system before probiotics should be recommended for use in the paediatric population.

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Appendix A

Parent Information Sheet

Study title:

A randomized double-blind placebo controlled trial to evaluate influence of probiotics on atopy, atopic disease and immunological responses.

The following information will describe the study and the role of you and your child as a participant. The investigator will answer any questions you may have about this form and about the study. Please read carefully and do not hesitate to ask anything about the information provided below.

Purpose of the Study

Allergic diseases are on the rise all over the world. Children may become allergic to various substances early in life. These allergies predispose them to developing asthma, eczema and allergic rhinitis. There is increasing evidence that the bacteria of the child's intestine plays a role in the development of these allergies, with some bacteria protecting the child from them.

We plan to invite 300 infants who are at risk of developing allergic disease to participate in this study. The purpose of this study is to assess the protective effect of adding probiotics, which is "helpful bacteria" found in Nan II ® (a milk formula) and in drinks like Yakult® and Vitagen®, to your baby's milk formula. One hundred and fifty babies will be given standard infant formula, and the other 150 babies will be given the milk formula containing the probiotics. Which formula your baby receives will be decided randomly. This means that there is a fifty percent chance of receiving either the standard milk formula, or the milk formula containing the probiotics.

The allergic status of your child will be determined through examination, skin prick test and blood tests. Stool samples will also be collected and analyzed. The relationship between allergy and stool bacteria will be studied.

Who can participate?

i. Either one parent or a sibling has a history of allergies, as this will put your unborn baby at higher risk of developing allergies. This will be confirmed by a doctor-diagnosis of asthma, allergic rhinitis or eczema AND a positive skin prick test/specific IgE test to any of a panel of common allergens.

ii. Parents agree to the child's participation in the study. The parents are willing to comply with procedures and are able to keep to scheduled clinic visits.

iii. Your child is born at above 35 weeks gestation and weighs above 2kg.

iv. Your child does not have major congenital malformations/major illness as judged by the doctor.

v. Your child is in otherwise good, stable health on the basis of medical history, physical examination, and the family appears to be able to successfully complete this trial.

Study Procedures

You will be approached by the nurse to participate in this study. Participation is entirely voluntary. We would like to remind parents that ***breast-feeding is the best form of nutrition for the child and we will invite you to participate in this trial only if you decide NOT TO TOTALLY breast-feed your child.***

If your child enters the study, he/she will receive either Nan I®, a commercially available milk formula, or Nan I with Lactobacilli and Bifidobacteria, which are the probiotics we are using. Which formula your child receives will be decided randomly. Once decided, you will be provided the same formula till your baby is 6 months old.

You are encouraged to breast feed your baby. If you agree to participate in this trial, you will be required to use the milk formula provided, and ensure that your child gets at least one 60ml feed with the formula everyday.

Feeding with this formula will be for the first 6 months, after which you can choose to use any formula. You will need to bring your child to visit the clinic at 1, 3, 6, 12 and 24 months of age. Each visit will last about 30 minutes. At each visit, milk formula sufficient until the next visit will be provided. A doctor will examine your child as well. Stool samples will be collected within 72 hours of age and at 1, 3, and 12 months clinic visits. You will be given instructions on how to do this closer to the time. Which formula you receive will be decided randomly, in a way similar to the tossing of a coin.

Blood samples will be collected at birth from the umbilical cord, and at 1 year of age. EMLA® cream, which is an anaesthetic cream, will be applied to your child's hand. About 30 minutes later, a sample of approximately 5 ml (1 teaspoon)of blood will be obtained from your child. If it is not obtainable, we would do a skin prick test for your child. You will not have to pay for any of these tests done for this study. Skin prick test will also be performed when your child is 2 years of age.

Hepatitis B vaccination will be provided free of charge to your child at 1 and 6 months. This is in keeping with the Singapore vaccination schedule. The nurses will also make telephone contacts with you once a month to check on the health of your child and to answer any queries you may have.

Prior Experience with the Probiotics, *Lactobacillus rhamnosus GG* and *Bifidobacterium longum*

Both *Lactobaccillus rhamnosus GG* and *Bifidobacterium longum* are probiotics of human origin. They do not cause disease in humans. They have been shown to be safe, tolerable and acceptable in neonates, resulting in normal growth with no harmful effects. These probiotics are widely used in different food products and infant formula.

Tests that will be performed

Cord blood (about 100 ml) will be drawn from the placenta at birth. The procedure will not affect your baby's health. The blood will be tested for both genetic and immune responses related to allergy and asthma. These tests will contribute towards research into predicting allergy and asthma.

