doi: 10.1111/j.1365-2222.2012.03975.x
Clinical & Experimental Allergy, **42**, 1071–1079

ORIGINAL ARTICLE **Epidemiology of Allergic Disease**

A protective effect of *Lactobacillus rhamnosus* HN001 against eczema in the first 2 years of life persists to age 4 years

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Clinical & Experimental Allergy

Summary

Background Using a double blind randomized placebo-controlled trial (Australian New Zealand Clinical Trials Registry: ACTRN12607000518460), we have shown that in a high risk birth cohort, maternal supplementation from 35 weeks gestation until 6 months if breastfeeding and infant supplementation until 2 years with *Lactobacillus rhamnosus* HN001 (HN001) (6×10^9 cfu/day) halved the cumulative prevalence of eczema by age 2 years. *Bifidobacterium animalis* subsp *lactis* HN019 (HN019) (9×10^9 cfu/day) had no effect.

Objective The aim of this study was to investigate the associations of HN001 and HN019 with allergic disease and atopic sensitization among these children at age 4 years, 2 years after stopping probiotic supplementation.

Methods The presence (UK Working Party's Diagnostic Criteria) and severity SCORing Atopic Dermatitis (SCORAD) of eczema and atopy (skin prick tests) and parent-reported symptoms of asthma and rhinoconjunctivitis were assessed using standard protocols and questions.

Results Four-hundred and seventy-four infants were eligible at birth of whom 425 (90%) participated in this follow-up. The cumulative prevalence of eczema by 4 years (Hazard ratio (HR) 0.57 (95% CI 0.39–0.83)) and prevalence of rhinoconjunctivitis at 4 years (Relative risk 0.38 (95% CI 0.18–0.83)) were significantly reduced in the children taking HN001; there were also nonsignificant reductions in the cumulative prevalence of SCORAD $\geq$ 10 (HR 0.74 (95% CI 0.52–1.05), wheeze (HR 0.79 (95% CI 0.59–1.07)) and atopic sensitization (HR = 0.72 (95% CI 0.48–1.06)). HN019 did not affect the prevalence of any outcome.

Conclusions and Clinical Relevance This study showed that the protective effect of HN001 against eczema, when given for the first 2 years of life only, extended to at least 4 years of age. This, together with our findings for a protective effect against rhinoconjunctivitis, suggests that this probiotic might be an appropriate preventative intervention for high risk infants.

Keywords allergy prevention, atopic sensitization, *Bifidobacterium animalis* subsp *lactis* HN019, eczema, *Lactobacillus rhamnosus* HN001, paediatrics, probiotics, randomized controlled trial, rhinitis

Submitted 21 September 2011; revised 19 January 2012; accepted 23 January 2012

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Cite this as: K. Wickens, P. Black, T. V. Stanley, E. Mitchell, C. Barthow, P. Fitzharris, G. Purdie and J. Crane, *Clinical & Experimental Allergy*, 2012 (42) 1071–1079.

Introduction

In 2008 we published the results of a randomized controlled trial investigating the effects of probiotic supplementation *Lactobacillus rhamnosus* HN001 (HN001) or *Bifidobacterium animalis* subsp *lactis* HN019 (HN019) in mothers and infants and found that only HN001 provided protection against eczema development by age 2 years [1].

The hygiene hypothesis [2] proposes that improvements in hygiene in the last 50 years have led to a reduced exposure to infective agents, including bacteria, with effects on the development of immune responses in infancy and childhood. Allergic diseases have been associated with dominant Th2 responses, thus it has been suggested that probiotic bacteria may protect against allergy through their stimulation of Th1 cytokines and associated suppression of Th2 cytokines. Our previous results, which showed that the effect of *L. rhamnosus* HN001 on eczema was the same regardless of atopic status, do not support a simple Th1/Th2 shift as the mode of action of probiotics in eczema. It is evident that other cell types, including immunomodulatory Treg cells, are involved in maintaining immunological tolerance, with tolerogenic dendritic cells also potentially involved in Treg differentiation in the gut. Cytokines including IL-10 and TGF-β have been shown to play a role in controlling allergic responses [3].

There have been a number of primary prevention studies investigating the effects of different species of probiotics taken by pregnant or breastfeeding mothers and/or their infants on the prevalence of eczema and sensitization by age 2 years [4–14]. Reaching a consensus on the role of probiotics as primary preventers of allergic disease has been hampered by heterogeneity between studies in probiotic species and dose, duration and timing of intervention, and definitions of outcomes measured. Nonetheless, the weight of evidence provided by a Cochrane review [15] is consistent with a protective effect for lactobacillus probiotics against eczema. A more recent meta analysis by Tang et al. [16], including more recent studies, concluded that there is a potential role for probiotics in the prevention of eczema, particularly IgE-associated eczema. Nevertheless there is little evidence that probiotics affect the development of atopic sensitization. Indeed one study [7] reported increased rates of sensitization in children taking probiotics, and in another the findings were equivocal depending on child's age and definition of atopy [12].

Herein, we report the follow-up of the children in our original study, investigating the effect of HN001 and HN019 on allergic disease and atopic sensitization at age 4 years, 2 years after cessation of the study probiotic or placebo.

