Received: 23 October 2024 | Accepted: 24 July 2025

DOI: 10.1002/jpn3.70189

JPGN

CLINICAL TRIAL

Nutrition

Immunological effects of alpha-lactalbumin-enriched low-protein infant formula: A randomized controlled trial

Ulrika T. Nilsson[1] | **Olle Hernell**[2] | **Bo Lönnerdal**[3] | **Lotte N. Jacobsen**[4] | **Maria Nunez-Salces**[4] | **Anne S. Kvistgaard**[4] | **Christina West**[2] | **Pia K. Åkeson**[1]

[1]Department of Clinical Sciences Malmö, Pediatrics, Lund University, Malmö, Sweden

[2]Department of Clinical Sciences, Pediatrics, Umeå University, Umeå, Sweden

[3]Department of Nutrition, University of California, Davis, Davis, California, USA

[4]Arla Foods Ingredients Group P/S, Viby, Denmark

Correspondence

Ulrika T. Nilsson, Department of Pediatrics, Skåne University Hospital, Ruth Lundskogs gata 3, Malmö 214 28, Sweden.
Email: Ulrika.tinghall_nilsson@med.lu.se

Funding information

Arla Foods Ingredients Group P/S, Viby J, Denmark; Region Skåne Council Foundation for Research and Development

Abstract

Objectives: Breast-fed (BF) have lower risk of infections during infancy compared to those formula-fed (FF). A higher content of alpha-lactalbumin (α-lac) in breast milk, which may promote a more favorable gut microbiota, could be one reason. In this study, we evaluated whether increased concentration of α-lac in low-protein infant formula affects the immune response and the incidence of infections during infancy.

Methods: In a double-blinded randomized controlled trial, healthy-term infants ($n = 245$) received low-protein infant formulas with α-lac-enriched whey (α-lac-EW; 1.75 g protein/100 kcal, 27% α-lac) or casein glycomacropeptide-reduced whey (CGMP-RW; 1.76 g protein/100 kcal, 14% α-lac), or standard formula (SF; 2.2 g protein/100 kcal, 10% α-lac) from 2 to 6 months. BF constituted a reference group. Cytokines and high-sensitivity C-reactive protein (hsCRP) were measured during intervention and infection-related morbidity, and treatment was evaluated until 12 months.

Results: Serum interleukin-6 (IL-6) was lower in BF than in all FF groups during intervention ($p < 0.001$). No other differences in cytokines (tumor necrosis factor alpha [TNF-α], transforming growth factor beta 1 [TGF-β1], TGF-β2, IL-1, IL-10, IL-12, interferon gamma [INF-γ]) or hsCRP were found among the study groups. Infection-related morbidity did not differ among study groups, except slight differences in the use of antibiotics during (α-lac-EW vs. CGMP-RW [$p = 0.008$]) and after intervention (α-lac-EW vs. BF [$p = 0.016$]).

Conclusions: Increased α-lac concentration in low-protein infant formula to levels similar to breast milk did not affect the cytokine profile and had minor effect on infection-related morbidity. The higher IL-6 concentrations in FF than in BF needs further investigation.

KEYWORDS

cytokines, gut microbiota, infant nutrition, infectious diseases

1 | INTRODUCTION

Breast milk is not only important for infant growth,[1] but also plays a key role in protecting the infant from infections, mainly gastrointestinal- and respiratory infections, and otitis media.[2-7] Multiple bioactive components in breast milk are involved in the maturation and function of the immune system of the infant. Their complex interactions and activity enhance the development of immunity in early life.[8,9] However, despite international recommendations,[10] less

than 50% of infants worldwide are exclusively breast-fed (BF) during the first 6 months of life, and depend on infant formula to varying degrees.[6]

Early life nutrition has a great impact on the establishment of the gut microbiota,[11-15] and hence on the development and function of the infant's immune system.[16,17] The gut microbiota is important for protection against pathogens and infections.[18] According to previous research the gut microbiota in formula-fed (FF) infants is more diverse and has a different composition compared to BF infants, with higher abundance of Bacteroidetes and Firmicutes.[12,19] Importantly, breast milk, in contrast to cow's milk, contains high concentrations of oligosaccharides that promote colonization of beneficial bacteria such as Bifidobacterium.[15] The different composition of the gut microbiota in FF compared to BF infants,[11,20,21] could be one reason for the increased susceptibility to infections during infancy in FF than in BF infants.

