

Effects of feeding probiotics during weaning on infections and antibody responses to diphtheria, tetanus and Hib vaccines

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Microbial exposure is necessary for the development of normal immune function, which has driven the idea of using probiotics for treatment and prevention of immune-mediated diseases in infancy and childhood. Mounting evidence indicates that probiotics have immunomodulatory effects. However, the mechanisms are still poorly understood. Specific antibody response is a valuable proxy for immune system maturation status in infancy. We aimed at determining the impact of *Lactobacillus* F19 (LF19) during weaning on infections and IgG antibody responses to routine vaccines. In a double-blind, placebo-controlled randomized intervention trial, infants were fed cereals with (n = 89) or without LF19 (n = 90) from 4 to 13 months of age. Infants were immunized with DTaP (diphtheria and tetanus toxoid and acellular pertussis), polio and Hib-conjugate vaccines at (3), 5½ and 12 months of age. We assessed the number of days with infections, antibiotic prescriptions and antibody concentrations to Hib capsular polysaccharide (HibPS), diphtheria toxin (D) and tetanus toxoid (T) before and after the second and third doses. Days with infectious symptoms did not differ between the groups. Days with antibiotic prescriptions were fewer in the LF19 group (p = 0.044). LF19 enhanced anti-D concentrations when adjusting for breastfeeding duration and colonization with LF19 (p = 0.024). There was an interaction of the intervention and colonization with LF19 on anti-T concentrations during the course of vaccination (p = 0.035). The anti-HibPS concentrations were higher after the first and second dose of Hib vaccine in infants breastfed < 6 months compared with those breastfed ≥ 6 months (p < 0.05), with no effect by LF19. In conclusion, feeding LF19 did not prevent infections, but increased the capacity to raise immune responses to protein antigens, with more pronounced effects in infants breastfed < 6 months.

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The human intestinal tissue harbors a large number of immune cells, and the gut lumen hosts a wide range of microbiota. Post-natal colonization is influenced by mode of delivery, hygiene, mother's microbiota and type of feeding

(1, 2). Major changes in gut microbial composition occur early in life, with critical stages at birth and during weaning due to drastic changes in nutritional and physiological conditions (3). Gnotobiotic animal models have shown that intestinal bacteria are necessary for the development of gut and systemic immune responses (4, 5), which has driven the idea of using probiotics for treatment and prevention of immune-mediated diseases in childhood.

Abbreviations: LF19, *Lactobacillus* F19; LGG, *Lactobacillus rhamnosus* GG; BF, breastfeeding; D, diphtheria toxin; T, tetanus toxoid; Hib, *Haemophilus influenzae* type B; HibPS, Hib capsular polysaccharide.

Specific antibody response serves as a valuable proxy for the complex immune responses. It depends on the functional interplay of antigen-presenting cells, antigen specific T helper cells, intercellular cytokine communication and the differentiation of antigen specific B cells into antibody-producing plasma cells. The effects of probiotics on specific antibody levels have been evaluated in vaccination trials. *Lactobacillus rhamnosus* GG (LGG) increased the immunogenicity of oral rotavirus vaccine (6), and oral *Salmonella* vaccine tended to augment specific IgA responses when given together with LGG, when compared with placebo (7). The effects of probiotics on parenteral vaccination in infancy have been evaluated in a few trials with immunoenhancing results (8, 9).

The aim of the present trial was to investigate the impact of feeding healthy, term infants LF19 during weaning on the number of days with infectious symptoms, antibiotic prescriptions and IgG antibody responses to the routine vaccines against *Haemophilus influenzae* type b (Hib), diphtheria and tetanus.

