

Early supplementation with *Lactobacillus rhamnosus* HN001 reduces eczema prevalence to 6 years: does it also reduce atopic sensitization?

K. Wickens¹, T. V. Stanley², E. A. Mitchell³, C. Barthow¹, P. Fitzharris⁴, G. Purdie⁵, R. Siebers¹, P. N. Black^{6†} and J. Crane¹

¹Wellington Asthma Research Group, Wellington School of Medicine and Health Sciences, University of Otago, Wellington, New Zealand, ²Department of Paediatrics, Wellington School of Medicine and Health Sciences, University of Otago, Wellington, New Zealand, ³Department of Paediatrics, University of Auckland, Auckland, New Zealand, ⁴Immunology Department, Auckland Hospital, Auckland, New Zealand, ⁵Dean's Department, Wellington School of Medicine and Health Sciences, University of Otago, Wellington, New Zealand and ⁶Department of Pharmacology & Clinical Pharmacology, University of Auckland, Auckland, New Zealand

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Summary

Background The role of probiotics in prevention of allergic disease is still not clear; efficacy may depend on the timing, dose, duration, and specific probiotic used. 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 from birth until 2 years with *Lactobacillus rhamnosus* HN001 (HN001) (6×10^9 cfu/day) halved the cumulative prevalence of eczema at 2 and 4 years. *Bifidobacterium animalis* subsp *lactis* HN019 (HN019) (9×10^9 cfu/day) had no significant effect.

Objective To determine whether differences in effects of HN001 and HN019 on eczema persist to age 6 years, and to investigate effects on sensitization.

Methods Standard procedures were used to assess eczema (The UK Working Party's Criteria), eczema severity (SCORAD), atopic sensitization [skin prick tests (SPT), total and specific IgE] and standard questions used for asthma, wheeze, and rhinoconjunctivitis.

Results HN001 was associated with significantly lower cumulative prevalence of eczema (HR = 0.56, 95% CI 0.39–0.80), SCORAD ≥ 10 (HR = 0.69, 0.49–0.98) and SPT sensitization (HR = 0.69, 95% CI 0.48–0.99). The point prevalence of eczema (RR = 0.66, 95% CI 0.44–1.00), SCORAD ≥ 10 (RR = 0.62, 95% CI 0.38–1.01) and SPT sensitization (RR = 0.72, 95% CI 0.53–1.00) were also reduced among children taking HN001. HN019 had no significant effect on any outcome.

Conclusion and Clinical Relevance This study provides evidence for the efficacy of the probiotic *L. rhamnosus* HN001 in preventing the development of eczema and possibly also atopic sensitization in high risk infants to age 6 years. The absence of a similar effect for HN019 indicates that benefits may be species specific.

Keywords asthma, atopic sensitization, *Bifidobacterium animalis* subsp *lactis* HN019, eczema, *Lactobacillus rhamnosus* HN001, paediatrics, probiotics, randomized controlled trial, rhinoconjunctivitis, wheeze

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Correspondence:

Dr Kristin Wickens, Wellington Asthma Research Group, Wellington School of Medicine and Health Sciences, University of Otago, P O Box 7343, Wellington South, Wellington, New Zealand.

E-mail: kristin.wickens@otago.ac.nz

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Introduction

It was Elie Metchnikoff who in 1908 first promoted lactic acid bacteria consumed in fermented dairy prod-

ucts, such as yoghurt, for the enhancement of health and longevity [1]. He hypothesized that gut flora could be modified by replacing harmful microbes with these beneficial microbes. Eighty years later in 1989, Strachan also suggested the potential value of microbial stimulation in promoting maturation of the immune system and subsequent reduction in development of

†P. N. Black: deceased.

allergic disease; this was named the 'hygiene hypothesis' [2]. A large number of subsequent studies of the effects of lactic acid producing bacteria, including both lactobacilli and bifidobacteria probiotic strains, on prevention of allergic disease have not always shown definitive benefits. Effects appear to depend upon the species and strain of probiotic, the dose, the timing of administration (prenatal or postnatal), and length of probiotic exposure. These studies were discussed in our previous articles [3, 4]. Of the few recent published trials [5, 6], one intervened with *Lactobacillus rhamnosus* GG from 36 weeks prenatally only and found no effect on eczema or skin prick test response at 2 years, suggesting that a post-natal exposure may be important [6]. Epigenetic effects of probiotics on gene expression may also contribute to variable findings [7]. Nevertheless, a 2007 Cochrane review [8] provides some evidence for beneficial effects of the probiotic, *L. rhamnosus*, in eczema prevention. There is less evidence for a beneficial effect of other probiotics in preventing allergic diseases or atopic sensitization [9].

In our own randomized controlled trial, we investigated the effects of probiotic supplementation (*L. rhamnosus* HN001 (HN001) or *Bifidobacterium animalis* subsp *lactis* HN019 (HN019) in mothers and infants and found that only HN001 provided statistically significant protection against eczema development by 2 years [3] and 4 years [4] with a weaker non-significant effect on atopy defined by skin prick test (SPT) occurring by 4 years [4]. Here, we report the effects of these probiotics on allergic disease development, including eczema, asthma, rhinoconjunctivitis, and atopic sensitization, including SPT, total and serum-specific IgE (ssIgE), to age 6 years. This is only the second randomized controlled trial to assess the primary preventative effects of probiotics on allergic disease to this age or beyond, and the first to investigate the long-term effects of two different probiotic species.