The blood sample taken at 1 year will be analyzed for evidence of allergies to common substances. These substances include egg, milk, fish, soy, peanuts and 2 dust mites. In the event that blood sample is not obtainable, a skin prick test with the substances will be done. Skin prick test to the above allergens will also be performed at 2 years of age. The stool samples will be analyzed for bacteria and bacteria products.

Discomforts and Risks

The blood test may cause some discomfort to your child. This discomfort is minimized by using an anaesthetic cream which is usually effective enough to remove any pain during needle puncture. The skin prick test may cause some itch at the test site, and hdrocortisone cream will be used to sooth the itch if necessary. There is no risk of a major allergic reaction as only a very small dose of allergen will be used, and this is a superficial skin test with minimal introduction of allergen to the person.

Possible Benefit to Participants

You will be provided with free milk formula for 6 months. Your child will be followed up by the doctors at the National University Hospital. We will perform tests for allergy in your child at 1 year of age.

Your child's 2nd and 3rd dose hepatitis B vaccination will be provided for free at NUH.

This study will provide doctors with information about allergies in young children and the possible protective effect of certain bacteria. This will help the doctors in designing strategies to prevent allergy.

Re-imbursement / Costs involved in Study

The study does not require you to make any additional visits to the clinic. Visits coincide with standard visits for infant vaccination.

You will be given S$20.00 on each scheduled clinic visit to cover transport costs.

Confidentiality

The blood samples taken will be used solely for the purposes of this study, and will be destroyed once this study is over. Your child will not be identified in any reports or publications resulting from the study.

Parties to Contact

The investigator or her designate will be happy to answer any questions you may have. If you have additional questions during the course of this study about the research or your child's rights as a research subject, you may address them to Dr Marion Aw or Dr Lynette Shek at 67724420 at NUH.

For an independent opinion, you may contact a member of the NHG Domain Specific Review Board (Attn: Dr Sujatha Sridhar) at 64713266.

Voluntary participation / Right to Withdrawal

Your child's participation in this study is voluntary. You may decide for your child not to participate and may discontinue participation at any time during the entire duration of the study without penalty or loss of benefits to which your child is otherwise entitled.

New Findings

You will be told of any significant new findings developed during the course of this study which may relate to your willingness to continue your participation.

CONSENT FORM

SQNU01 - A randomized double-blind placebo controlled trial to evaluate influence of probiotics on atopy, atopic disease and immunological responses

I have read the patient information sheet and understand the contents. My questions have been answered. I voluntarily consent for my child to be enrolled in the above study.

This study has been explained to me in _____ (state language if not in English) by _____ (name of translator) on _____ (date)

PARENT'S/GUARDIAN'S PARTICULARS

Parent/Guardian's Name:……Parent/Guardian's Identity No:……

Parent/Guardian's signature……Date:……(dd/mm/yy)

INVESTIGATOR'S PARTICULARS

I have explained the nature and purpose of the study to the child's parent (s)/guardian.

Investigator's Name:……..

Investigator's signature:……..Date:……...(dd/mm/yy)

WITNESS'S OR TRANSLATOR'S PARTICULARS

Witness's Name:…….

Witness's signature:……..Date:…….....(dd/mm/yy)

To be completed once the information is available:
Child's particulars

Child's Name……Birth Certificate No……

Completed by: ……..Date:……

Version 4, dated 25/10/2005

Appendix B

Screening Form

A Randomised Double-Blind Placebo-Controlled Clinical Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

GENERAL INFORMATION *(Please fill in the boxes with the appropriate number/details)*

1. Gender ☐ 1. Male 2. Female

2. Ethnic group ☐ 1. Chinese 2. Malay
3. Indian 4. Other, specify _____

3. Gestation ☐☐ weeks + ☐ days

4. Mode of delivery ☐ 1. LSCS
2. Vaginal delivery

5. Time of delivery ☐☐ : ☐☐ hr

PHYSICAL EXAMINATION *(Screened by the neonatologist at birth)*

6. Weight ☐☐ . ☐☐ kg

7. Height ☐☐☐ . ☐ cm

8. Head Circumference ☐☐ . ☐ cm

9. Heart ☐ 1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

10. Respiratory ☐ 1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Version Date 1.1: 6 July 2004

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

11. Abdominal ☐ 1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

12. Neurologic ☐ 1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

13. Other abnormalities ☐ 1. Yes, specify _____
2. No

BABY'S POST-NATAL HISTORY *(Please fill in the boxes with the appropriate number)*