Methods

In the original randomized controlled trial of high risk infants (Australian New Zealand Clinical Trials Registry: ACTRN12607000518460), daily supplementation with either HN001 (6×10^9 cfu/day), HN019 (9×10^9 cfu/day) or placebo was from 35 weeks gestation until birth, continuing to 6 months after birth in mothers if breastfeeding, and from birth till 2 years in all infants [1]. Numbers (percentages) of eligible infants completing the trial to 2 years were 144/157 (91.7%) in the HN001 group, 152/158 (96.2%) in the HN019 group and 150/159 (94.3%) in the placebo group. After all infants had turned 2 years, parents were unblinded to study group and provided with a summary of the study findings. Study nurses remained blinded to participant study group. Ethical approval for a 4 year follow-up was granted by the New Zealand Multi-region Ethics Committee.

Four year assessments

Parents who gave informed consent were invited to attend our research facilities when the child was aged over 4 years (Mean age: 4 years 4 months; SD = 0.13 years; Range: 4 years 0 months-5 years 0 months).

Outcome measures. Data on allergic disease and atopic sensitization were collected using the same standard protocols as in the original study [1], except for eczema where adjustments had been made to the UK Working Party's Diagnostic Criteria on atopic dermatitis [17] in the earlier analysis [1] to allow for the different distribution of eczema in infants i.e. the infant protocol included as part of the criteria the presence of eczema on cheeks and outer arms or legs. At age 4 years, eczema was assessed based on the same protocol [17] but using the distribution patterns recommended for this age. Eczema was determined as present if there was a history of scratching or rubbing since the child turned 2 years plus two or more of the following: (1) a generally dry skin since turning 2 years, (2) a history of asthma or hayfever ever, (3) flexural involvement since 2 years around the eyes, sides or front of neck, elbow or knee flexures or fronts of ankles and (4) visible atopic eczema present at any of these sites. The UK Working Party's criterion [17] for onset below 2 years was not included to prevent a bias towards our earlier findings resulting in a less specific definition than that used at 2 years. Cumulative eczema prevalence to 4 years combined the original eczema prevalence data to age 2 years with eczema (defined as above) at 4 years.

Eczema severity was assessed using SCORAD (SCORing Atopic Dermatitis) [18] and as previously, analysed as a dichotomous variable with a cut-off of $\geq$ 10.

Skin prick tests were performed on the child's forearm according to ASCIA guidelines [19] to egg white, peanut, cow's milk, cat pelt, *Dermatophagoides pteronyssinus* and mixed grass pollen (Hollister-Stier, Spokane, WA, USA). Antihistamine medication was withheld as appropriate (depending on the half-life of the antihistamine) before the test. Stallergenes 1 mm lancets (Antony, France) were used to puncture the skin for 1 s, after applying the allergens and positive (histamine 10 mg/mL) and negative controls. The histamine response was read at 10 min and allergen responses at 15 min. A mean wheal diameter $\geq$ 3 mm to one or more allergens after subtraction of the negative control defined a child as atopic.

Study nurses were trained in the standard use of these protocols.

In addition, standard ISAAC questions [20] were used to assess the prevalence of current asthma symptoms ('Has your child had wheezing or whistling in the chest in the last 12 months?'), rhinoconjunctivitis (as a positive response to both these questions: 'in the past 12 months, has your child had a problem with sneezing or a runny or a blocked nose when he/she DID NOT have a cold or the flu?' and if yes, 'In the past 12 months, was this nose problem accompanied by itchy watery eyes?').

Body mass index was defined as weight (kg)/height $(m)^2$. Obesity, overweight and underweight were defined according to the gender and age specific cut-offs provided by Cole et al. [21, 22].

Fecal sample analysis. Fecal samples from a subset of children (n = 153) were analysed. Total DNA was extracted from 1.4 g samples of fecal material stored at −80°C using the STAR Buffer and High Pure PCR Template Preparation kits (Roche Molecular Systems, New Zealand). Real-time PCR was performed using a LightCycler 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany) (software version 4.05). Samples were analysed in duplicate using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche).

The HN001 primer sequences used were Forward 5′-CGCTTAGGACTCAGGATACA-3′ and Reverse 5′-GCTTGCGTCAGATTTTCAGTA-3′, according to published sequences (GenBank acc no. NZ_ABWJ00000000). Primers used to detect HN019 were derived from Malinen et al. [23], PCR conditions used to amplify fecal DNA templates were pre-incubation at 95°C for 10 min; followed by 45 cycles of denaturation at 95°C for 10 s, annealing (69°C for HN001 primers and 60°C for HN019 primers) for 10 s and extension at 72°C for 30 s; with a final cooling at 40°C for 30 s.

Specificity of the primers was assessed by performing real-time PCR on known bifidobacterial and lactobacillus strains (data not shown). The HN001 primers were species-specific and could discriminate the HN001 strain from other strains, including *L. rhamnosus* GG. However, a positive finding using the HN001 primers could not rule out that the fecal sample contained a closely related HN001-like strain. The HN019 primers were specific to the *B. animalis* subsp. *lactis* species but could not discriminate between *B. animalis* subsp. *lactis* strains. Sensitivity experiments, whereby known quantities of HN001 or HN019 bacteria were added to PCR-negative fecal samples prior to DNA extraction, showed that both primer sets could detect down to approximately 1×10^3 cfu HN001 or HN019 per g fecal material.