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 [3]. Study outcomes were assessed and reported at 2 [3] and 4 years [4]. 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 throughout.

Ethical approval for a 6-year follow-up was granted by the New Zealand Multi-region Ethics Committee (WGT/01/08/095) and parents gave informed consent.

Study nurses assessed eczema prevalence at 6 years, as at age 4 years, according to the UK Working Party's Diagnostic Criteria [10] modified to exclude the criteria for onset below 2 years to prevent a bias towards our findings at age 2 years. Thus eczema was determined as present if there was a history of an itchy rash since turning 4 years, plus 2 or more of the following: (i) a generally dry skin since 4 years, (ii) a history of asthma or hayfever ever, (iii) flexural involvement since 4 years around the eyes, sides or fronts of the neck, elbow or knee flexures, or fronts of ankles, (iv) visible atopic eczema present at any of these sites.

Other outcome measures were also assessed using the same protocols as previously [3, 4]. Eczema severity was assessed using SCORAD (Scoring Atopic Dermatitis) [11]. To exclude trivial rash, a SCORAD value ≥ 10 was considered positive. ASCIA (Australasian Society of Clinical Immunology and Allergy) guidelines [12] were used to measure skin prick test (SPT) reactions to egg white, peanut, cow's milk, cat pelt, *Dermatophagoides pteronyssinus*, and mixed grass pollen (Hollister-Stier, Spokane, WA, USA) using Stallergenes 1 mm lancets (Antony, France). SPT sensitization was defined as a mean weal diameter ≥ 3 mm to one or more allergens after subtraction of the negative control. After the administration of topical anaesthesia, 3.5 mL of blood was sampled for total and ssIgE to the same allergens as measured on skin prick tests. SsIgE was analysed using the Phadia ImmunoCAP ssIgE fluorescence enzyme immunoassay (Phadia AB, Uppsala, Sweden). Total IgE was analysed using Roche Cobas e601 total IgE electrochemiluminescence immunoassay (Roche Diagnostics GmbH, D-68298 Mannheim, Germany).

At the same time, a second 3.5 mL blood sample was collected for serum cytokine levels. Serum thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) levels were quantified by sandwich enzyme immunoassay technique using the Quantikine human CCL17/TARC and human MDC immunoassay kits (R&D Systems, Inc., Minneapolis, MN, USA).

As previously, standard questions from the International Study of Asthma and Allergies in Childhood (ISAAC) were used to assess the prevalence of wheeze symptoms and rhinoconjunctivitis [13]. At 6 years, children were considered old enough to reliably assess asthma, using the ISAAC question for a history of asthma plus wheeze or inhaler use in the last 12 months.

Body mass index (BMI) was defined as weight (kg)/height (m^2) with gender and age-specific cut-off values to define obesity [14], overweight [14], and underweight [15].

Fractional exhaled nitric oxide (FE_{NO}) was measured using the chemiluminescence method. Measurements were taken prior to bronchodilator challenge using a Niox[®] MINO machine (Aerocrine AB, Solna, Sweden) and the six second exhalation mode to American Thoracic Society and European Respiratory Society standards while children were seated [16].

Spirometry was performed using an EasyOn-PC spirometer and Easyware software (ndd Medizintechnik AG, Zurich, Switzerland) according to American Thoracic Society and European Respiratory Society guidelines [17], but without the criterion for the nurse to exclude poor quality curves. Instead, children were asked to repeat manoeuvres until an acceptable machine-based test grade was achieved (grade A, B, C) or until the child tired. The test was performed with children seated upright without wearing a nose clip. Spirometry reference values were from Stanojevic *et al.* [18] Bronchodilator challenge was performed on all children regardless of their pre-bronchodilator test grade after withholding beta-agonists for 6 h if short acting and 12 h if long acting. Salbutamol (200 mcg) was administered to children using a Volumatic[®] spacer (Glaxo Wellcome GmVH&Co. KG, Bad Oldesloe, Germany) and 15 min later spirometry was repeated. A positive response to bronchodilator was defined as $\geq 12\%$ change from baseline FEV₁.

Statistical analysis

An intention to treat statistical analysis was performed using SAS Version 9.2 (SAS Institute, Cary, NC, USA). The effect of each probiotic on the cumulative prevalence of eczema, SCORAD (≥ 10), and wheeze occurring between 3 months and 6 years, and SPT sensitization occurring at 2, 4, or 6 years, were summarized using Kaplan–Meier plots and hazard ratios estimated with Cox's proportional hazards models. For outcomes collected at 6 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 (≥ 10), wheeze, current asthma, rhinoconjunctivitis, bronchodilator response, SPT sensitization, and sIgE ≥ 0.35 kU/L in serum. Exhaled nitric oxide and total IgE levels were log transformed. Then analysis of variance was used to investigate study group differences in geometric mean levels of these outcomes and mean differences in % FEV₁/FVC and FEV₁. All analysis of lung function measures was limited to children with acceptable curves (machine graded as A, B, or C) and FEV₁ was adjusted for height and gender. All health outcome analysis was adjusted for the presence of a family history of allergic disease in the mother only, father only, or both parents. To assess whether the effects differed by SPT sensitiza-

tion, the interaction between sensitization and study group was added to the models. A *P*-value < 0.05 was considered significant in all analyses.