14. Admission to ☐ 1. Post-natal ward
2. Special Care Nursery
3. Neonatal ICU

15. Antibiotics used ☐ 1. Yes, specify _____
2. No

16. Post-natal complications ☐ 1. Yes, specify _____
2. No

17. Date of first formula feed ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

18. Time of first formula feed ☐☐ : ☐☐ hr

19. Date of discharge ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

MOTHER'S ANTENATAL HISTORY *(Please fill in the boxes with the appropriate number/details)*

20. Did the mother have any illness during pregnancy? ☐ 1. Yes
2. No

S/N	*Trimester	Duration	Diagnosis	Medication given

*** Fill in the period of trimester in the box. eg 1st, 2nd or 3rd trimester.**

21. Has the mother ever smoked when she was pregnant with this child in:

Trimester 1 ☐ 1. Yes
2. No

Trimester 2 ☐ 1. Yes
2. No

Trimester 3 ☐ 1. Yes
2. No

22. Post-natal complications in mother

☐ 1. Yes, *Specify complications* _____
2. No

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

FAMILY HISTORY *(Please fill in the boxes with the appropriate number)*

23. How many brothers and sisters does this child have? ☐☐

24. Birth order of this child ☐☐

25. Anyone in the house smoke? ☐ 1. Yes
2. No ***(Skip Question 26 & 27))***

26. Who is this person? ***(may tick more than one box)***
☐ Father ☐ Mother ☐ Others, specify _____

27. Frequency of smoking exposure ☐ 1. Daily
2. More than once per week
3. Others, specify _____

28. Do you keep pets? ☐ 1. Yes, *specify what animal* _____
2. No

29. Anyone in the house has the following? ***(may tick more than one box)***

a. Asthma ☐ Father ☐ Mother ☐ Siblings, specify _____

b. Allergic rhinitis (Hayfever) ☐ Father ☐ Mother ☐ Siblings, specify _____

c. Allergic Dermatitis (Eczema) ☐ Father ☐ Mother ☐ Siblings, specify _____

If Yes to any of the above, please give details of diagnosis (frequency, duration of atopy and medication)

_____

_____

_____

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

PARENTS' PARTICULARS *(Please fill in the boxes with the appropriate number)*

30. Total monthly family income
☐ 1. Below $2000
2. $2000-$3999
3. $4000-$5999
4. More than $6000

31. Highest level of education completed

a. Father
☐ 1. Primary
2. Secondary
3. Tertiary

b. Mother
☐ 1. Primary
2. Secondary
3. Tertiary

c. Type of Housing
☐ 1. Public housing
2. Private housing (Condominium)
3. Landed property

DISPENSING OF MILK FORMULA *(Please fill in the boxes with the appropriate number)*

32. Dispensing of milk formula
☐ 1.Yes, specify _____ cans
2. No, specify reason _____

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Appendix B - *Follow-up Form*

A Randomised Double-Blind Placebo Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

To be completed on month 1, month 3, month 6, month 12 & month 24 visits

Date of visit ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

Type of visit ☐
1. Month 1
2. Month 3
3. Month 6
4. Month 12
5. Month 24

ADVERSE EVENT *(Please fill in the box with the appropriate number)*

1. Did the child encounter any problems or side effects after consuming the milk formula?

☐ 1. Yes, ***complete the adverse event form***
2. No

COMPLIANCE REVIEW *(Please fill in the boxes with the appropriate number)*

2. Was the Diary chart returned? ☐
1. Yes
2. No, specify _____

3. Did the child drink the trial milk formula at least 60ml per day since last visit? ☐
1. Yes
2. No, specify _____

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Version Date 1.2: 7 September 2004

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

PHYSICAL EXAMINATION *(Please fill the boxes with the appropriate number)*

4. Weight ☐☐.☐☐ kg

5. Height ☐☐☐.☐ cm

6. Head Circumference ☐☐.☐ cm

7. Heart ☐
1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

8. Respiratory ☐
1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

9. Abdominal ☐
1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

10. Neurologic ☐
1. Normal
2. Abnormal, specify _____
3. Not done, specify _____

11. Other abnormalities ☐
1. Yes, specify _____
2. No

12. SCORAD ☐☐

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

FEEDING HISTORY *(Please fill in the boxes with the appropriate number)*

13. How was your child fed? ☐
    1. Total breastfeeding with at least 60ml of trial formula on most days (>80% of the time) ***(Skip to Q15)***
    2. Breastfeeding and formula feeding
    3. Total formula feeding

14. Was your child fed any other formula? ☐
    1. Yes, specify _____
    2. No

PRESENCE OF ATOPIC DISEASE *(Please fill in the boxes with the appropriate number)*

15. Has your child had wheezing in the chest since the last visit?
    ☐ 1. Yes ☐☐ times
    2. No

16. Has the child had a problem with sneezing or a runny nose when he/she DID NOT have a cold or flu?
    ☐ 1. Yes, at age ☐☐ . ☐ months
    2. No