Statistical analysis

Analysis was undertaken using SAS version 9.1 (SAS Institute, Cary, NC, USA). The effect of each probiotic on the cumulative prevalence of eczema, SCORAD ($\geq$ 10) and wheeze occurring between 3 months and 4 years, and atopic sensitization occurring at 2 and 4 years, were summarized using hazard ratios estimated with Cox's proportional hazards models. Kaplan Meier plots were used to present these results graphically for eczema and SCORAD. For outcomes collected at 4 years, relative risks were estimated, using a generalized linear model with a log link and binomial distribution, for the effect of each probiotic on the following variables: eczema, SCORAD ($\geq$ 10), wheeze, rhinoconjunctivitis and atopic sensitization. To determine if a probiotic effect was IgE associated, each outcome variable was analysed in the presence or absence of atopy. To assess whether or not the effects differed by atopic sensitization, the interaction of study group with atopic sensitization was added to the models. Analysis was intention-to-treat.

Results

At age 4 years, the response rate (numbers (%)) of eligible participants at birth were 143/159 (90.0%) in the placebo group, 136/157 (86.6%) in the HN001 group and 146/158 (92.4%) in the HN019 group.

Table 1 shows the risk of developing the study outcomes over the period from birth to 4 years for each study probiotic. Figures 1 and 2 present the data for the cumulative prevalence of eczema and SCORAD $\geq$ 10 as Kaplan Meier plots. Children in the HN001 group were significantly less likely to have developed eczema by 4 years. They also had some protection against developing SCORAD $\geq$ 10, wheeze and atopic sensitization by age 4 years but these reductions did

Table 1. Hazard ratios (95% CIs) for the 4 year cumulative prevalence of eczema, SCORAD $\geq$ 10, wheeze and atopic sensitization in infants taking L*actobacillus rhamnosus* HN001 and B*ifidobacterium animalis* subsp *lactis* HN019. (Percents represent the proportion with each outcome*)

N = 474	Placebo (*N* = 159)	*L. rhamnosus* HN001 (*N* = 157)	*P* value	*B. animalis* subsp *lactis* HN019 (*N* = 158)	*P* value	*P* value†	*P* value for interaction‡
Eczema	1.00 (49.3%)	0.57 (0.39–0.83) 32.7%	**0.003**	0.79 (0.56–1.11) 40.3%	0.17	**0.01**	
IgE associated eczema§	1.00 (27.8%)	0.69 (0.41–1.18) 18.0%	0.18	0.79 (0.50–1.26) 24.8%	0.33	0.37	0.76
Non-IgE associated eczema§	1.00 (21.1%)	0.67 (0.38–1.19) 17.2%	0.17	0.61 (0.33–1.09) 13.1%	0.10	0.19	
SCORAD $\geq$ 10	1.00 (46.4%)	0.74 (0.52–1.05) 38.7%	0.09	0.99 (0.71–1.38) 45.0%	0.95	0.17	
IgE associated SCORAD§	1.00 (27.8%)	0.85 (0.52–1.41) 21.3%	0.53	1.05 (0.67–1.64) 29.2%	0.84	0.71	0.70
Non-IgE associated SCORAD§	1.00 (18.8%)	0.83 (0.47–1.47) 18.9%	0.53	0.78 (0.43–1.41) 14.6%	0.41	0.68	
Wheeze	1.00 (63.7%)	0.79 (0.59–1.07) 54.9%	0.13	1.01 (0.76–1.34) 64.2%	0.94	0.21	
IgE associated wheeze§	1.00 (27.8%)	0.81 (0.49–1.36) 19.7%	0.43	0.89 (0.56–1.41) 27.0%	0.62	0.73	0.85
Non-IgE associated wheeze§	1.00 (37.6%)	0.75 (0.50–1.12) 36.1%	0.16	0.98 (0.66–1.46) 36.5%	0.93	0.29	
Atopic sensitization¶	1.00 (41.1%)	0.72 (0.48–1.06) (31.9%)	0.09	0.93 (0.64–1.33) (40.0%)	0.67	0.23	-

*Proportions based on total *n* in each study group.
†*P* value χ^2 test for differences between the three study groups.
‡*P* value χ^2 test for differences between study groups dependent on sensitization status.
§*n* = 392 (based on all those completing skin prick tests at 2 and 4 years). Hazard ratios stratified by atopic status.
¶*n* = 441 (based on all those completing skin prick tests at age 2 or 4 years).
Significant *P* values are in bold.