We also investigated the effect that the use of non-study probiotics had on the relationship between each probiotic and each outcome. Up to age 2 years, we collected information only on any non-study probiotic consumption. At 4 and 6 years, we collected more detail about the species of probiotic used. As suggested in the literature [8], we hypothesized that only probiotics of the *L. rhamnosus* species (predominantly GG and HN001) are likely to prevent eczema and thus be likely to dilute a true effect of the probiotic intervention. Thus, the variable for cumulative non-study probiotic consumption between birth and 6 years was defined to include the use of any non-study probiotic between birth and 2 years or the use of a probiotic supplement containing but not restricted to *L. rhamnosus* between 2 and 6 years. We also defined a variable that described the use of *L. rhamnosus* as a supplement between the ages of 4 and 6 years and used this to test for interaction effects at 6 years.

When skin prick testing was not performed, the SPT sensitization status was randomly imputed [19] using probabilities of SPT sensitization from logistic regression models including terms for SPT sensitization status at other times, eczema, SCORAD (≥ 10), wheeze, study group, non-study probiotic use, gender, ethnicity, parental allergic disease, and family history of treated asthma. The results of multiple imputations (1000) were averaged [19].

Results

Among randomized children eligible at birth, 144/159 (90.6%) in the placebo, 134/157 (85.4%) in the HN001, and 144/158 (91.1%) in the HN019 group completed questionnaires at the 6-year assessment [mean age: 6.2 years (SD: 0.22, range: 5.9–7.4)]. Fewer participants completed measures requiring physical assessment: For eczema and SCORAD, there were 129/159 (81.1%) children in the placebo, 112/157 (71.3%) in the HN001 and 133/158 (84.2%) in the HN019 groups. Skin prick tests were performed on 120/159 (75.5%) children in the placebo, 109/157 (69.4%) in the HN001, and 129/158 (81.6%) in the HN019 groups. Blood samples were taken from 98/159 (61.6%) children in the placebo, 92/157 (58.6%) in the HN001, and 106/158 (67.1%) in the HN019 groups at age 6 years. Response rates to skin prick tests or blood sampling did not differ greatly between those SPT-positive at 4 years (85% and 73% respectively) and those negative (90% and 73% respectively). There were no significant differences between study group in key variables at birth [3].

Table 1. Incidence of eczema, skin prick test (SPT) sensitization and SCORAD ≥ 10 by study group (based on number completing at each age)

	Placebo	<i>Lactobacillus rhamnosus</i> HN001	<i>Bifidobacterium animalis</i> subsp <i>lactis</i> HN019	P
Eczema				
Before age 2	41/156 (26.3%)	22/154 (14.3%)	37/155 (23.9%)	0.03
Between age 2 and 4	32/140 (22.9%)	23/129 (17.8%)	23/141 (16.3%)	0.35
Between age 4 and 6	9/127 (7.1%)	5/112 (4.5%)	12/132 (9.1%)	0.37
SPT sensitization				
At age 2	42/146 (28.8%)	30/141 (21.3%)	35/149 (23.5%)	0.30
Between age 2 and 4	17/136 (12.5%)	13/122 (10.7%)	23/139 (16.6%)	0.35
Between age 4 and 6	11/116 (9.5%)	8/107 (7.5%)	10/125 (8.0%)	0.85
SCORAD ≥ 10				
Before age 2	59/156 (37.8%)	36/154 (23.4%)	57/155 (36.8%)	0.01
Between age 2 and 4	11/159 (6.9%)	19/157 (12.1%)	11/158 (7.0%)	0.17
Between age 4 and 6	6/127 (4.7%)	2/112 (1.8%)	5/132 (3.8%)	0.69

P-value <0.05 are significant.

Table 2. Hazard ratios (95% CIs) for the 6-year cumulative prevalence of eczema, SCORAD ≥ 10 , wheeze, and atopic sensitization in infants taking *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* subsp *lactis* HN019 (all adjusted for mother only, father only, or both parents with allergic disease)

	Placebo	<i>L. rhamnosus</i> HN001	P-value	<i>B. animalis</i> subsp <i>lactis</i> HN019	P-value	P-value*	P-value for interaction [†]
Eczema (<i>n</i> = 465 [‡])	1.00	0.56 (0.39–0.80)	0.001	0.83 (0.61–1.14)	0.26	0.006	0.80
	82/156	50/154		73/155			
SCORAD ≥ 10 (<i>n</i> = 465 [‡])	1.00	0.69 (0.49–0.98)	0.04	0.98 (0.71–1.35)	0.89	0.08	0.51
	76/156	57/154		74/155			
Wheeze (<i>n</i> = 465 [‡])	1.00	0.81 (0.62–1.07)	0.14	1.00 (0.77–1.31)	0.99	0.23	1.00
	102/156	87/154		102/155			
Skin prick test sensitization (<i>n</i> = 442 [‡])	1.00	0.69 (0.48–0.99)	0.04	0.91 (0.65–1.26)	0.56	0.12	
	72/149	51/142		69/151			
Non-study probiotics used (<i>n</i> = 121 [§])	1.00	1.27 (0.63–2.56)	0.50	0.74 (0.34–1.61)	0.45	0.45	0.02
	13/35	20/43		13/43			
Non-study probiotics not used (<i>n</i> = 311 [§])	1.00	0.58 (0.37–0.89)	0.02	1.03 (0.71–1.50)	0.86	0.06	
	56/109	31/96		56/106			

*P-value for differences between the three study groups.