17. How many fever episodes (>38.5˚C) has your child have since the last visit? ☐☐ episodes

18. How many diarrhea episodes did your child have since the last visit? ☐☐ episodes

19. How many antibiotic courses (for ≥3 consecutive days) did your child has since the last visit?
    ☐☐ episodes

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

EXPOSURE TO ALLERGENS *(Please fill in the boxes with appropriate number)*

20. Does your child attend day care? ☐ 1. Yes, at age ☐☐ months
2. No

21. No. of people in the household ☐

22. Ever kept pets since the last visit? ☐ 1. Yes, specify what animal_____
2. No

23. Anyone in the house smoke? ☐ 1. Yes
2. No ***(Skip to Q25)***

24. Who is this person? ***(may tick more than one box)***
☐ Father ☐ Mother ☐ Others, specify _____

25. Frequency of smoking exposure ☐ 1. Daily
2. More than once per week
3. Others, specify _____

26. Ever exposed to pet? ☐ 1. Yes, specify _____
2. No

DISPENSING OF MILK FORMULA *(Please fill in the boxes with appropriate number)*

27. Dispensing of milk formula ☐ 1.Yes, specify _____ cans
2. No, specify reason _____

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

SQNU01: PRELIMINARY ADVERSE EVENT FORM

A Randomised Double-Blind Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form to CTERU after completion

Subject Initials ☐☐☐☐ **Trial No.** ☐☐☐☐ **Date of Notification** ☐☐/☐☐/☐☐☐☐ **DD MM YYYY**

ADVERSE EVENTS

1= Abdominal pain > 3 days	**5= Diarrhoea > 3 days**	**9= Fever**	**13= Vomiting > 3 days**
2= Asthma	**6= Eczema**	**10= Oral Thrush**	**14= Wheezing**
3= Colic > 6 days	**7= Extensive Diaper Dermatitis (nappy rash)**	**11= Undiagnosed rash (specify area)**	**15= Others**
4= Constipation > 1 week	**8= Extensive Seborrhoeic Dermatitis**	**12= Upper Respiratory Tract Infection**	

ADVERSE EVENTS *Please fill in the box with the above-defined adverse event.*	**DATE OF ONSET** *DD-MM-YYYY*	**END DATE** *DD-MM-YYYY* *Please tick box if continue post trial.*	**DURATION OF EVENT IF LESS THAN 24 HOURS**	**SOURCE OF MEDICAL CONSULTATION** **1=Prompt Doctor 2=Polyclinic 3=General Practitioner 4=Paediatrician 5=Others** *(Please provide name of GP or Pediatrician)*	**FREQUENCY** **1=Single episode 2=Intermittent**	**SEVERITY Is this a serious adverse event?** **1=Yes 2=No** *If Yes, please fill in the Serious adverse event form*	**OUTCOME** **1=Resolved 2=Unresolved 3=Unknown 4=Fatal 5=Others** Specify ***If fatal,*** *please fill in the serious adverse event form*	**ACTION TAKEN** **With study formula:** **1=None 2=Stop & restart 3=Discontinue** *If discontinue, please fill in the study summary form*	**ACTION TAKEN** *If re-start, state the number of days between stopping and restarting the formula*	**ACTION TAKEN** **Was medication used to treat event** **1=Yes** *(please complete page 2)* **2= No**
1. ☐ Specify _____		☐	___hrs___mins	☐	☐	☐	☐ Specify_____	☐		☐
2. ☐ Specify _____		☐	___hrs___mins	☐	☐	☐	☐ Specify_____	☐		☐
3. ☐ Specify _____		☐	___hrs___mins	☐	☐	☐	☐ Specify_____	☐		☐
4. ☐ Specify _____		☐	___hrs___mins	☐	☐	☐	☐ Specify_____	☐		☐

Investigator's Signature: _____ **Date:** _____/____/_____ **(DD/MM/YYYY)**

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

SQNU01: PRELIMINARY ADVERSE EVENT FORM

A Randomised Double-Blind Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Adverse Event Number	Medication or other treatment	Dose	Frequency	Route	Start Date	Stop Date	Indication

Form Completed By _____ **Date:** _____/____/_____ **(DD/MM/YYYY)**

Investigator's Signature: _____ **Date:** _____/____/_____ **(DD/MM/YYYY)**

Appendix B - *Serious Adverse Event Form*

SQNU01: SERIOUS ADVERSE EVENT FORM

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

A Randomised Double-Blind Controlled Trial to Compare Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please complete this form and fax (065) 6324 2790 within 24 hours of awareness to Clinical Trials & Epidemiology Research Unit, Singapore. Please fill in neatly.