Material and methods

Participants

This is a double-blind, placebo-controlled, randomized intervention trial on the effects of feeding infants LF19 daily from 4 to 13 months of age on infections and immunological development. The trial was conducted from August 2000 to November 2003 in Umeå, a university town in northern Sweden with 110,000 inhabitants. Inclusion criteria were healthy, term infants (gestational age 37–42 wk) with birth weight > 2500 g who were vaginally delivered, no atopic manifestation or medication that could have affected the gut microbiota, i.e., antibiotics, or prior probiotic intake. Written and oral information was provided before enrolment and written consent was signed by parents. Approval of the trial was obtained from the ethics committee of the Medical Faculty of Umeå University.

Intervention

When the trial was designed and initiated, data on the clinical and immunomodulatory effects as well as safety aspects of probiotics were scarce. *Lactobacillus paracasei* ssp. *paracasei* strain F19 (LF19) was chosen as it fulfilled the criteria of a probiotic and doses between 10^8 and 10^{10} CFU/day had been administered without side effects (10, 11). Furthermore, this strain was demon-

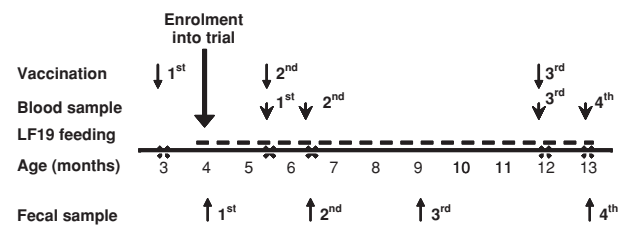


Fig. 1. Schedule for enrolment, administration of vaccines, blood and fecal samplings.

strated to induce immunomodulatory effects *in vitro* (12).

Infants were randomized to one of two groups: cereals supplemented with LF19 or the same cereals without LF19 from 4 months (Fig. 1), which at the time of the study was the recommended age for introducing complementary feeds. The cereals contained milk protein and were mainly rice based from 4 to 6 months, and wheat based from 6 to 13 months of age. One serving contained 1×10^8 colony-forming units (CFU) of strain LF19. The recommended intake was at least one serving of cereals daily until 13 months of age. Instruction on how to prepare the cereals was given. Parents were asked to refrain from feeding the infant other products with probiotics. There was no interference with their dietary habits in other respects. The cereals (Semper AB, Stockholm, Sweden) were delivered in cartons colored blue or yellow. Parents and investigators were blinded as to which carton contained LF19 until laboratory analyses were completed.

Immunizations

Infants were immunized at their well-baby clinics with DTaP (diphtheria and tetanus toxoid and acellular pertussis), polio and Hib-conjugate vaccines (Infanrix® Polio + Hib, GlaxoSmith Kline, Rixensart, Belgium) following the national immunization program. The first dose had been given at 3 months of age. The following doses were given at 5½ and 12 months (Fig. 1).

Data collection and outcome variables

Parents recorded daily in a diary the number of breastfeedings as long as the infant was breast-fed, the number of servings of cereals, the number and consistency of stools and any symptom of illness (vomiting, runny nose, cough, earache and rashes) and fever ($> 38.0^\circ\text{C}$).

Diarrhea was defined as ≥ 3 abnormally loose stools per 24 h. The research nurse reviewed the diaries with the parents monthly and interviewed

them following a questionnaire about any illness, doctor's diagnoses and prescriptions and use of any drug or supplement.

Serology

Venous blood was collected at 5½, 6½, 12 and 13 months of age in Vacutainer® tubes (Becton Dickinson, Plymouth, UK) (Fig. 1). After centrifugation within hours, plasma and serum were stored at -20°C until analysis. IgG antibodies to Hib capsular polysaccharide (Hib-PS) were assessed in serum by enzyme immunoassay (EIA) (13) with a minor modification; 2 mg of skim milk powder (Difco™, 232100 (0032-17) lot 3007113, Becton Dickinson, Plymouth, UK) in 400 ml phosphate-buffered saline (PBS) was used for dilution.

Antibodies to tetanus toxoid (T) were quantified using a commercial EIA kit, Virion/Serion Tetanus IgG (Institut Virion/Serion, Würzburg, Germany). Neutralizing antibodies to diphtheria toxin (D) were quantified using the Vero cell neutralization test (14), with the equine WHO standard DI02 as reference.

