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Maternal supplementation alone with *Lactobacillus rhamnosus* HN001 during pregnancy and breastfeeding does not reduce infant eczema

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Abstract

Background: In a randomized placebo-controlled trial, we previously found that the probiotic *Lactobacillus rhamnosus* HN001 (HN001) taken by mothers from 35 weeks of gestation until 6 months post-partum if breastfeeding and their child from birth to age 2 years halved the risk of eczema during the first 2 years of life. We aimed to test whether maternal supplementation alone is sufficient to reduce eczema and compare this to our previous study when both the mother and their child were supplemented.

Methods: In this 2-centre, parallel double-blind, randomized placebo-controlled trial, the same probiotic as in our previous study (HN001, 6×10^9 colony-forming units) was taken daily by mothers from 14-16 weeks of gestation till 6 months post-partum if breastfeeding, but was not given directly to the child. Women were recruited from the same study population as the first study, where they or their partner had a history of treated asthma, eczema or hay fever.

Results: Women were randomized to HN001 (N = 212) or placebo (N = 211). Maternal-only HN001 supplementation did not significantly reduce the prevalence of eczema, SCORAD ≥ 10 , wheeze or atopic sensitization in the infant by 12 months. This contrasts with the mother and child intervention study, where HN001 was associated with reductions in eczema (hazard ratio (HR): 0.39, 95% CI 0.19-0.79, $P = .009$) and SCORAD (HR = 0.61, 95% 0.37-1.02). However, differences in the HN001 effect between studies were not significant. HN001 could not be detected in breastmilk from supplemented mothers, and breastmilk TGF- β /IgA profiles were unchanged.

Conclusion: Maternal probiotic supplementation without infant supplementation may not be effective for preventing infant eczema.

KEYWORDS

atopic sensitization, bacteria in breastmilk, breastmilk proteins, eczema, *Lactobacillus rhamnosus* HN001, probiotics

1 | INTRODUCTION

We have previously found that the probiotic, *Lactobacillus rhamnosus* HN001 (HN001), when taken daily by pregnant women from 35 weeks of gestation to 6 months post-partum if breastfeeding and by their infant daily from birth to age 2 years reduces the prevalence of eczema at age 2,¹ 4² and 6 years.³ In the same trial, the probiotic *Bifidobacterium animalis* subs. *lactis* HN019 did not provide significant protection against eczema or atopic sensitization, highlighting the differential efficacy of probiotic species. That study and others⁴ have included both maternal and infant supplementation, making it difficult to distinguish whether the beneficial effect of probiotics was through maternal transfer of protective factors and/or via direct supplementation to the infant.

The rationale for this study was based on evidence that suggests that protection against eczema in the infant can be achieved when probiotic exposure is solely through maternal supplementation.⁴ This could be through a probiotic effect on the composition of breastmilk which has been shown to be important for infant gut bacterial colonization⁵ and/or maternal transmission of bacterial metabolites, such as short-chain fatty acids, to the foetus through the placenta.⁷ Probiotic-mediated modification of maternal gut microbiota and associated metabolites⁸ may also provide infant protection against allergic disease such as eczema.

Furthermore, few studies have found a probiotic effect on atopic sensitization, perhaps because probiotic supplementation has commenced too late in pregnancy to influence the development of atopy. Evidence showing foetal production of IgE antibodies may occur before the end of the first trimester and allergen-specific IgE antibodies towards the end of the second trimester, suggests that an earlier intervention might maximize foetal probiotic effects.⁹

For these reasons, we studied the effects of maternal-only HN001 supplementation on infant allergic disease prevalence commencing in the first trimester of pregnancy through to 6 months post-partum while breastfeeding. To enable comparisons between eczema outcomes at age 12 months, we also report our previously unpublished 12-month allergic disease outcomes from our earlier probiotic study, where both mother and infant received HN001 supplementation¹ and compare HN001 effects on allergic disease development between the two studies. To assess whether HN001 altered the immunologic profile of breastmilk, between-group differences in TGF- β 1, TGF- β 2 and immunoglobulin A were analysed in breastmilk samples.

The trial is registered at the Australian New Zealand Clinical Trials Registration No. ACTRN12612000196842 and received ethical approval from the New Zealand Multi-Region Ethics Committee (MEC/11/09/077).