Subject initials	Trial no.	Date of reporting event
		DD / MM / YYYY

DETAILS OF SERIOUS EVENT

1. Date of onset of event ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

2. What was the events (please provide diagnosis if known) _____

_____

_____

_____

_____

3. Why was the event serious? ☐

1. Death
2. Persistent or significant disability/incapacity
3. Requires hospitalisation
4. Prolong hospitalisation
5. Fatal or immediately life-threatening
6. Is a congenital anomaly
7. Others, specify _____

Concomitant medication / Other Treatment	Dose	Frequency	Route	Date Started	Date Stopped	Indication for using drugs
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐ (DD / MM / YYYY)

Version Date 1.1: 6 July 2004

Subject initials	Trial no.	Date of reporting event
		DD / MM / YYYY

4. What in your opinion caused the event? ☐
   1. Study formula
   2. Concurrent disorder
   3. Others, specify _____

5. Outcome ☐
   1. Recovered
   2. Recovered with residual effects
   3. Not yet recovered
   4. Died due to this adverse event
   5. Died, other causes, specify _____
   6. Unknown
   7. Others, specify _____

6. Stopped study formula? ☐
   1. Yes
   2. No

7. Need to break code? ☐
   1. Yes, specify why _____
   2. No

8. Investigator's comment (if any) : _____

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐ (DD / MM / YYYY)

Appendix B - *Weaning Practices Form*

SQNU01: WEANING PRACTICES FORM

A Doubled-Blind, Randomised Placebo-Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

TO BE COMPLETED ONLY WHEN THE CHILD HAS BEEN WEANED*(Please fill in the boxes with the appropriate number)*

1. Has your child been weaned? ☐ 1. Yes 2. No

Age at weaning: ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 2. Month 6 3. Month 12 4. Month 24

2. Has your child taken

a. Egg yolk ☐ 1. Yes 2. No

Age of first intake ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 2. Month 6 3. Month 12 4. Month 24

b. Egg white ☐ 1. Yes 2. No

Age of first intake ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 2. Month 6 3. Month 12 4. Month 24

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Version Date 1.2: 7 September 2004

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

WEANING PRACTICES (CONT.)

2. Has your child taken

c. Fish ☐ 1. Yes
2. No

Age of first intake: ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 3. Month 12
2. Month 6 4. Month 24

2. Has your child taken

d. Soy products ☐ 1. Yes
2. No

Age of first intake: ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 3. Month 12
2. Month 6 4. Month 24

2. Has your child taken

e. Peanuts ☐ 1. Yes
2. No

Age of first intake: ☐☐ . ☐ months

Type of visit ☐ 1. Month 3 3. Month 12
2. Month 6 4. Month 24

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Appendix B - *Skin Prick Test Form*

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

SQNU01: SKIN PRICK TEST FORM

A Randomised Double-Blind Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

(Please fill in the boxes with the appropriate number/details)

SKIN PRICK TEST

1. Is skin prick test done? ☐ 1. Yes
   2. No, specify _____

2. Type of visit ☐ 1. Month 12
   2. Month 24

3. Date of skin prick test done ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

4. Details of Allergens:

Serial	Allergens	Test Done 1. Yes 2. No	Results 1. Positive 2. Negative	Erythema (mm)	Wheal (mm)
a)	Cow's Milk	☐	☐	☐☐x☐☐	☐☐x☐☐
b)	Egg White	☐	☐	☐☐x☐☐	☐☐x☐☐
c)	Egg Yolk	☐	☐	☐☐x☐☐	☐☐x☐☐
d)	Fish (Cod)	☐	☐	☐☐x☐☐	☐☐x☐☐
e)	Soy Protein	☐	☐	☐☐x☐☐	☐☐x☐☐
f)	Peanut	☐	☐	☐☐x☐☐	☐☐x☐☐
g)	Shrimp	☐	☐	☐☐x☐☐	☐☐x☐☐
h)	Der p	☐	☐	☐☐x☐☐	☐☐x☐☐
i)	Blo t	☐	☐	☐☐x☐☐	☐☐x☐☐
j)	Others, specify _____	☐	☐	☐☐x☐☐	☐☐x☐☐
k)	Others, specify _____	☐	☐	☐☐x☐☐	☐☐x☐☐

Remarks _____

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Appendix B - *Blood /Skin Prick Test Form*