Fecal sampling procedures

Fecal samples were collected at entry and at 6½, 9 and 13 months of age (Fig. 1). The samples were collected from diapers by a spatula into sterile tubes and stored at home in the freezer before transportation to the hospital where they were stored at -70°C until analysis.

Microbiological procedures

Fecal samples were serially diluted and placed on Rogosa agar (15). Lactobacilli with colony- and Gram-staining morphology similar to that of LF19 were analyzed by randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for identification (16).

Statistics

Based on a study on the effects of nucleotides on specific antibody responses by Pickering et al., we needed 70 infants in each group to detect a 50% difference in antibody concentration to HibPS 4 wk after the second dose ($\alpha = 5\%$ and power 80%) (17). We included 90 infants in each group, which allowed a 20% drop-out rate. Primary outcome measures were the IgG antibody responses to D, T and Hib-PS. Secondary outcome measures were days with infections and antibiotic prescriptions. Data were analyzed on

an intention-to-treat basis. We used chi-squared test for categorical and Student's t-test for numerical data. Morbidity and vaccine response data were skewed and transformed to logarithms. Morbidity data were further analyzed by an ANCOVA. Vaccine response data were analyzed by longitudinal repeated measures analysis of variance. When the assumption of sphericity was violated, the Huynh-Feldt correction was used. Only infants with samples from all four samplings were included. This analysis generates an estimated marginal mean, i.e., a mean that adjusts for the covariates, in this case breastfeeding duration and colonization with LF19. An ANOVA was calculated at all samplings. Indicator variables representing intervention, colonization with LF19 and duration of breastfeeding were constructed. Each combination was evaluated in a linear regression model, using infants breastfed ≥ 6 months receiving placebo as reference. Differences were considered significant at the level of $p < 0.05$. Multiple comparisons were Bonferroni adjusted. All statistical analyses were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Participants and compliance

We enrolled 180 infants. After excluding one infant due to exclusion criteria (delivery by caesarean section), 89 infants were randomized to receive LF19 and 90 to receive placebo, of which 84 and 87 infants completed the study respectively (Fig. 2). The eight dropouts were the result of withdrawal of consent by the parents. Cow's milk protein allergy was diagnosed by an oral food challenge in six and three infants in the LF19 and placebo groups respectively ($p > 0.05$). These infants remained in the study but stopped consuming the cereals that contained milk powder. Thus, 171 infants completed the

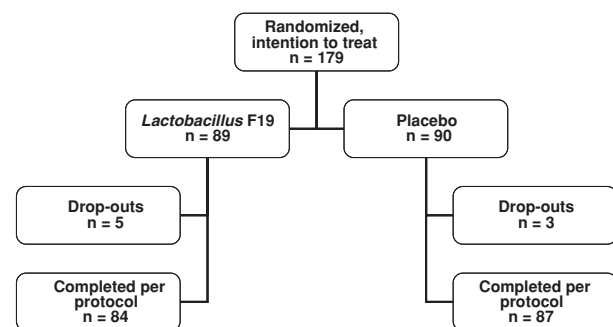


Fig. 2. Trial profile.

Table 1. Characteristics of subjects

	<i>Lactobacillus</i> F19	Placebo
Subjects (n)	84	87
Boys (%)	39	45
Gestational age (wk)*	40.2 (1.2)	39.9 (1.3)
Birth weight (g)*	3725 (516)	3632 (503)
Older siblings (%)	45	56
Attending daycare (%)	10	11
Smoking at home (%)	12	18

*Values are mean (s.d.).

trial and were included in the intention-to-treat analysis. There were no statistically significant differences between the groups with respect to gender, gestational age, birth weight, siblings, daycare attendance or smoking at home (Table 1).