2 | METHODS

We have previously published the study methods for this study.¹⁰ These were based on our earlier mother and child intervention

study,¹ with the exception that in the current intervention study only mothers took the probiotic, and we brought forward the start of the intervention from 35 to 14-16 weeks of gestation. Briefly, the study was a 2-centre, parallel double-blind, randomized, placebo-controlled trial with an allocation ratio of 1:1. We investigated the effects of HN001 (6×10^9 colony-forming units daily) taken by the mother from 14 to 16 weeks of gestation and until 6 months post-partum if breastfeeding on the development of eczema and atopic sensitization in the infant by age 12 months. The placebo powder was corn-derived maltodextrin, and these capsules had identical appearance and smell to the probiotic capsules. Both active and placebo capsules were supplied by Fonterra Co-operative Group Ltd, who were also responsible for randomization and quality control of probiotic viability.

Randomization was stratified by study centre and performed in blocks of random length according to a computer-generated randomized list. At enrolment, research staff provided eligible women with the next available sequentially numbered capsule bottle. Both participants and research staff were blind to treatment group.

After collection of the used bottles of capsules, remaining capsules were counted by researchers with no participant contact. Adherence was calculated as the proportion of capsules taken divided by the number of days till 6 months post-partum or till breastfeeding ceased (if before 6 months).

Pregnant women were recruited between November 2012 and December 2014 in Auckland and Wellington, New Zealand. In our earlier study, recruitment was between March 2004 and August 2005. In both studies, the woman or the biologic father had a history of treated asthma, eczema or hay fever, but the women were otherwise healthy and intending to breastfeed.

All outcomes were defined as in our earlier study and measured in both studies at age 6 and 12 months. Eczema was measured in the infant at ages 6 and 12 months using the UK Working Party's Diagnostic Criteria for atopic dermatitis, modified for use in infants.¹⁰ Eczema severity was assessed at the same time points using SCORAD,¹⁰ defined dichotomously as a score of ≥ 10 . Wheeze was collected via questionnaire as a history of wheezing or whistling in the chest since the previous visit.¹¹ Skin prick tests were performed at age 12 months using standardized protocols with Stallergenes 1-mm lancets (Antony, France), positive control (histamine 10 mg/mL), negative control (phenolated glycerol-saline), *Dermaphagoides pteronyssinus*, cat fur, 5 grass mix, (Alyostal Stallergenes, Antony, France), egg white, peanut and cow's milk (ALK-ABELLO, Madrid, Spain). The positive control was measured at 10 minutes and allergens and the negative control at 15 minutes. A mean wheal diameter of 3 mm or greater, after subtraction of any negative control response, was considered positive.

Post-colostrum breastmilk was collected into sterile containers by mothers at 4-7 days post-partum and stored long term at -80°C . To examine the effect of supplementation on immunomodulatory factors in breastmilk, TGF- β 1 and TGF- β 2 (TGF- β Luminex Performance Assay, R&D systems) were measured in 157 samples from each treatment group and IgA (Human IgA ELISA, Mabtech) was measured in 160 samples from each treatment group. Values were

extrapolated for two samples outside the assay range. To investigate whether supplemented HN001 was being transferred to the breast-milk, total DNA was extracted from the milk samples of 16 active and 16 placebo mothers selected because of high treatment compliance. Methods for DNA extraction are given in full here as they are not included in the published methods paper. The extraction used DNAzol (Life Technologies) with the following modifications: 2 mL of thawed milk was centrifuged at $5000 \times g$ for 30 minutes at 4°C , and the cell pellet was washed twice with ice-cold PBS and resuspended in lysozyme (10 mg mL^{-1}) with gentle pipetting and then incubated for 15 minutes at room temperature with rocking before the addition of 0.5 mL DNAzol and subsequent inversion to mix. Glass beads (0.1 mm diameter) were added, and the sample was vortexed vigorously for 3×10 seconds bursts, before heating at 95°C for 15 minutes. Samples were centrifuged for 10 minutes at $10\,000 \times g$, and the supernatant was collected and the DNA precipitated using ethanol as recommended (Life Technologies). Real-time PCR studies utilized the Rotor-Gene SYBR Green PCR Kit (Qiagen) and PCR primers specific for *Lactobacillus rhamnosus* HN001² or Staphylococcus (not strain-specific¹²) using the following conditions; pre-incubation for 10 minutes at 95°C , then 50 cycles of denaturation at 95°C for

10 seconds, annealing at 65°C for 10 seconds, extension at 72°C for 10 seconds, followed by melt curve analysis.