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

SQNU01: BLOOD/SKIN PRICK TEST FORM

A Randomised Double-Blind Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

To be collected at birth and month 12

BLOOD TEST DATA *(Please fill in the boxes with the appropriate number/details)*

1. Type of visit ☐ 1. At birth
   2. Month12

2. Is blood taken? ☐ 1. Yes
   2. No, specify reason _____

3. Type of blood taken ☐ 1. Cord blood ***(taken at birth only)***
   2. Total IgE ***(taken at 1 year of age) OR***
   3. Skin prick test ***(go to skin prick test section, pg2)***

4. Date of blood collection ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

5. Total Immunoglobulin E (IgE) ☐☐ · ☐☐ mg/dl

Please tick accordingly

6. Allergens used:

a. Cow's milk ☐ 1. Yes Level _____KU/L
   2. No

b. Egg white ☐ 1. Yes Level _____KU/L
   2. No

c. Fish ☐ 1. Yes Level _____KU/L
   2. No

d. Soy protein ☐ 1. Yes Level _____KU/L
   2. No

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Version Date 1.1: 6 July 2004

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

e. Peanut ☐ 1. Yes 2. No Level : _____KU/L

f. Dust mite 1 ☐ 1. Yes 2. No Level : _____KU/L

g. Dust mite 2 ☐ 1. Yes 2. No Level : _____KU/L

Remarks _____

_____

SKIN PRICK TEST *(Please fill in the boxes with the appropriate number/details)*

7. Is skin prick test done? ☐ 1. Yes 2. No, specify _____

8. Date of skin prick test done ☐☐ / ☐☐ / ☐☐☐☐ (DD/MM/YYYY)

Please tick accordingly

9. Allergens used:

a. Cow's milk ☐ 1. Yes 2. No ☐ Positive *E _____ ☐ Negative *W _____

b. Egg white ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

c. Fish ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

d. Soy protein ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

Continued on next page

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

e. Peanut ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

f. Dust mite 1 ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

g. Dust mite 2 ☐ 1. Yes 2. No ☐ Positive E _____ ☐ Negative W _____

*** E: Erythema**

W: Wheal

Remarks _____

_____

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐ DD / MM / YYYY

Appendix B - *Atopic Disease Form*

CLINICAL TRIALS & EPIDEMIOLOGY RESEARCH UNIT

SQNU01: ATOPIC DISEASE FORM

A Doubled-Blind, Randomised Placebo-Controlled Trial to Evaluate Influence of Probiotics on Atopy, Atopic Disease and Immunological Responses

Please return this form after completion to Clinical Trials & Epidemiology Research Unit, Singapore.

Subject initial ☐☐☐☐ Trial no. ☐☐☐☐☐

TO BE COMPLETED ONLY WHEN THE CHILD HAS BEEN DIAGNOSED WITH THE ATOPIC DISEASE I.E. ALERGIC RHINITIS, ASTHMA AND ECZEMA *(Please fill in the boxes with the appropriate number)*

1. Has this child been diagnosed with **allergic rhinitis**?

☐ 1. Yes — 1st diagnosed at age ☐☐ . ☐ months

2. No

Type of visit ☐

1. Month 1
2. Month 3
3. Month 6
4. Month 12
5. Month 24

2. Has this child been diagnosed with **asthma**?

☐ 1. Yes — 1st diagnosed at age ☐☐ . ☐ months

2. No

Type of visit ☐

1. Month 1
2. Month 3
3. Month 6
4. Month 12
5. Month 24

3. Has this child been diagnosed with **eczema**?

☐ 1. Yes — 1st diagnosed at age ☐☐ . ☐ months

2. No

Type of visit ☐

1. Month 1
2. Month 3
3. Month 6
4. Month 12
5. Month 24

Investigator's signature _____ Date ☐☐ / ☐☐ / ☐☐☐☐
DD MM YYYY

Version Date 1.1: 6 July 2004

Appendix C

Diary Chart

PROMPT –Probiotics in milk for the prevention of atopy trial

(SQNU01 STUDY)

3 monthly Diary Chart

Name ……

BC/NRIC ……

Trial no……

IMPORTANT: Please remember to bring this Diary Chart with you at your next appointment

Instructions:

- Tick the dates on the calendar once your child has drunk the trial milk formula (at least 60ml).
- Put a cross if the trial milk formula is not given & write the reason in the "remark" section.
- If you have any problem, please do not hesitate to contact the following staff:

Ms Hor Chuen Yee : 91994920 (Hp)

For example:

Yr: 03 Mth: Nov

Date	✓ or x	Remark
1st	✓	
2nd	✓	
3rd	✓	
4th	x	Forgot
5th	x	Forgot
6th	✓	
7th	✓	

- Please list any side effects or problems
- Record the date they were first noticed & ended
- State type of treatment given