[†]P-value for interaction between study group and non-study probiotic use.

[‡]*n* is number completing at least one time-point.

[§]Missing data due to loss-to-follow-up before 6 years.

Bold values denote the cumulative prevalence to make the difference.

Table 1 shows the incidence of eczema, SCORAD ≥ 10 , and SPT sensitization at each age. Among the children who developed eczema for the first time at 6 years, fewer were in the HN001 group than the HN019 or placebo groups, although the difference between groups was not significant. This pattern was also reflected in the number of children whose SCORAD values moved above the ≥ 10 cut-off or became sensitized for the first time at 6 years. More children had lost their eczema by 6 years than had developed eczema (27, 19, and 22 children in the placebo, HN001 and HN019 groups, $P = 0.57$). Similarly, SCORAD values dropped to below 10 by 6 years for a greater number of children (27, 27, and 35 in the placebo,

HN001, and HN019 groups, respectively, $P = 0.32$). For SPT sensitization, only a few had lost their sensitization by 6 years (2, 3, and 9 in the placebo, HN001, and HN019 groups, respectively, $P = 0.07$).

Table 2 shows the cumulative prevalence of outcomes measured before 2 years, at age 4 or at age 6 years. Children in the HN001 group had significant reductions in risk for the cumulative prevalence of eczema by age 6 years compared to placebo (Table 2) and compared to HN019 [HR 0.67 (95% CI 0.47–0.96, $P = 0.03$), and SCORAD ≥ 10 compared to placebo (Table 2). These effects did not depend on the SPT sensitization status of the child. Children taking HN001 also had a > 30% significant risk reduction in the cumula-

tive prevalence of SPT sensitization compared with children taking the placebo. This effect was significant for children allergic to aeroallergens [HR = 0.65 (95% CI 0.44–0.95), $P = 0.03$], but not for those allergic to food allergens [HR = 0.70 (95% CI 0.41–1.20), $P = 0.20$]. There was evidence that the risk reduction for overall SPT sensitization differed according to the use of non-study probiotics (P for interaction = 0.02) and present only among those who did not take non-study probiotics. The risk of all outcomes did not differ depending on which parent had allergic disease. Children in the HN019 group showed no similar risk reductions. These effects are shown graphically as Kaplan–Meier plots for eczema, SCORAD ≥ 10 , and SPT sensitization (Fig. 1). To investigate non-response bias in SPT assessments, analyses were performed with imputed SPT sensitization status for missing values. The imputed and unimputed hazard ratios and relative risks were the same to within 0.03, except when non-study probiotics were used where they differed by up to 0.18 (data not shown).

Table 3 shows the relative risks for outcomes assessed at 6 years. With the smaller numbers where outcomes were present at this time-point only, the $> 30\%$ reductions in risk for eczema and SCORAD ≥ 10 among children in the HN001 group did not quite reach significance. Neither probiotic affected the prevalence of wheeze, current asthma, rhinoconjunctivitis, or bronchodilator response. The levels of FEV₁, %FEV₁/FVC ratios, and exhaled nitric oxide were similar across all three study groups. Neither SPT sensitization status of the child nor the use of non-study probiotics showed significant interactions with these risk estimates. There was no difference in levels of TARCs or MDCs between study groups (data not shown).

Among the subset participating in skin prick tests ($n = 358$), HN001 significantly lowered the risk of sensitization, particularly among those not using non-study probiotics, but there was little effect of HN019. The HN001 effect was significant among those allergic to aeroallergens (RR = 0.68, 95% CI 0.49–0.95, $P = 0.02$) with no significant effect among those allergic to food allergens (RR = 0.80, 95% CI 0.35–1.83, $P = 0.60$). Fewer children ($n = 296$) provided a blood sample and the presence of ssIgE antibody reaction to at least one allergen using the cut point of 0.35 kU/L was not significantly reduced in either probiotic group. An additional analysis was conducted using the food-specific 95% positive predictive values for food challenge response proposed by Sampson and Ho [20] and retaining the cut point of 0.35 kU/L for aeroallergens. This showed a lower rate for HN001 (RR = 0.74, 95% CI 0.53–1.04) that approached significance ($P = 0.08$). HN001 also provided some protection against total IgE which was significant in children not using non-study