The power calculation was based on our earlier study of HN001 and eczema (reported in Table 2). Based on a hazard ratio (HR) of 0.39 for eczema from the 6- and 12-month data, with a sample size of 200 in each group, the study had 87% power at the 5% level of significance.⁸

Intention-to-treat analyses were used to assess the effect of HN001 on the 12-month cumulative prevalence of eczema, SCORAD ≥ 10 and wheeze with the HR from a Cox's proportional hazards model. These analyses were performed for both studies and differences in the associations between HN001, and outcomes were compared between studies. The point prevalence of atopy at age 12 months was compared using relative rates and a chi-squared test in the current study only, as atopic sensitization was not measured at 12 months in the earlier study.

3 | RESULTS

The flow of participants through the study is summarized in Figure 1. Among randomized participants, 96% (203/212) in the

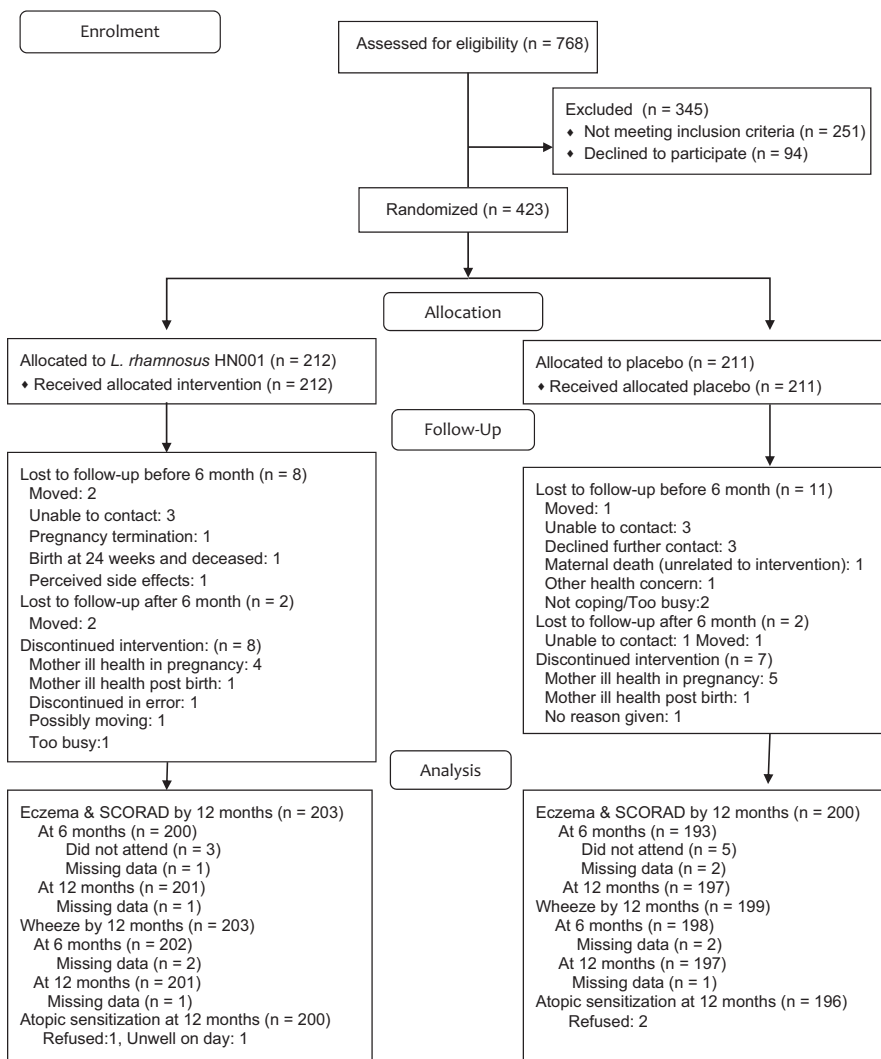


FIGURE 1 Participant flow chart

HN001 group and 95% (200/211) in the placebo group completed our primary outcome of eczema occurring before 12 months. Final assessments were between May 2014 and May 2016. The median adherence to allocated treatment did not differ by study group (HN001: 88.8% (IQR: 80.5%-96.3%), placebo: 89.2% (IQR 79.4%-95.9%).