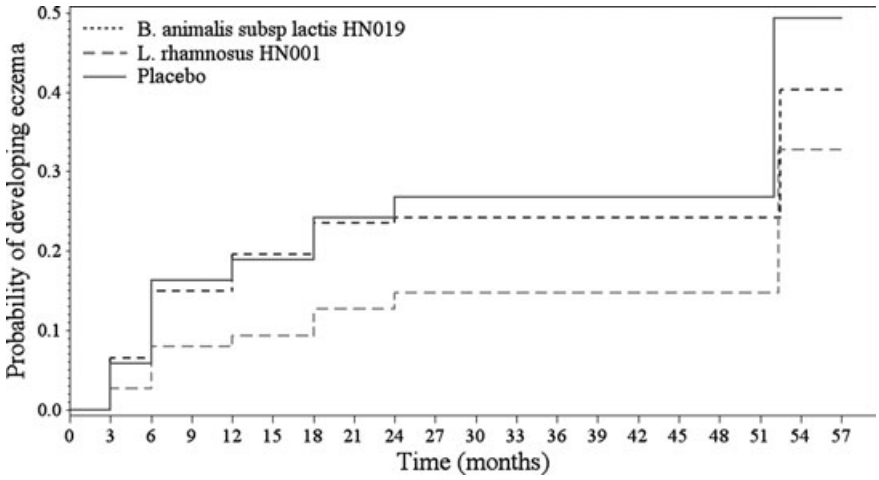

Fig. 1. Kaplan-Meier plot showing 4 year cumulative prevalence of eczema in children taking the placebo, L*actobacillus rhamnosus* HN001, or B*ifidobacterium animalis* subsp *lactis* HN019.

not reach significance. There was no significant effect of HN019 on any outcome. The overall effect of study group on any outcome was not significantly different between IgE-sensitized and non-sensitized children.

Table 2 shows the probiotic associations for outcomes that were collected at age 4 years. Children who had taken HN001 had significantly reduced risks of having eczema and rhinoconjunctivitis in the last 12 months. HN019 was not associated with any outcome. IgE-sensitization did not modify the effect of either probiotic on any outcome.

We previously reported that there was no difference between study groups for gender, ethnicity, cesarian delivery, birth weight, length and head circumference, breastfeeding duration, smoking in pregnancy or in the household, pet ownership, family history of allergic disease or antibiotic use before 2 years [1]. Between 2 and 4 years, more children in the *B. lactis* HN019 compared to the placebo group ($P = 0.03$) had received more than two courses of antibiotics. The rates of antibiotic use in the placebo and HN001 groups were similar.

Based on questionnaire data, 24% of children ($n = 102$) had been given non-study commercially available probiotic drinks or supplements (as a powder or capsule) between the ages of 2 and 4 years but usage was not related to study group. Exclusion of children using non-study probiotics from the analysis made little difference to the relative risk estimates.

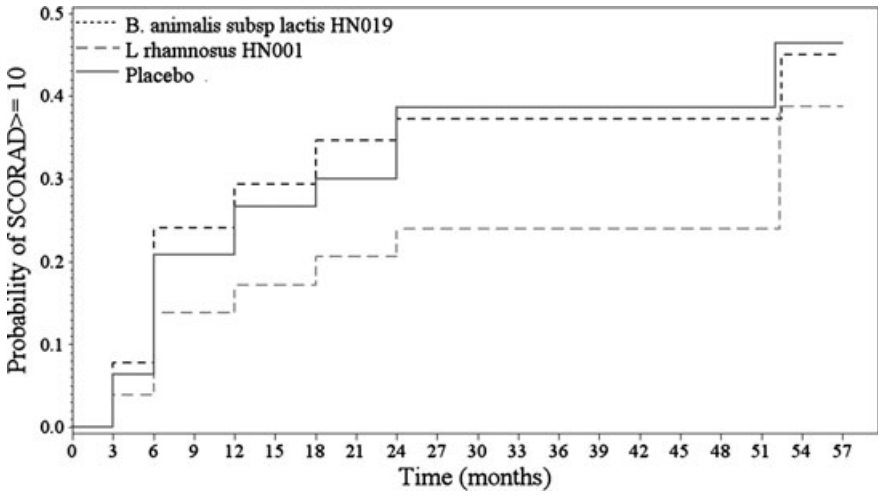

Fig. 2. Kaplan-Meier plot showing 4 year cumulative prevalence of SCORAD $\geq$ 10 in children taking the placebo, L*actobacillus rhamnosus* HN001, or B*ifidobacterium animalis* subsp *lactis* HN019.

Table 2. Relative risks (95% CIs) of eczema, SCORAD $\geq$ 10, current wheeze, rhinoconjunctivitis and atopic sensitization at 4 years in infants taking L*actobacillus rhamnosus* HN001 and B*ifidobacterium animalis* subsp *lactis* HN019. (Percents represent the proportion with each outcome*)