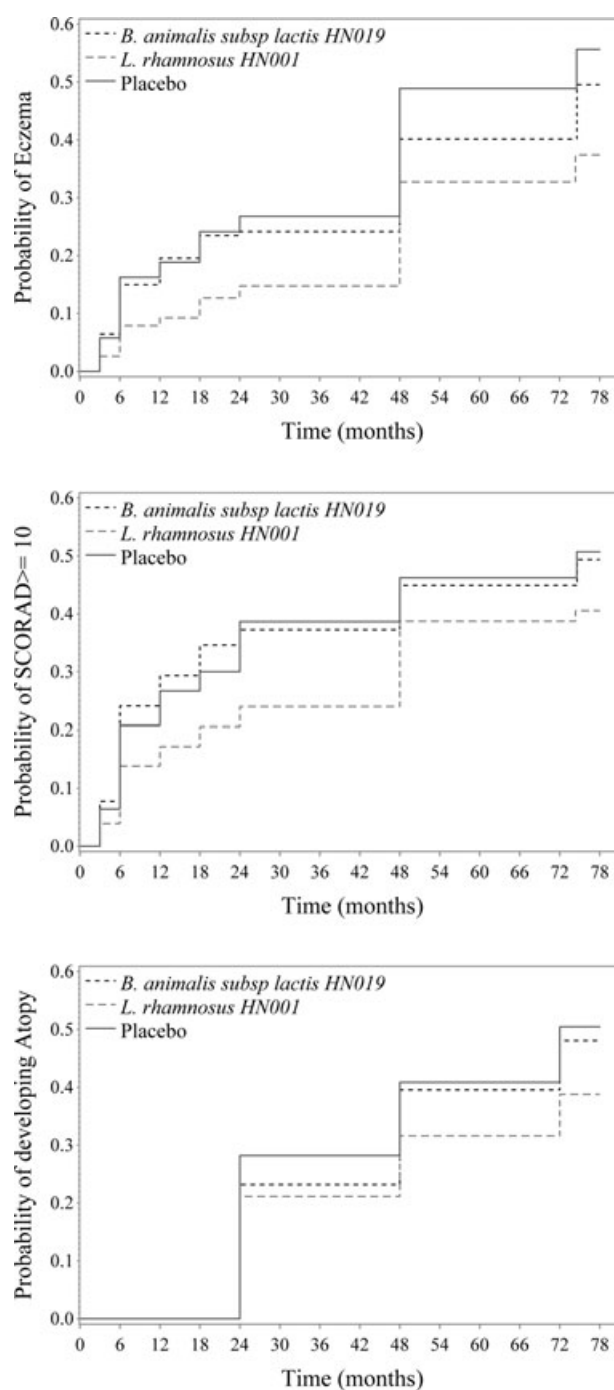


Fig. 1. Six-year cumulative prevalence of eczema in children taking the placebo, *Lactobacillus rhamnosus* HN001, or *Bifidobacterium animalis* subsp. *lactis* HN019. Six-year cumulative prevalence of SCORAD ≥ 10 in children taking the placebo, *L. rhamnosus* HN001, or *B. animalis* subsp. *lactis* HN019. Six-year cumulative prevalence of SPT sensitization in children taking the placebo, *L. rhamnosus* HN001, or *B. animalis* subsp. *lactis* HN019.

probiotics. After stratification of the data by the presence or absence of eczema at 6 years, these differences persisted only among children with eczema [Total IgE

Table 3. Relative risks (95% CIs) of current health outcomes (eczema, SCORAD ≥ 10 , wheeze, asthma, rhinoconjunctivitis), bronchodilator response, mean %FEV₁/FVC, mean FEV₁, and geometric mean exhaled nitric oxide at 6 years in infants taking *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* subsp *lactis* HN019 (all adjusted for mother only, father only, or both parents with allergic disease)

	Placebo	<i>L. rhamnosus</i> HN001	<i>P</i> -value	<i>B. animalis</i> subsp <i>lactis</i> HN019	<i>P</i> - value	<i>P</i> - value*	<i>P</i> - value†
Current eczema (<i>n</i> = 374‡)	1.00 43/129	0.66 (0.44–1.00) 26/112	0.05	0.90 (0.64–1.28) 41/133	0.56	0.12	0.51
Current SCORAD ≥ 10 (<i>n</i> = 374‡)	1.00 36/129	0.62 (0.38–1.01) 20/112	0.055	0.79 (0.52–1.21) 30/133	0.28	0.14	0.51
Wheeze last 12 months (<i>n</i> = 422§)	1.00 41/144	0.90 (0.61–1.32) 35/134	0.59	0.96 (0.66–1.39) 40/144	0.83	0.87	0.81
Current asthma (<i>n</i> = 422§)	1.00 35/144	0.92 (0.60–1.41) 31/134	0.71	1.03 (0.69–1.54) 37/144	0.89	0.87	0.30
Rhinoconjunctivitis last 12 months (<i>n</i> = 422§)	1.00 22/144	1.03 (0.60–1.78) 22/134	0.90	0.93 (0.54–1.61) 21/144	0.80	0.93	0.53
Bronchodilator response $\geq 12\%$ (<i>n</i> = 174¶)	1.00 6/60	1.36 (0.51–3.68) 8/52	0.54	1.29 (0.49–3.41) 9/62	0.61	0.81	0.08
% FEV ₁ /FVC (<i>n</i> = 215)	<i>n</i> = 73	<i>n</i> = 64		<i>n</i> = 78			
Mean% (95% CI)	84.1 (82.6–85.7)	82.7 (81.0–84.3)	0.20	82.9 (81.4–84.4)	0.27	0.38	0.30
Mean differences (95% CI)		–1.47 (–3.72 to 0.78)		–1.20 (–3.35 to 0.94)			
FEV ₁ (L)‡‡ (<i>n</i> = 215)	<i>n</i> = 73	<i>n</i> = 64		<i>n</i> = 78			
Mean (95% CI)	1.27 (1.23–1.31)	1.24 (1.20–1.28)	0.17	1.27 (1.24–1.31)	0.82	0.34	0.84
Mean differences (95% CI)		–0.04 (–0.10–0.12)		–0.006 (–0.06 to 0.05)			
eNO (<i>n</i> = 327**)	<i>n</i> = 111	<i>n</i> = 104		<i>n</i> = 112			
Geometric means (95% CI)	13.7 (11.6–16.1)	13.1 (11.0–15.6)	0.81	13.0 (10.6–15.8)	0.55	0.69	0.56
Geometric mean ratios (95% CI)	1.00	0.96 (0.75–1.22)		0.95 (0.73–1.23)			