The characteristics of the study population are shown in Table 1.

In this mother-only intervention study, we did not observe any significant protective effect of HN001 on eczema, SCORAD $\geq$ 10, wheeze or atopic sensitization in the child by age 12 months (Table 2). This contrasts with our earlier study, where HN001 was given to both mother and child. In that study, HN001 was strongly protective against eczema developing by age 12 months (hazard ratio (HR): 0.39 95% CI 0.19-0.79) and a HR of 0.61 (95% CI 0.37-1.02) for having a value of SCORAD $\geq$ 10. The differences in HN001 effect between the two studies were not significant for eczema ($P = .07$), SCORAD $\geq$ 10 ($P = .15$) or wheeze ($P = .92$) (Table 2).

HN001 had no effect on the levels of the breastmilk proteins, TGF- β 1, TGF- β 2 and IgA (Figure 2).

HN001 was only weakly detected in one of the 32 breastmilk samples screened, and this was from the placebo group. In control extractions where the probiotic was added directly to breastmilk, HN001 could be readily detected down to a concentration of 4×10^4 cfu mL $^{-1}$. To confirm that DNA from endogenous Gram-positive bacteria had been extracted, we also screened all samples for *Staphylococcus*, a species common in breastmilk.⁶ Fifteen samples, from both active and placebo groups, were *Staphylococcus* positive.

4 | DISCUSSION

We are the first group to compare the effects of a probiotic supplementation of mothers only with the effects when both mother and infant are supplemented on eczema and atopic sensitization in the infant. We used historic unpublished data and compared this to a recent data collection. Both studies used the same probiotic which was administered daily in the same dose to both mothers and children (in the earlier study), and both studies selected participants from the same high-risk population. Our findings suggest that infant exposure to HN001 via the mother may be insufficient to provide the eczema protection we observed in our previous study where HN001 was additionally administered to infants from birth till 2 years, although the differences we found between studies were not significant ($P = .07$).

A study of prenatal exposure only, this time to *Lactobacillus rhamnosus* GG (1.8×10^{10} cfu/day) from 36 weeks of gestation until delivery, also reported no effect on eczema¹³ and no transfer of this probiotic to maternal breastmilk.¹⁴ On the other hand, maternal-only exposure to *Lactobacillus rhamnosus* GG (GG), *L. acidophilus* La-5 and *Bifidobacterium animalis* subsp *lactis* Bb-12 in milk from 36 weeks of gestation to 3 months post-natally significantly halved the cumulative incidence of eczema in the infant by 2 years.¹⁵ Only

GG was transiently present in faecal samples of these infants, indicating a differential ability of probiotic species to transfer from mother to child.¹⁶ The odds of having eczema (OR = 0.51) for these indirectly supplemented infants were identical to that in our earlier study which supplemented both mothers and infants.¹ In another mothers-only study, a combination of *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 or *L. paracasei* ST11 and *B. longum* BL999 in sachets from 2 months before delivery and during the first 2 months of breastfeeding significantly reduced the odds ratios for persistent eczema by 70% and 83%, respectively.¹⁷ Arguably, a probiotic combination may provide better stimulation of the Th1 arm of the immune system than our single probiotic.¹⁸ Synergy between lactobacilli and bifidobacteria has been previously reported.¹⁹

In contrast to the above mother-only studies,^{15,17} our findings suggest that direct infant probiotic supplementation is necessary to ensure eczema prevention but does not clearly answer the question as to whether maternal supplementation is also required. In a recent meta-analysis,⁴ when only the infant is supplemented, results for eczema have been inconclusive, indicating the need for pre- and/or post-natal maternal supplementation. Rautava et al¹⁷ conclude that eczema prevention is effective only if probiotics are administered prenatally to the mother.

In our earlier study, median levels of IgA and TGF- β 1 in breastmilk were slightly higher in the HN001 group compared to placebo.²⁰ Although the assays for each study were sourced from different suppliers with differences in the detection limit for the IgA kit, it is also feasible that the differences between the studies could be due to chance.