	Placebo N = 143	*L. rhamnosus* HN001 N = 136	*P* value	*B. animalis* subsp *lactis* HN019 N = 146	*P* value	*P* value†	*P* value‡
Eczema§	1.00 (39.3%)	0.69 (0.49–0.98) (27.1%)	**0.04**	0.85 (0.62–1.16) (33.3%)	0.30	0.11	
IgE associated eczema#	1.00 (19.9%)	0.76 (0.48–1.20) (12.3%)	0.24	0.84 (0.57–1.23) (17.3%)	0.36	0.45	1.00
Non-IgE associated eczema#	1.00 (19.1%)	0.78 (0.47–1.29) (16.4%)	0.33	0.85 (0.52–1.37) (15.8%)	0.50	0.60	
SCORAD $\geq$ 10§	1.00 (31.4%)	0.79 (0.54–1.16) (24.8%)	0.23	0.93 (0.65–1.32) (29.1%)	0.67	0.48	
IgE associated SCORAD $\geq$ 10#	1.00 (19.1%)	0.69 (0.41–1.13) (10.7%)	0.14	0.83 (0.56–1.24) (16.6%)	0.37	0.30	0.36
Non-IgE associated SCORAD $\geq$ 10#	1.00 (11.8%)	1.20 (0.66–2.18) (15.6%)	0.55	1.06 (0.57–1.97) (12.2%)	0.85	0.83	
Wheeze last 12 months¶	1.00 (32.9%)	0.83 (0.58–1.19) (27.2%)	0.31	1.02 (0.74–1.42) (33.6%)	0.90	0.45	
IgE associated wheeze#	1.00 (17.7%)	0.86 (0.53–1.38) (12.3%)	0.53	0.94 (0.63–1.41) (17.3%)	0.77	0.81	0.86
Non-IgE associated wheeze#	1.00 (16.9%)	0.84 (0.49–1.42) (15.6%)	0.51	1.09 (0.67–1.76) (18.0%)	0.74	0.60	
Rhinoconjunctivitis last 12 months¶	1.00 (15.4%)	0.38 (0.18–0.83) (5.9%)	**0.02**	0.80 (0.45–1.43) (12.3%)	0.45	**0.03**	
IgE associated rhinoconjunctivitis#	1.00 (8.8%)	0.46 (0.16–1.30) (3.3%)	0.14	0.86 (0.42–1.77) (7.9%)	0.69	0.27	0.89
Non-IgE associated rhinoconjunctivitis#	1.00 (6.6%)	0.34 (0.09–1.20) (2.5%)	0.09	0.67 (0.25–1.79) (4.3%)	0.42	0.19	
Atopic sensitization#	1.00 (35.3%)	0.81 (0.57–1.17) (28.7%)	0.26	1.04 (0.76–1.43) (36.7%)	0.81	0.35	

*Proportions based on total n in each study group.

†P value x^2 test for differences between the 3 study groups.

‡P value χ^2 test for differences between study groups dependent on sensitization status.

§n = 410 (based on all with physical assessment completed at 4 years).

¶n = 425 (based on all with questionnaire data at 4 years).

#n = 397 (based on all those completing skin prick tests at 4 years). Relative risks stratified by atopic status.

Significant P values are in bold.

Of the 78 children who developed eczema between 2 and 4 years, more were in the placebo (n = 32), than in the HN001 (n = 23) or HN019 (n = 23) group but the difference across the three groups was not significant (P = 0.31). There was also no significant difference among the three study groups for those children whose eczema had resolved between 2 and 4 years (13 in the placebo, 8 in the HN001 and 9 in the HN019 group, P = 0.49). There were 41 children where SCORAD $\geq$ 10 was present for the first time at 4 years, 11 in the placebo group, 19 in the HN001 group and 11 in the HN019 group (P = 0.17). A total of 60 children previously with SCORAD $\geq$ 10 on at least one occasion before 2 years did not satisfy this condition at 4 years.

There was no difference between the study groups ($P = 0.81$) of these children (placebo ($n = 20$), HN001 ($n = 18$) and HN019 ($n = 22$)).

At 4 years, there was no effect of either study probiotic on the child's weight, height, or body mass index defined continuously or categorized into obese, overweight, normal or underweight (Table 3).

Analysis of the fecal DNA samples showed that only 13/153 (8.5%) children had detectable levels of *B. animalis* sbsp. *lactis* strains (a group that includes HN019) in feces 2 years after discontinuing probiotic supplementation but HN001 (or HN001-like strains) was detectable in samples from 50/153 (33%) children. Although the detection of HN001 was lower in both probiotic groups than in the placebo group, these differences were not significant (Table 4).

Discussion

At 2 years we showed that HN001 was associated with a 50% reduction in eczema prevalence [1]. We have now shown that the effect of HN001 persisted to 4 years, 2 years after the cessation of study capsules. There were fewer new cases of eczema since age 2 years in the HN001 (and HN019) groups than the placebo group, indicating that the earlier protective effect did not simply result in delay in onset of the disease once supplementation had ceased. However, a similar earlier reduction in cumulative SCORAD $\geq$ 10 by 2 years in children taking HN001 had weakened. The size of the effect of HN001 on atopic sensitization by age 4 years was similar to that found at 2 years but with greater numbers in the cumulative analysis, the confidence intervals had narrowed.

Few studies on probiotic supplementation [24–26] have investigated their effect on the prevention of allergic disease beyond 2.5 years. Our findings for a protective effect on eczema at 2 and 4 years are remarkably similar to the original study by Kalliomaki et al. [4] which used a similar probiotic (*L. rhamnosus* GG) and showed significant relative risk reductions at age 2, 4 and 7 years of 49%, 43% and 36% respectively. A second study of *L. rhamnosus* GG by a German group [8] could not replicate these findings, possibly due to lack of power. A larger study [5] of four different probiotics (including *L. rhamnosus* GG) plus a prebiotic found a significant reduction in eczema, especially atopic eczema, at 2 years but no effect on any allergic disease at 5 years (except in children born by cesarian section).