**P*-value for differences between the three study groups.

†*P*-value for interaction between study group and non-study probiotic use.

‡*n* with eczema and SCORAD assessed at 6 years.

§*n* with questionnaire data at 6 years.

¶*n* with acceptable curves pre- and post-bronchodilator.

|| *n* with acceptable curve pre-bronchodilator.

***n* with successful eNO completed at 6 years.

‡‡ Adjusted for height and gender.

kU/L 107.1 (95% CI 50.6–226.7) in HN001 group vs. 247.3 (137.8–443.7) in placebo group, *P* = 0.08]. Total IgE levels were similar to placebo for children in the HN019 group and were not affected by whether the child had eczema. None of these effects were dependent on which parent had allergic disease. There were no similar effects on SPT sensitization, specific, or total IgE in the HN019 group. (Table 4)

Table 5 shows that neither probiotic influenced the weight, height or BMI of children at 6 years.

Discussion

Our earlier findings of a protective effect of HN001 on the cumulative prevalence of eczema before age 2 and 4 years are confirmed for eczema occurring before age 6 years, where the effect of this probiotic was to reduce the cumulative prevalence by over 40% (Table 2).

Importantly, our data shows that HN001 did not simply provide protection against eczema during the 2-year intervention period, but that protection persisted for 4 years after cessation of HN001 supplementation. HN001 also reduced the risk of having eczema (and SCORAD ≥ 10) at 6 years (Table 3). These children are mostly those with eczema that had persisted from a younger age and include few new cases. Thus, our study was not powered to look at incident eczema.

Among children taking HN001, there were also 30% reductions in the cumulative prevalence of having a SCORAD value ≥ 10 , and developing SPT sensitization. There would appear to be a consolidation of effect on SPT sensitization, with the earlier findings at 2 years and 4 years strengthening and becoming significant (*P* = 0.04) by 6 years, perhaps due to larger numbers attaining SPT sensitization by 6 years. However, there was also a significant reduction compared to placebo in

Table 4. Relative risks (95% CIs) for skin prick test sensitization and specific IgE, and geometric mean total IgE at 6 years in infants taking *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* subsp *lactis* HN019 (all adjusted for mother only, father only or both parents with allergic disease)

	Placebo	<i>L. rhamnosus</i> HN001	<i>P</i> -value	<i>B. animalis</i> subsp <i>lactis</i> HN019	<i>P</i> -value	<i>P</i> -value*	<i>P</i> -value†
Skin prick test sensitization (<i>n</i> = 358‡)	1.00 56/120	0.72 (0.53–1.00) 38/109	0.048	0.88 (0.66–1.16) 53/129	0.35	0.13	
Non-study <i>L. rhamnosus</i> used (<i>n</i> = 48)	1.00 6/17	1.40 (0.64–3.07) 9/17	0.40	0.82 (0.29–2.32) 4/14	0.70	0.45	0.12
Non-study <i>L. rhamnosus</i> not used (<i>n</i> = 310)	1.00 50/103	0.63 (0.44–0.91) 29/92	0.01	0.87 (0.65–1.16) 49/115	0.35	0.03	
Specific IgE ≥ 0.35 kU/L (<i>n</i> = 296§)	1.00 51/98	0.84 (0.62–1.13) 41/92	0.24	1.12 (0.87–1.44) 62/106	0.37	0.11	
Non-study <i>L. rhamnosus</i> used (<i>n</i> = 39)	1.00 5/13	1.42 (0.63–3.22) 9/15	0.40	1.12 (0.43–2.88) 5/11	0.82	0.65	0.23
Non-study <i>L. rhamnosus</i> not used (<i>n</i> = 257)	1.00 46/85	0.76 (0.55–1.06) 32/77	0.11	1.12 (0.87–1.44) 57/95	0.39	0.04	
Total IgE (kU/L) (<i>n</i> = 292¶)	<i>n</i> = 98	<i>n</i> = 91		<i>n</i> = 103			
Geometric means (95% CI)	104.3 (75.5–142.8)	66.8 (47.1–94.8)	0.07	105.7 (76.1–146.9)	0.95	0.10	
Geometric mean ratios (95% CI)	1.00	0.64 (0.40–1.04)		1.01 (0.64–1.61)			
Non-study <i>L. rhamnosus</i> used (<i>n</i> = 39)	<i>n</i> = 13	<i>n</i> = 15		<i>n</i> = 11			
Geometric means (95% CI)	44.0 (14.6–132.4)	58.6 (19.9–173.2)	0.71	79.6 (22.6–279.7)	0.47	0.77	0.12
Geometric mean ratios (95% CI)	1.00	1.33 (0.28–6.28)		1.81 (0.34–9.52)			
Non-study <i>L. rhamnosus</i> not used (<i>n</i> = 253)	<i>n</i> = 85	<i>n</i> = 76		<i>n</i> = 92			
Geometric means (95% CI)	121.5 (85.4–172.9)	70.6 (48.8–102.0)	0.03	108.3 (77.4–151.6)	0.64	0.08	
Geometric mean ratios (95% CI)	1.00	0.58 (0.35–0.96)		0.89 (0.55–1.44)			