The low rate of detection of HN001 in breastmilk, collected 4-7 days post-partum, suggests that the maternally ingested probiotic was not available to the developing infant in measurable quantities via breastmilk and is in accord with another study where a molecular approach was used to screen for administered probiotic bacteria in breastmilk,²¹ but contrast with the findings of others that utilized culture-dependent screening methods^{22,23}. However, 10 (31%) (5 HN001, 5 placebo) of our mothers contributing samples to this analysis had received antibiotics perinatally to which HN001 is very sensitive. This may have affected the abundance of the bacteria in breastmilk at this time point.

The lack of effect of HN001 in the current study and its absence from the breastmilk of the most compliant mothers suggests that we need to consider possible mechanisms that rely, at least in part, on direct infant exposure. We have shown in our earlier study that HN001 does not alter gut composition, other than the supplemented probiotic, or diversity (manuscript under review), even when directly taken by the child, but this does not exclude a possible effect on the immune system occurring through the gut. This report suggests that direct child supplementation may be important for eczema prevention, but the absence of a plausible mechanism to explain a probiotic effect hinders our ability to move the field forward and delays the process of refining clinical guidelines.

TABLE 1 Characteristics of study population and exposures during study

Baseline (14-16 wk of gestation)	HN001 (N = 212)	Placebo (N = 211)
Previous pregnancy	67.5% (143/212)	73.8% (155/210)
Median (IQR) weeks gestation at enrolment	15.00 (14.4-15.6)	15.00 (14.6-15.4)
Age (y), median (interquartile range)	34 (30-36) (N = 212)	34 (31-37) (N = 210)
Ethnicity	N = 212	N = 211
Maori	10.9% (23)	16.6% (35)
Pacific	3.8% (8)	1.9% (4)
Asian	7.1% (15)	7.6% (16)
European	78.3% (166)	73.5% (155)
Other	0.0% (0)	0.5% (1)
Household income	N = 207	N = 204
0-49 k	8.0% (17)	7.6% (16)
50-99 k	30.4% (63)	32.4% (66)
100-149 k	35.8% (74)	34.8% (71)
150 + k	25.6% (53)	25.0% (51)
Maternal or paternal history of treated eczema	68.9% (146/212)	71.6% (151/211)
Maternal history of treated allergic disease ^a	87.3% (185/212)	82.9% (175/211)
Paternal history of treated allergic disease ^a	64.2% (136/212)	66.8% (141/211)
Maternal systemic antibiotic use during pregnancy prior to enrolment	14.6% (31/212)	11.4% (24/210)
Median (IQR) maternal BMI (kg/m ²)	25.1 (23.0-28.6) (N = 211)	25.8 (23.0-29.6) (N = 210)
Pregnancy (between enrolment and birth)		
Any maternal smoking	2.0% (4/202)	1.0% (2/197)
Maternal systemic antibiotic use enrolment to birth	30.9% (62/201)	30.7% (62/202)
Maternal systemic antibiotic use during labour and birth	36.8% (78/212)	42.7% (90/211)
Non-study probiotic use	7.0% (14/201)	4.0% (8/198)
Caesarean delivery	27.7% (57/206)	25.4% (51/201)
Median (IQR) weeks gestation at birth	39.7 (38.7-40.7) (n = 205)	39.6 (38.7-40.4) (n = 201)
Post-birth		
Maternal systemic antibiotic use till 6 mo	41.4% (84/203)	43.2% (85/197)
Maternal non-study probiotic use till 6 mo	12.1% (24/199)	12.4% (24/194)
Duration of breastfeeding	N = 204	N = 197
>0-3 mo	6.9% (14)	7.6% (15)
>3-6 mo	9.8% (20)	9.6% (19)
>6 mo	83.3% (170)	82.7% (163)
Child		
Male gender	55.8% (115/206)	57.4% (116/202)
Ethnicity		
Maori	17.7% (36/204)	16.8% (33/197)
Pacific	3.4% (7/204)	4.6% (9/197)
Asian	9.3% (19/204)	9.1% (18/197)
European	68.6% (140/204)	68.0% (134/197)
Other	1.0% (2/204)	1.5% (3/197)
Antibiotic use from birth till study end (12 mo)	51.3% (102/199)	53.3% (104/195)
Non-study probiotic use from birth till study end (12 mo)	20.5% (41/200)	17.4% (34/195)
Pets in household at birth	52.7% (106/201)	51.5% (102/198)

^aDefined as having treated asthma, eczema or hay fever.