Breastfeeding and consumption of trial product

‘Exclusive breastfeeding’ refers to feeding only breast milk and no other liquids or solids with the exception of supplements or medicine. ‘Breastfeeding’ means the infant receives complementary feedings in addition to breast milk (18). There was no difference in the rate of breastfeeding between the groups. Infants were exclusively breastfed for a mean duration of 3.7 (1.4 SD) months and 3.8 (1.1 s.d.) months in the LF19 and placebo groups respectively ($p > 0.05$). The mean total duration of breastfeeding was 7.8 (3.3 s.d.) months in both groups. At 6 months of age, 82% and 79% of the infants were breastfed in the LF19 and placebo groups respectively ($p > 0.05$). There was no difference in the mean intake of cereals between the groups but a wide variation between infants. Mean daily intake was 0.8 (0.5 s.d.) and 0.9 (0.4 s.d.) servings daily in the LF19 and placebo groups respectively. There were no differences between groups in frequency or consistency of stools.

Gut microbiota

LF19 was isolated in feces at entry in one infant in the LF19 group and during intervention in 4/87 infants in the placebo group; in one of them on two occasions. In the LF19 group, LF19 was isolated in 90% of the infants at 6½ months. The corresponding figures at 9 and 13 months were 85% and 71% respectively. In 64% of the infants, LF19 was isolated at all time points during intervention, but in two infants LF19 was not isolated at any time point.

Table 2. Morbidity parameters in the *Lactobacillus* F19 (LF19) and placebo groups

Parameter	LF19	Placebo	p-value*
n	84	87	
Days with fever	7.2 (6.0–8.8)	6.1 (5.1–7.7)	0.249
Days with diarrhea	2.4 (1.9–3.1)	3.0 (2.3–3.8)	0.228
Days with respiratory illness	39.5 (31.5–49.5)	33.8 (26.4–43.9)	0.360
Days with antibiotics	1.6 (1.3–1.9)	2.2 (1.7–2.7)	0.044

Data are presented as means (95% CI) in the original scale.

*t-test on data transformed to natural logarithms.

Morbidity parameters

There was no difference between the groups in the number of days with fever, respiratory illness or diarrhea (Table 2). Adjusting for seasonal variation, siblings, daycare attendance or smoking at home did not affect the outcome. Infants in the LF19 group had fewer days with antibiotics compared with placebo ($p = 0.044$). Prophylactic antibiotics were prescribed following a urinary tract infection treatment during the period of follow-up investigations. Days with prophylaxis were excluded from the analysis. When included, the difference between the groups became more evident.

Vaccine responses

Swedish infants are immunized against diphtheria, tetanus, pertussis, polio and Hib by administration of combination vaccines according to a 3–5–12 months schedule (Fig. 1). We monitored the IgG responses to two protein antigens, i.e., diphtheria and tetanus toxoid and one polysaccharide antigen (HibPS) to study the potential effects of LF19 on T-cell-dependent humoral immunity. As expected, the antibody response was markedly stronger after the third than the second dose for all antigens (Table 3). However, no statistically significant differences were observed between the two groups. Breastfeeding could have influenced the antibody response (19). It was not possible to compare formula-fed and breastfed infants as all infants but four were initially breastfed. Instead, breastfeeding duration, i.e., breastfeeding < 6 months (BF < 6 months) or breastfeeding ≥ 6 months (BF ≥ 6 months) was adjusted for. Furthermore, to control for the variation in intake of LF19, colonization with LF19 in fecal samples was included in the statistical model, dichotomized as colonized (LF19 isolated in all three fecal samples during intervention) or not colonized (LF19 isolated in less than three fecal samples).