Table 3. Anthropometric measures at 4 years by study group

	Placebo ($n = 139$)	*Lactobacillus rhamnosus* HN001 ($n = 129$)	*Bifidobacterium animalis* subsp *lactis* HN019 ($n = 140$)	*P* value
Mean weight (Kg) (95% CI)	18.26 (17.92–18.61)	18.43 (18.07–18.79)	18.38 (18.04–18.72)	0.79
Range of weights	13.90–25.35	14.00–23.10	13.95–24.70	
Mean height (cm) (95% CI)	106.1 (105.4–106.8)	106.6 (105.8–107.4)	106.3 (105.5–107.0)	0.64
Range of heights	96.30–120.70	93.25–115.10	92.25–117.35	
Mean BMI (95% CI)	16.17 (15.98–16.36)	16.18 (15.98–16.38)	16.23 (16.04–16.42)	0.89
Range of BMI	13.98–19.32	13.26–19.19	13.06–21.63	
	n (%)	*n* (%)	*n* (%)	
Obesity* ($N = 6$)	2 (1.4)	1 (0.8)	3 (2.1)	0.93
Overweight† ($N = 57$)	20 (14.4)	18 (14.0)	19 (13.6)	
Normal weight ($N = 343$)	117 (84.2)	109 (84.5)	117 (83.6)	
Underweight‡ ($N = 2$)	0 (0)	1 (0.8)	1 (0.7)	

*Definition of obesity [21]: For girls 4–5 years BMI > 19.1; For boys 4–5 years BMI > 19.3.

†Definition of overweight [21]: For girls 4–4.5 years BMI > 17.3; For girls 4.5–5 years BMI > 17.2; For boys 4–4.5 years BMI > 17.6; For boys 4.5–5 years BMI > 17.5.

‡Definition of underweight [22]: For girls 4–4.5 years BMI < 13.34; For girls 4.5–5 years BMI < 13.21; For boys 4–4.5 years BMI < 13.52, For boys 4.5–5 years BMI < 13.41.

Table 4. Relative risks (95% CIs) of HN001 and HN019 detection in fecal samples at 4 years in infants taking L*actobacillus rhamnosus* HN001 and B*ifidobacterium animalis* subsp *lactis* HN019

	Placebo $N = 56$	*L. rhamnosus* HN001 $N = 43$	*P* value	*B. animalis subsp lactis* HN019 $N = 54$	*P* value	*P* value*
HN001 positive	1.00 (42.9%)	0.63 (0.36–1.09) (25.6%)	0.08	0.70 (0.45–1.10) (27.8%)	0.10	0.12
HN019 positive	1.00 (7.1%)	1.17 (0.56–2.43) (9.3%)	0.70	1.15 (0.62–2.12) (9.3%)	0.69	0.90

*P value x^2 test for differences between the three study groups.

Our study also showed significant protective effects of HN001 on rhinoconjunctivitis at 4 years. This is in sharp contrast to the findings of Kalliomaki et al. [24, 25] who at 4 and 7 years found non-significant increased risks of allergic rhinitis in association with *L. rhamnosus* GG and Kuitunen et al. [26] who found no effect of a mixture of probiotics (plus a prebiotic) on allergic rhinitis by 5 years. Arguably, rhinitis is difficult to distinguish from symptoms due to infections at age 4 years and this may have resulted in misclassification of this outcome in our study. However, this misclassification is likely to be non-differential and bias the findings towards the null hypothesis, suggesting that this is an unlikely explanation for our findings. Further, there is preliminary evidence from some studies reviewed in Vliagoftis et al. [27] that some probiotics may reduce symptom severity and medication use among children with allergic rhinitis but more rigorous larger studies are required.

There have been reports of an increased risk of asthma-like symptoms in relation to supplementation with *L. acidophilus* at age 6–12 months [7] and *L. rhamnosus* GG by 7 years [25]. In one of these studies [7] there was no difference between study groups once the children had reached 2.5 years [28], and Kuitunen et al. [26] showed that a probiotic plus prebiotic mixture did not affect asthma prevalence at age 5 years. Our finding of a slight protective effect of HN001 on asthma symptoms at 4 years is also reassuring.

In considering a possible mechanism of probiotic action, the findings from two studies of probiotic administration to the infant only [7, 9] failed to show an effect on eczema suggesting that probiotics given to the mother may drive the reduction in eczema development. Two small studies with maternal intervention only (both from 36 weeks gestation and continuing during breastfeeding) [14, 29] have shown effects at 2 years that are as strong as those seen when probiotics were also administered directly to the infant but these results were not replicated in a more recent study [30]. In a study [11] with a maternal-only probiotic intervention from the first trimester of pregnancy till the end of exclusive breastfeeding the protective effect on eczema at age 1 year was only marginal, but there was a significantly lower rate of sensitization in children whose mothers were sensitized.

Although these clinical trials suggest that probiotics may be more efficacious if infant exposure is via the placenta or breast milk than when given directly to the infant, the reason for this is unclear. Prenatal supplementation may affect the cytokine profile of cord blood. In a previous report of our study, we found higher IFN-γ, a Th1 cytokine, in cord blood of neonates whose mothers took probiotics from 35 weeks gestation [31]. In addition, probiotics given to the mother, by influencing maternal gut microbiota and altering the maternal gut barrier may affect the transfer of allergens across the maternal gut, thereby influencing the cytokine profile of breast milk [32]. In support of this, anti-inflammatory cytokines such as TGF-β2, have been found to be elevated in colostrum [11], and in our own study, TGF-β1 and IgA were elevated in breast milk of mothers taking probiotics [31]. This suggests that there may be a critical period during fetal development or soon after birth when the immune system is amenable to modification by probiotics.