**P*-value for differences between the three study groups.†*P*-value for interaction between study group and non-study probiotic use.‡*n* = all those completing skin prick tests at 6 years.§*n* = all with blood test completed at 6 years.¶*n* = all with total IgE measures.All *P*-value < 0.05 are significant.

the point prevalence of SPT sensitization at 6 years which strengthened after the removal of children who had taken a non-study *L. rhamnosus* probiotic. If the 95% positive predicted values for food allergens proposed by Sampson and Ho [20] were used the protective effect of HN001 on sslgE approached significance ($P = 0.08$). Using these values, Hill et al. [21] showed SPTs to be more sensitive in diagnosing peanut and egg allergy measured by challenge than sslgE antibody level, suggesting that the SPT measure may be the better predictor of clinical reaction. Although most other probiotic trials have found no effect on SPT or sslgE sensitization, one study [22] found an increased risk of

SPT sensitization among children taking *L. acidophilus*. In other studies of lactobacillus, SPT sensitization was significantly reduced among the subset of children with maternal allergic disease [23] or maternal sensitization [24]. In our study, there was no significant difference in risk dependent on which parent had allergic disease.

Mean total IgE level was also non-significantly lower among children in the HN001 than the placebo group ($P = 0.07$). This effect was stronger among children with current eczema, although due to reduced numbers this also did not reach significance ($P = 0.08$). In the subgroup who did not use non-study probiotics this difference reached significance ($P = 0.03$). Kukkonen et al.

Table 5. Anthropometric measures at 6 years by study group

	Placebo (n = 127)	<i>L. rhamnosus</i> HN001 (n = 112)	<i>B. animalis</i> subsp <i>lactis</i> HN019 (n = 133)	P-value*
Mean weight (kg) (95% CI)	22.4 (21.9–22.9)	22.6 (22.1–23.2)	22.6 (22.1–23.1)	0.80
Range of weights (kg)	16.8–30.2	15.9–30.8	16.7–35.2	
Mean height (cm) (95% CI)	118.6 (117.7–119.5)	119.3 (118.4–120.3)	119.0 (118.1–119.9)	0.57
Range of heights (cm)	107.1–133.9	106.0–130.0	105.4–136.3	
Mean BMI (95% CI)	15.9 (15.6–16.1)	15.8 (15.6–16.1)	15.9 (15.7–16.2)	0.82
Range of BMI	13.3–19.9	12.4–19.6	12.7–23.0	
Obesity [†] (N = 3), n (%)	3 (2.3)	0 (0)	0 (0)	0.34
Overweight [‡] (N = 26), n (%)	9 (6.8)	9 (8.0)	8 (6.3)	
Normal weight (N = 341), n (%)	120 (90.2)	102 (91.1)	119 (93.7)	
Underweight [§] (N = 2), n (%)	1 (0.8)	1 (0.9)	0 (0)	

*P-value for differences between the three study groups.

[†]Definition of obesity [14]: for girls: 6.0–6.5 years BMI > 20.1; 6.5–7.0 years BMI > 20.5, 7.0–7.5 years BMI > 21.0; for boys: 6.0–6.5 years BMI > 20.2, 6.5–7.0 years BMI > 20.6, 7.0–7.5 years BMI > 21.1.

[‡]Definition of overweight [14]: for girls 6.0–6.5 years BMI > 17.5, 6.5–7.0 years BMI > 17.8; 7.0–7.5 years BMI > 18.0; for boys 6.0–6.5 years BMI > 17.7; for boys 6.5–7.0 years BMI > 17.9; for boys 7.0–7.5 BMI > 18.2.

[§]Definition of underweight [15]: for girls 6.0–6.5 years BMI < 12.90; 6.5–7.0 years BMI < 12.91; 7.0–7.5 years BMI < 12.95; for boys 6.0–6.5 years BMI < 13.10, 6.5–7.0 years BMI < 13.08, 7.0–7.5 years BMI < 13.09.

[5] report a similar reduction in 37% in total IgE levels in a group of 5-year-old children who had taken *L. rhamnosus* GG in the first 6 months of life, but do not report effects on sIgE.