Table 3. Antibody concentrations to the capsular polysaccharide of *Haemophilus influenzae* (HibPS), diphtheria toxin and tetanus toxoid in the *Lactobacillus* F19 (LF19) and placebo groups

Immune indicator	Before 2nd dose 5½ months	4 wk after 2nd dose 5½ months	Before 3rd dose 12 months	4 wk after 3rd dose 13 months
IgG anti-HibPS (µg/ml)				
LF19 (n = 79)	0.24* (0.18–0.30)	1.56 (1.03–2.25)	1.08 (0.78–1.44)	24.67 (18.37–32.00)
Placebo (n = 75)	0.19 (0.15–0.25)	1.88 (1.27–2.83)	1.02 (0.75–1.42)	27.52 (20.58–34.14)
IgG anti-diphtheria toxin (IU/ml)				
LF19 (n = 80)	0.01 (0.01–0.01)	0.19 (0.14–0.25)	0.06 (0.05–0.08)	1.60 (1.29–1.96)
Placebo (n = 75)	0.01 (0.01–0.01)	0.21 (0.15–0.30)	0.06 (0.05–0.08)	1.92 (1.54–2.45)
IgG anti-tetanus toxoid (IU/ml)				
LF19 (n = 81)	0.09 (0.08–0.11)	0.97 (0.77–1.21)	0.27 (0.22–0.32)	4.36 (3.76–5.00)
Placebo (n = 76)	0.10 (0.08–0.11)	0.92 (0.74–1.14)	0.23 (0.19–0.28)	4.35 (3.63–5.07)

*Geometric mean (95% confidence interval).

No significant effects of LF19 and breastfeeding duration were demonstrated on the anti-D response, but there was an interaction of time (5½, 6½, 12 and 13 months), breastfeeding duration and colonization on anti-D concentrations ($p = 0.003$, Huynh–Feldt). When adjusting for breastfeeding duration as a continuous variable, the anti-D concentrations were greater in infants colonized with LF19 throughout intervention ($p = 0.024$) and in the entire LF19 group ($p = 0.048$). Follow-up analyses demonstrated that the enhanced anti-D concentrations occurred 4 wk after the second dose ($p = 0.044$), with a trend towards enhancement also prior to the third dose in infants colonized with LF19 throughout the intervention period ($p = 0.072$) (Fig. 3a). Linear regression with indicator variables – including intervention, breastfeeding duration and colonization with LF19 – showed that infants breastfed <6 months carrying LF19 throughout the intervention had significantly higher anti-D concentrations both 4 wk after the second dose ($p = 0.018$) and prior to the third dose ($p = 0.048$). Thus, the enhancing effect of LF19 was more evident in infants breastfed <6 months than in infants breastfed ≥6 months. This divergence could not be explained by differences in intake of study product, LF19 colonization patterns or growth between infants breastfed <6 and ≥6 months.

No significant effect of the intervention was demonstrated on the anti-T concentrations. Adjusting for breastfeeding duration and colonization with LF19 in the analysis of anti-T concentrations, revealed an interaction of intervention, time and colonization ($p = 0.035$, Huynh–Feldt). Follow-up analyses did not reach statistical significance at any time point (Fig. 3b).

There was no effect of LF19 on anti-HibPS concentrations, but there was an interaction between time and breastfeeding duration ($p =$

0.003, Huynh–Feldt), with no further effect by colonization with LF19. Follow-up analyses verified that the anti-HibPS response was higher in infants BF < 6 months compared with those BF ≥ 6 months prior to the second dose ($p < 0.001$), 4 wk after the second ($p = 0.024$) and prior to the third dose (0.040). However, the response to the third dose was the same, regardless of breastfeeding duration ($p > 0.05$) (Fig. 3c).

Discussion

In the present trial, probiotic intake was initiated during weaning in an attempt to maintain healthy gut microbial composition and prolong the preventive effects of breastfeeding on infections (20). Probiotics did not prevent infections but slightly reduced days with antibiotic prescriptions. However, regular consumption of LF19 increased the capacity to raise the immune response to D with more marked effects in infants BF < 6 months, with a similar trend in the response to T. By contrast, there was no effect of LF19 on the immune response to the polysaccharide antigen, but a strong influence on anti-HibPS concentrations by breastfeeding duration.