A number of studies have reported a relationship between gut microbiota and allergy in young children. In a cross sectional study, allergic children were less often colonized with lactobacilli than non-allergic children [33], and, prospectively, differences in gut microbiota have been shown to precede atopy [34]. In the current study, HN001 or closely related strains were detected in 33% of fecal samples although strains belonging to the *B. animalis* sbsp. *lactis* sub-species, including HN019 were rarely detected. This difference reflects the changes in bowel flora over time reported in a cross sectional study [35], where fecal bifidobacterium levels were greater in infants than those in adults, and lactobacillus levels were equivalent in infancy and adulthood. Our study does not support long term colonization of the distal gut due to earlier probiotic supplementation, despite earlier probiotic consumption affecting fecal levels of these probiotics [1]. Thus, it is unlikely that any probiotic influence on the infant gut barrier, such as reduced permeability, would have been sustained over time. For HN001 to have long-term effects, as found at age 4 years, it is possible that early gut microbiota may have generalized effects in shaping the immune system, and that these effects persist over time. Further, as immune modulating effects of bacteria may be strain (or species) specific [36, 37], it is not surprising that effects for the two probiotics studied differ. Gill et al. [38] investigated the effects of the same probiotics as used in our study on cytokine production in mice and showed IFN-γ levels significantly higher in mice fed HN001 but no different from control in mice fed HN019.

This is the only study to separately evaluate two different probiotics, and show an effect for HN001 but not HN019. The different effects we found for each probiotic at age 2 years persisted to 4 years, highlighting the importance of the particular probiotic in allergic disease prevention. Another strength of our study is the high response rate (> 86% of eligible infants in each group) and follow-up beyond infancy. The only other studies with follow-up beyond infancy are by Kalliomaki [24, 25] and Kuitunen [26].

A potential weakness of our study is that at age 4 years there may have been misclassification around

the measurement of allergic rhinitis and asthma, with the latter including both transient wheezers and children with established disease.

We cannot entirely exclude the possibility that bias due to unblinding of participants to study group and main study findings prior to the 4 year assessments may have contributed to our findings for HN001 and eczema. Informing parents that HN001 'protected against eczema developing before 2 years' may have influenced responses to the question about eczema ('Since your child turned 2 years old, has s/he ever had eczema?'), However our definition of eczema [17] did not use this question but was based on questionnaire responses about symptoms (itchy rash), site of symptoms, skin dryness, history of other allergic disease and visible atopic eczema assessed by a nurse who was blinded to the study group, suggesting the protection of HN001 against eczema at 4 years is more likely to be due to a treatment effect than bias. Consistent with a protective effect, the nurse assessment of visible atopic eczema was also reduced, non-statistically significant, in the HN001 group (RR = 0.80, 95% CI 0.58–1.10). The finding of a protective effect against rhinoconjunctivitis at 4 years, which was not examined at 2 years, is unlikely to have resulted from being informed that HN001 provided protection against eczema before 2 years, thus supporting our contention that the effect is real.

In conclusion, our study has shown that the use of HN001 in the first 2 years of life may continue to protect against eczema to age 4 years, 2 years after the cessation of probiotic supplementation. This is the first study to show a protective effect against the development of rhinitis symptoms but, given that this has not been shown in other studies, more trials with large sample sizes and long follow-up periods are needed to further clarify this effect. The precise pathways for effects on allergic disease remain elusive and require more work, including the possibility that effects are mediated via epigenetic mechanisms.

Acknowledgements

We are very grateful for the ongoing support from the Wellington and Auckland families who have generously given their time for this study. We also thank Ms Alex Nicholson, Ms Marilyn Appleton, Ms Helen Nagels, Ms Barbara Rotherham and Ms Phillipa Lampshire for data collection, clerical and database support; and Dr James Dekker and Mr Dinuk Peiris for performing PCR on fecal samples. Funding was provided by Fonterra Cooperative Group, New Zealand. EA Mitchell is supported by Cure Kids. None of the authors has a conflict of interest.

References

1 Wickens K, Black PN, Stanley TV *et al*. A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Imunol* 2008; **122**:788–94.

2 Strachan D. Hay fever, hygiene, and household size. *BMJ* 1989; **299**:1259–60.

3 McLoughlin RM. Mills KHG Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. *J Allergy Clin Immunol* 2011; **27**:1097–107.

4 Kalliomaki M, Salminen S, Arvilommi H *et al*. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001; **357**:1076–9.

5 Kukkonen K, Savilahti E, Haahtela T *et al*. Probiotics and prebiotics galactooligosaccharides in the prevention of allergic disease: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:192–8.

6 Abrahamsson T, Jakobsson T, Bottcher M *et al*. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:1174–80.

7 Taylor A, Dunstan J, Prescott S. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. *J Allergy Clin Immunol* 2007; **119**:184–91.

8 Kopp M, Hennemuth I, Heinzmann A *et al*. Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: no clinical effects of *Lactobacillus GG* supplementation. *Pediatrics* 2008; **121**:e1–7.

9 Soh S, Aw M, Gerez I *et al*. Probiotic supplementation in the first 6 months of life in at risk Asian infants - effects on eczema and atopic sensitization at 1 year. *Clin Exp Allergy* 2009; **39**: 571–8.