In general, there have been two mechanisms proposed for a probiotic effect on atopy. Firstly, probiotics may enhance gut barrier function [25]. Secondly, probiotics may influence the immune system providing a more balanced Th1/Th2 immune profile [26]. By age 2 years, the presence in the gut of HN001 and HN019 among infants in this study corresponded closely to study group [3], but by age 4 years this relationship had ceased. We have not looked at this at age 6 years but would not expect to find that the study probiotics affected levels of these probiotics in the gut at 6 years, given the lack of effect at 4 years. Thus, a long-term effect of HN001 against atopic sensitization at 6 years suggests that this probiotic had immunomodulatory effects during the first 2 years of life which have persisted to the sixth year. Early evidence for an immunomodulatory effect of HN001 is found in the cord blood of these same children where a higher proportion of HN001-supplemented infants had detectable IFN γ compared to placebo [27]. A study of HN001-supplemented (vs. non-supplemented) pigs sensitized to *Ascaris suum* allergen (ASA) found the HN001 group had greater levels of the Th1 cytokine IFN γ and the regulatory IL-10 cytokine and less severe skin reactions to an intradermal test, but only at high doses (> 50 μ g/mL) of ASA [28]. A pre-post intervention trial in healthy adult humans also supports an immunomodulatory effect of HN001 [29]. TGF- β in breast milk has been shown to be protective against immunological outcomes of allergy [30] and was shown to be elevated in the breast milk of

mothers in our study who received probiotics [27]. These effects could be modulated through epigenetic mechanisms [7, 31]. It is perhaps more difficult to explain our findings of a protective effect of HN001 against eczema, which was not dependent on response to skin prick tests, suggesting that the preventive effect on eczema may not be IgE mediated.

Any potential effect on outcomes of non-study *L. rhamnosus* probiotics, which were used about equally across all study groups, had the potential to dilute any true effect of the study probiotics. Thus, our finding of a strengthening of effect of HN001 on atopic status (measured by SPT) after removal of children who had used these non-study probiotics suggests that there may be at least a weak effect of all probiotics containing any strain of *L. rhamnosus* on atopic status. Most of the studies of *L. rhamnosus* have used the GG strain with some showing a protective effect against eczema at 2 [32], 4 [33], and 7 years [34] which was not paralleled by an equivalent protection against atopic sensitization.

In contrast to the reported protective effect for eczema, Kalliomaki et al. [34] found an elevated relative risk for asthma of 3.44 (95% CI 0.98–12.1) among 7-year-old children taking *L. rhamnosus* GG, but lower mean concentrations of eNO among these children at 4 years [33]. These findings have not been substantiated by our findings showing neither probiotic was associated with an increased risk of current asthma, asthma symptoms or lung function abnormality at age 6 years. Consistent with a Finnish cohort which found no effect of a mixture of probiotics taken in early life on eNO at 5 years [5], we also found little difference in eNO level between study groups.

At age 4 years, we reported a strong protective effect of HN001 against symptoms consistent with rhinoconjunctivitis [4]. This has not been supported by our 6 years findings, suggesting that our earlier findings may have occurred by chance or resulted from a systematic bias due to misclassification of upper respiratory tract infections (URTI) as rhinoconjunctivitis. This would suggest that HN001 might confer protection against URTI at 4 years. However, we found no evidence that either study probiotic protected against URTIs occurring before age 2 years (Wickens K, Black PN, Stanley TV, Mitchell E, Fitzharris P, Tannock GW, Purdie G, Crane J and Probiotic Study Group, unpublished data) although others have reported a protective effect against URTI [35]. Alternatively, the effect at 4 years could have been real but transitory.

A strength of our study is the long-term follow-up to age 6 years. The only other study with a longer follow-up is the original Finnish study, which also showed a similar protective effect (RR = 0.64, 95% CI 0.45–0.92) against eczema persisting to 7 years using *L. rhamnosus* GG [34]. In contrast, a second Finnish group using a combination of probiotics (including *L. rhamnosus* GG) plus a prebiotic found the protective effect against atopic eczema by 2 years [36] only persisted to 5 years in the group of children delivered by caesarean section [37]. The effect on all eczema reported in their earlier article was also no longer present at 5 years.

There may be limitations in our study due to unblinding of parents after completion of the 2-year follow-up, but given our definition of eczema [10] was primarily symptom (itch) rather than disease based (eczema) and our findings for the objective measures of SCORAD and skin prick sensitization were consistent with that of eczema, we do not believe that bias due to unblinding of parents is a likely contributor to our findings. Further the nurses remained blinded at all age assessments.

There is also the possibility that parental knowledge of early SPT results, together with unblinding of participants, may have resulted in a selection bias at 6 years. However, the association between atopic status at 4 years and participation in SPTs or blood tests at 6 year was similar between study groups. Some of our positive associations may be due to multiple testing of the data.

While evidence, including a Cochrane review [8], supports a role for probiotics containing *L. rhamnosus* in the prevention of eczema, our study is the first to show a significant effect of a probiotic on skin prick test responses by 6 years. The protection provided by HN001 was much weaker for HN019 indicating that not all probiotics are the same. This provides further support for a role of HN001, not just in eczema prevention, but possibly also in long-term prevention of atopic sensitization.

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Conflicts of interest

None of the authors has a conflict of interest.

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