In a Swedish prospective birth cohort study, 45% of the infants were colonized with lactobacilli at 6 months of age, with higher rates of colonization in breastfed compared with weaned infants at that age. At 12 months of age, only 17% of the infants carried lactobacilli in feces irrespective of breastfeeding status (21). In the present trial, LF19 was isolated in 90% and 71% of the infants in the LF19 group at 6½ and 13 months respectively. The decreasing colonization rates might reflect the establishment of a more diversified microbiota at 13 months or a developing resistance to colonization by the host.

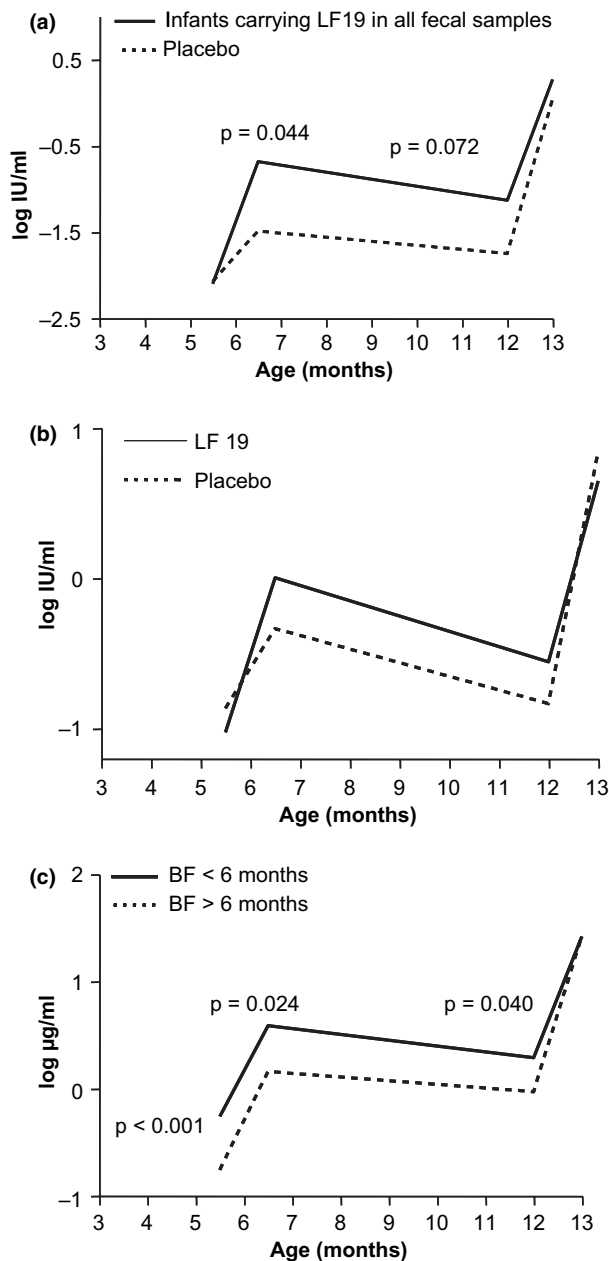


Fig. 3. Estimated marginal means of antibody concentrations at time of and 4 wk after administration of the second and third dose of combination vaccines. (a) Neutralizing IgG antibody concentrations to diphtheria toxin in infants carrying LF19 in all fecal samples during the intervention ($n = 54$) and all infants in the placebo group ($n = 75$). Duration of breastfeeding was adjusted for in the repeated measures ANOVA. (b) Specific IgG antibody concentrations to tetanus toxoid in the LF19 ($n = 81$) and placebo groups ($n = 76$). Duration of breastfeeding and colonization with LF19 were adjusted for in the repeated measures ANOVA. (c) Specific IgG antibody concentrations to HibPS in infants breastfed < 6 months (BF < 6 months) ($n = 27$) and breastfed ≥ 6 months (BF ≥ 6 months) ($n = 127$).

In view of these findings (21), non-breastfed infants and infants during weaning might benefit most from probiotic intake.