Side effects/ Problems	Date started	Date ended	Treatment given

Note: If your child develops any problems which you are concerned about, please do not hesitate to contact the study staff at : 91994920 (Hp)

From: **to:**

Please answer Q1 to Q6 accordingly. Since the last visit,	**Yes**	**No**
1. Has your child had wheezing in the chest?	☐	☐
2. Has your child had a problem with sneezing or a runny nose when he/she **DID NOT** have a cold or flu? If Yes, specify : _____ age	☐	☐
3. How many fever episodes (>38.5°C) has your child had? Specify : _____ times		
4. How often does your child pass stool? Specify : _____ times		
5. How many diarrhea episodes did your child have? Specify : _____ times		
6. How many antibiotic courses (for > 3 consecutive days) did your child have? Specify : _____ times		
7. What other medication has your child been given? Specify : _____		

Yr:____ Mth:____ Yr:____ Mth:____ Yr:____ Mth:____ Yr:____ Mth:____

Date	✓ or ✗	Remark	Date	✓ or ✗	Remark	Date	✓ or ✗	Remark	Date	✓ or ✗	Remark
1st			1st			1st			1st		
2nd			2nd			2nd			2nd		
3rd			3rd			3rd			3rd		
4th			4th			4th			4th		
5th			5th			5th			5th		
6th			6th			6th			6th		
7th			7th			7th			7th		
8th			8th			8th			8th		
9th			9th			9th			9th		
10th			10th			10th			10th		
11th			11th			11th			11th		
12th			12th			12th			12th		
13th			13th			13th			13th		
14th			14th			14th			14th		
15th			15th			15th			15th		
16th			16th			16th			16th		
17th			17th			17th			17th		
18th			18th			18th			18th		
19th			19th			19th			19th		
20th			20th			20th			20th		
21st			21st			21st			21st		
22nd			22nd			22nd			22nd		
23rd			23rd			23rd			23rd		
24th			24th			24th			24th		
25th			25th			25th			25th		
26th			26th			26th			26th		
27th			27th			27th			27th		
28th			28th			28th			28th		
29th			29th			29th			29th		
30th			30th			30th			30th		
31st			31st			31st			31st		

Appendix D

Phone contacts

PROMPT: Questions for Tel Interview

Trial ID: _____

Date called: _____

Called by: _____

Circle Answers

1. Did baby drink the trial formula at least 60ml/day? (**for cases <6mth**)	1. Yes 2. No, specify _____days missed
2. Has your child had wheezing in the chest since (last call)?	1. Yes, specify _____days 2. No
3. Has your child had a problem with recurrent sneezing or a runny nose or blocked nose when baby did not have a cold or flu?	1. Yes, specify _____days 2. No
4. How many fever episodes (>38.5^{o}C, >1/2 day) has your baby had since the last call?	1. 0 2. 1-2 3. 3-4 4. 5 or more
5. How many antibiotic courses (for >3 consecutive days) did baby have since the last call?	1. 0 2. 1-2 episodes 3. 3-4 episodes 4. >5 episodes
6. How often does your child pass stool since the last call?	1. < once a week 2. once a week 3. once in 3-4 days 4. 1-2 times a day 5. 2-4 times a day 6. > 4 times a day
7. How many diarrhea episodes did your child have since the last call? (each recurrence = 1 episode)	1. 0 2. 1-2 episodes 3. 3-4 episodes 4. > 5 episodes
8. Did your child have any rashes that need medication since the last call? Eczema?	1. Yes, Dx: _____ Med: _____ _____ 2. No
9. What other medication has your baby been given since the last call?	Med: _____ Duration: _____days Med: _____ Duration: _____days

Mar 05

Appendix E

SCORAD (SCOring Atopic Dermatitis) index

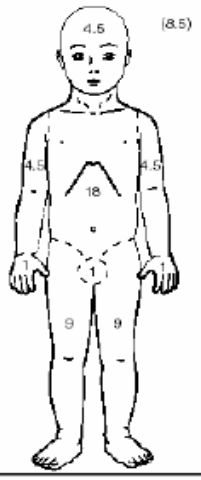
Based on SCORAD European Task Force on atopic dermatitis

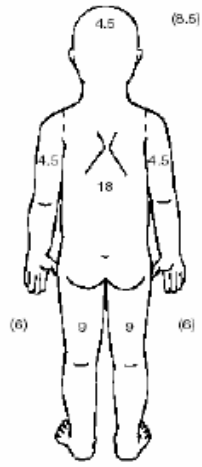
Name of assessor | Date of visit

Last name | First name | Date of birth

Topical steroid used | Amount/month (G)

Potency (brand name) | Number of flares/month





Mark the inflamed areas on the diagram and calculate percent of body surface affected.