TABLE 2 The hazard ratio and cumulative prevalence of eczema, SCORAD ≥ 10 and wheeze in studies of both mother and child intervention and mother-only intervention, and point relative rate and prevalence of atopic sensitization at age 12 mo in mother-only intervention study

	Mother/child study		Mother-only study		P value for interaction
	HR (95% CI) prevalence	P value	HR (95% CI) prevalence	P value	
Eczema	N = 304		N = 403		
Placebo	1.00 18.9%		1.00 21.5%		
HN001	0.39 (0.19-0.79) 9.2%	.009	0.83 (0.53-1.29) 18.0%	.40	.07
SCORAD≥ 10	N = 304		N = 403		
Placebo	1.00 26.7%		1.00 39.0%		
HN001	0.61 (0.37-1.02) 17.2%	.06	0.95 (0.69-1.31) 37.9%	.74	.15
Wheeze	N = 316		N = 402		
Placebo	1.00 32.0%		1.00 45.0%		
HN001	0.89 (0.59-1.36) 29.2%	.60	0.89 (0.66-1.20) 40.4%	.45	.92
Atopic sensitization	Not measured at 12 mo		RR (95% CI)		
Placebo	–		1.00 14.3%		
HN001	–		1.02 (0.63-1.64) 14.5%	.95	

HR, Hazard ratio; RR, Relative rate. Significant P values are in bold.

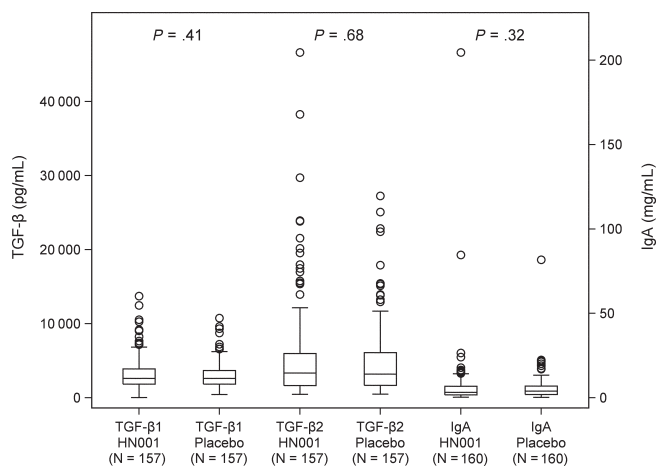


FIGURE 2 Concentration of TGF- β 1, TGF- β 2 and IgA in breastmilk collected at day 4-7 post-partum. Data are represented as Tukey box and whisker plots. P-values are Wilcoxon rank-sum tests

5 | CONCLUSION

To conclude, we have found that HN001 supplementation of the mother only, even from early pregnancy, is insufficient to provide protection to the infant against eczema by 1 year. This is perhaps not surprising given that HN001 was not detected in breastmilk and did not modulate breastmilk immune factors. Our findings indicate that maternal HN001 supplementation without infant supplementation

may not be effective in preventing eczema in infants. Only once a study of HN001 supplementation in infants only is completed, can we conclusively decide whether infant HN001 supplementation alone is effective.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

AUTHOR CONTRIBUTIONS

KW conceived of the idea, raised the funds, coordinated the project, analysed the data and drafted the manuscript. JC had overall responsibility for the study. CB, EM, TS, GP, JK, RMM PS, PA, RM, PF and JC helped with study design and development, and EM led the Auckland arm of the study. CB was largely responsible for the development and management of the study in Wellington, with assistance by FH, KW and JK. JR managed the Auckland team. PB

and JK developed the database and helped with study design. CB, JK, FH and JR also helped with data collection. GP advised on the data analysis. LvdE and EF-B were responsible for breastmilk protein analysis. IF was responsible for measuring HN001 in breastmilk. KW takes responsibility for the content of the manuscript. All authors reviewed and commented on the manuscript.

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