The effects of breastfeeding on specific antibody responses are conflicting. Breastfed infants produced higher antibody concentrations in response to Hib conjugate vaccine than did formula-fed infants (22) and exclusive breastfeeding for 13 wk or more protected against invasive Hib disease (23). In contrast, compared with formula-fed counterparts, Australian breastfed infants had lower anti-HibPS concentrations both before and after completed immunization with Hib conjugate vaccine (24). Several species of commensal bacteria in the gut and respiratory tract display antigens that cross-react with Hib (25). We speculate that the greater anti-HibPS concentrations in infants BF < 6 months originate in a more diverse microbiota with consequently earlier stimulation of the developing immune system. In addition, specific antibodies and other factors with anti-inflammatory and anti-proliferative properties in breast milk could possibly reduce the activation of the immune system (19).

Recently, it was demonstrated that perinatal administration of a mix of four probiotic strains and prebiotic galacto-oligosaccharides (synbiotics) to infants at high risk of allergy increased the prevalence of infants reaching protective anti-HibPS concentrations after primary immunization compared with placebo (9). There were no differences between groups in IgG concentrations to D, T or Hib. However, the samples analyzed were few, 32 in the synbiotic and 29 in the placebo group. Thus, the statistical power to detect more subtle differences was low. In comparison, we found that LF19 increased the capacity to raise immune responses to D and T with no differences in the proportion of infants reaching protective levels to any of the antigens (data not shown). Indeed, different probiotic strains have different immunomodulatory capacities. Furthermore, nature and dosage of antigen, number of vaccine doses, infant age and level of maternal antibodies at the time of immunization affect the outcome (26).

The preventive effects of probiotics to infants and children attending daycare on infections have been evaluated in randomized trials; *Lactobacillus reuteri* and *Bifidobacterium lactis* (Bb 12) reduced the number of days and episodes with diarrhea in infants of similar age as in the present trial. The former also reduced absence from daycare and courses of antibiotics. Neither probiotic affected respiratory illnesses. All infants were weaned when entering the trial (27). In a large-scale Finnish randomized, LGG intake slightly reduced the incidence of respiratory infections and courses of antibiotics compared

with placebo, although adjusting for age reduced the differences (28). Infants receiving formula supplemented with Bb 12 and *Streptococcus thermophilus* had a lower use of antibiotics compared with placebo but there was no effect on fever, emesis or loose stools. A total of 22% of these infants were breastfed at entry (29). In the present trial, where the majority of infants were breastfed, there were no differences between the groups with respect to days with infectious symptoms but a slight reduction in days with antibiotic prescriptions. The lack of preventive effect by LF19 might be explained by the protective effect of breastfeeding on infections (20). In another trial, LGG reduced the incidence of diarrhea in undernourished Peruvian children, but only in non-breastfed infants and children. Adjusting for breastfeeding in our trial did not affect the outcome which could be due to a lower incidence of infections in the healthy well-nourished infants in our setting compared with the Peruvian setting and not enough statistical power to detect subtle differences. The authors suggest that probiotics alter the microbiota and stimulate some of the protective effects of breastfeeding on infections but that the impact of the microbiota induced by breastfeeding is still superior to probiotic prophylaxis (30). Our trial supports this view.

Conclusions

In summary, regular consumption of LF19 during weaning did not prevent infections. However, feeding LF19 during the course of vaccination to diphtheria, tetanus and Hib increased the capacity to raise immune responses to the protein antigens, with more marked effects in infants BF < 6 months. On the other hand, there was no effect of LF19 on the immune response to the polysaccharide antigen, but a strong influence on anti-HibPS concentrations by breastfeeding duration. Thus, our results suggest that probiotics may modulate vaccine responses. The significance of these findings should be further explored in studies addressing the effects of probiotics in conjunction with formula feeding or breastfeeding on humoral immune responses.

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statistician Hans Stenlund for invaluable advice on statistical procedures.

Conflict of interests

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