(Do not include dry but non-inflamed areas).

Figures in brackets for children under two years

A: Extent (Please indicate the area involved)

For a typical affected area

B: Intensity

Criteria	Intensity
Redness	
Swelling or roughness	
Oozing/crust	
Scratch marks	
Thickening of skin and deeper skin creases	
Dryness of unaffected skin	

Means of calculation

Intensity items
(average representative area)

0 = absence

1 = mild

2 = moderate

3 = severe

C: Subjective symptoms pruritus + sleep loss

SCORAD A/5 + 7B/2 + C

Visual analog scale
(average for the last 3 days or nights)

Pruritis (0 to 10) | 0 10

Sleep loss (0 to 10) | 0 10

Recommended treatment:

Remarks:

Curriculum Vitae SOH Shu E

ACADEMIC QUALIFICATIONS:

2005	Bachelor of Science (2nd Upper class Honours), National University of Singapore
2000	GCE 'A' levels, Victoria Junior College
1998	GCE 'O' levels, Dunman High School

SCHOLARSHIPS AND AWARDS:

2006-2009	NUS Research Scholarship
2007	World Allergy Organization Travel Grant
2005	Dean's List AY2004/2005

RELEVANT COURSES ATTENDED:

2007	Workshop in Advanced Biostatistics: Hierarchical Data Analysis, TTSH
	Proper Conduct of Research for Study Coordinators (Advanced II), NHG
	Proper Conduct of Research (Intermediate I), NHG
	Proper Conduct of Research (Basic III), NHG
2006	Clinical Trial Management Course, NHG and Republic Poly
	Basic Biostatistics & SPSS Workshop, CTERU

PUBLICATIONS:

1. **SE Soh**, M Aw, I Gerez, YS Chong, M Rauff, YPM. Ng, HB Wong, N Pai, BW Lee, LPC Shek. 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. *Clinical & Experimental Allergy 2009; 39:571–578.*
2. BW Lee, LPC Shek, I Gerez, **SE Soh**, HPS Van Bever. Food Allergy - Lessons From Asia. *WAO Journal 2008;129-133.*
3. Wang XS, Shek LP, Ma S, **Soh SE**, Lee BW, Goh DYT. Time trends of co-existing atopic conditions in Singapore school children: prevalence and related factors. *Paediatric Allergy & Immunology 2009 Apr 21. [Epub ahead of print]*
4. KW Koh, **SE Soh**, GT Seah. Strong antibody responses to Mycobacterium tuberculosis PE-PGRS protein Rv3812 are associated with latent and active tuberculosis. *Infect Immun. 2009 Aug;77(8):3337-43.*
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ORAL AND POSTER PRESENTATIONS:

1. **Soh S E**, L P C Shek, M M Aw, D L Lim, B W Lee, Y S Chong and M H J Rauff. Eczema in the first year of life in high risk infants participating in a double-blind placebo controlled trial on the protective effect of supplementation of probiotics - preliminary data. *Annual Singapore Paediatric Congress ASPC 2006, Singapore.*
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3. IFA Gerez, **SE Soh**, JY Soh, PZ Ng, E Morales, S Ma, BW Lee, L Shek. Prevalence of Peanut & Tree-nut Allergy In Singapore Teenagers - Estimates from a Questionnaire Survey, Allergy Testing and Food Challenges. *World Allergy Congress 2007, Bangkok, Thailand.*
4. IFA Gerez, **SE Soh**, JY Soh, PZ Ng, E Morales, S Ma, BW Lee, L Shek. Prevalence of Shellfish Allergy In Singapore Children – Preliminary Data from a Questionnaire Survey, Allergy Testing and Food Challenges. *15th Annual PAPP (Philippine Academy of Pediatric Pulmonologists) and APAPARI (Asia-Pacific Association of Pediatric Allergy, Respirology and Immunology) Meeting, Manila, Philippines, 2007.*
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9. Chua KL, **Soh SE**, Ma S on behalf of the APAPARI Group. Paediatric Asthma Mortality and Hospitalization Trends Across Asia-Pacific – Relationship with Asthma Drug Utilization Patterns. *Asia Pacific Association of Pediatric Allergy, Respirology & Immunology APAPARI, Singapore, 2008.*
10. Wong YZI, **Soh SE**, Chng SY, Shek LPC, Goh DYT, Van Bever HPS, Lee BW. Compliance with Topical Nasal Medication – An Evaluation in Children. *Asia Pacific Association of Pediatric Allergy, Respirology & Immunology APAPARI, Singapore, 2008.*
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