10 Kim JY, Kwon JH, Ahn SH *et al*. Effect of probiotic mixture (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacilus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial. *Pediatr Allergy Immunol* 2010; **21**: e386–93.

11 Huurre A, Laitinen K, Rautava S *et al*. Impact of maternal atopy and probiotic supplementation during pregnancy on infant sensitization: a double-blind placebo-controlled study. *Clin Exp Allergy* 2008; **38**:1342–8.

12 Niers L, Martin R, Rijkers G *et al*. The effects of selected probiotic strains on the development of eczema (the PandA study). *Allergy* 2009; **64**:1349–58.

13 West CE, Hammarstrom M-L, Hernell O. Probiotics during weaning reduce the incidence of eczema. *Paediatr Allergy Immunol* 2009; **20**:430–7.

14 Dotterud C, Storro O, Johnsen R *et al*. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. *Br J Dermatol* 2010; **163**:616–23.

15 Osborn D, Sinn J. Probiotics in infants for prevention of allergic disease and food hypersensitivity (Review). *Cochrane Database Syst Rev* 2007 Oct 17; (4) CD006475.

16 Tang M, Lahtinen S, Boyle R. Probiotics and prebiotics: clinical effects in allergic disease. *Curr Opin Pediatr* 2010; **22**:626–34.
17 Williams H. So how do I define atopic eczema? A practical manual for researchers wishing to define atopic eczema. 1996 [cited. Available from: http://www.nottingham.ac.uk/dermatology/eczema/contents.html (accessed 14 June 2008).
18 European Task Force on Atopic Dermatitis. Severity scoring of atopic dermatitis: the scorad index. *Dermatology* 1993; **186**:23–31.
19 ASCIA skin prick testing working party. Skin prick testing for the diagnosis of allergic disease. A manual for practitioners. 2009. Available from http://www.allergy.org.au/images/stories/ascia_spt_manual_2009.pdf (accessed 14 June 2008).
20 Asher MI, Keil U, Andersen HR *et al*. International study of asthma and allergies in childhood (ISAAC): rationale and methods. *Eur Respir J* 1995; **8**:483–91.
21 Cole TJ, Bellizzi MC, Flegal KM *et al*. Establishing a standard definition for child overweight and obesity world wide: international survey. *BMJ* 2000; **320**:1240–3.
22 Cole TJ, Flegal KM, Nicholls D *et al*. Body mass index cut offs to define thinness in children and adolescents: international survey. *BMJ* 2007; **335** (7612):194–7. doi: 10.1136/bmj.39238.39944455
23 Malinen E, Kassinen A, Rinttila T *et al*. Comparison of real-time PCR with SYBR Green 1 or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected fecal bacteria. *Microbiology* 2003; **149**:269–77.
24 Kalliomaki M, Salminen S, Poussa T *et al*. Probiotics and prevention of atopic disease: 4 year follow-up of a randomised placebo-controlled trial. *Lancet* 2003; **361**:1869–71.
25 Kalliomaki M, Salminen S, Poussa T *et al*. Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:1019–21.
26 Kuitunen M, Kukkonen K, Juntunen-Backman K *et al*. Probiotics prevent IgE-associated allergy until age 5 years in cesarian-delivered children but not in the total cohort. *J Allergy Clin Immunol* 2009; **123**:335–41.
27 Vliagoftis H, Kouranos VD, Betsi GI *et al*. Probiotics for the treatment of allergic rhinitis and asthma: systematic review of randomized controlled trials. *Ann Allergy Asthma Immunol* 2008; **101**:570–9.
28 Prescott SL, Wiltschut J, Taylor A *et al*. Early markers of allergic disease in a primary prevention study using probiotics: 2.5-year follow-up phase. *Allergy* 2008; **63**:1481–90.
29 Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breastfeeding might confer immunological protection against atopic disease in the infant. *J Allergy Clin Immunol* 2002; **109**:119–21.
30 Boyle RJ, Ismail IH, Kivivuori S *et al*. *Lactobacillus* GG treatment during pregnancy for the prevention of eczema: a randomized controlled trial. *Allergy* 2011; **66**:509–16.
31 Prescott S, Wickens K, Westcott L *et al*. Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-y and breast milk transforming growth factor-B and immunoglobulin A detection. *Clin Exp Allergy* 2008; **38**:1606–14.
32 Dattner AM. Breastfeeding and atopic dermatitis: protective or harmful? facts and controversies. *Clin Dermatol* 2010; **28**:34–7.
33 Bjorksten B, Naaber P, Sepp E *et al*. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999; **29**:342–6.
34 Kalliomaki M, Kirjavainen P, Eerola E *et al*. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001; **107**:129–34.
35 Mariat D, Firmesse O, Levenez F *et al*. The *Firmicutes/Bacteroidetes* ratio of the human microbiota changes with age. *BMC Microbiol* 2009; **9**:123. doi:10.1186/1471-2180-9-123.
36 Vaarala G. Immunological effects of probiotics with special reference to lactobacilli. *Clin Exp Allergy* 2003; **33**:1634–40.
37 Ozdemir O. Any benefits of probiotics in allergic disorders? *Allergy Asthma Proc* 2010; **31**:103–11.
38 Gill H, Rutherford K, Prasad J *et al*. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br J Nutr* 2000; **83**:167–76.