Alpha-lactalbumin (α-lac) is the main whey protein in breast milk, comprising 25% of total protein, compared to the much lower (3%–5%) concentration in cow's milk, and thus in standard infant formula.[22] Bioactive peptides derived from α-lac have been suggested to promote the establishment of a favorable gut microbiota composition, more similar to that of BF infants.[23-25] In preclinical studies, acute diarrheal illness, caused by infection with enteric pathogens, was prevented by feeding an α-lac-enriched formula.[23,26] Possible bactericidal effects of α-lac peptides against several bacteria species,[27] as well as potential antiviral and various immune-modulating effects of α-lac peptides have also been reported.[22,28,29] Many interactions between and within different parts of the immune system are regulated by cytokines. We and others have previously reported different cytokine profiles in FF and BF infants,[30] and that addition of bioactive components to infant formula can alter the immune response.[31-34] We recently reported effects on growth and metabolism during[35] and after the intervention[36] from the ALFoNS study where infants were fed low-protein infant formula with increased concentrations of α-lac during early infancy.

In this study, as secondary outcomes of the ALFoNS study, we investigated if increased α-lac concentration in low-protein formula, closer to that of breast milk, affects the cytokine profile during intervention and decreases infection-related morbidity and related treatment during and after intervention to reduce the gap between FF and BF infants.

What is Known

- Breast-fed (BF) infants have fewer infections during infancy, mainly respiratory and gastrointestinal, than those being formula-fed (FF).
- Alpha-lactalbumin (α-lac) concentration in infant formula is much lower than in breast milk.
- Preclinical studies indicate that enrichment of infant formula with α-lac reduces the risk of gastrointestinal infections.

What is New

- Increasing α-lac concentration in low-protein infant formula did not change the inflammatory response or the incidence of infections compared to those fed standard infant formula.
- The higher concentration of interleukin-6 in FF than in BF infants during intervention remains to be further investigated.

2 | METHODS

2.1 | Ethics statement

The study was approved by the Regional Ethical Review Board in Lund (ref: 2014/14) and registered at ClinicalTrials.gov (NCT02410057). Written parental informed consent was obtained before inclusion.

2.2 | Design and study population

This double-blinded randomized controlled trial (RCT) was conducted in Malmö/Lund and Umeå, Sweden. Infants were recruited by invitation letters to all families with a 4-week-old infant between December 2014 and November 2019.

Full-term infants with birth weight 2500–4500 g, absence of chronic illness or disease that could affect normal growth and nutrition, and either exclusively BF or exclusively FF at inclusion date, were included in the study. Infants who had received probiotics (*Limosilactobacillus reuteri*) could be included provided a "washout" period of 7 days, while antibiotic treatment and delivery by cesarean section were exclusion criteria.

2.3 | Intervention

In total, 245 FF infants were stratified by sex and assigned into random blocks of 6 or 12 with computerized randomization to receive low-protein infant formulas with either α-lac-enriched whey (α-lac-EW; 1.75 g protein/100 kcal, 27% α-lac), or casein glucomacropeptide-reduced whey (CGMP-RW; 1.76 g protein/100 kcal, 14% α-lac), or standard infant formula (SF; 2.2 g protein/100 kcal, 10% α-lac), from 2 to 6 months. BF infants ($n = 83$) served as a reference. The study formulas were blinded at the production site (Laiterie de Montaigu); and whey protein fractions provided by Arla Foods Ingredients

Group P/S, Denmark. Nutritional composition is presented in Supporting Information S1: Table S1. Families, study nurses, and investigators were blinded to group allocation and unblinding of investigators occurred when all infants had completed the intervention (March 2020) and statistical analyses up to 6 months of age had been completed.

2.4 | Data collection

Background data of infants and parents were obtained at inclusion, as previously presented.[35] At monthly study visits from baseline (6–8 weeks) to 6 months and at follow-up at 12 months of age, anthropometric measurements were performed, and 3-day-dietary record collected.[35,36] In symptom diaries morbidity was reported on a daily basis. Evaluated outcomes were; days of fever (≥38.0°C), respiratory tract infection, gastroenteritis, diagnosis of otitis media, rash or eczema, use of any medication, doctor's visits and hospitalization. Morbidity and treatment data are presented as cumulative incidence; the proportion of participants presenting a specific outcome at least once, and as longitudinal prevalence; the proportion of days with a specific outcome. Study visit was rescheduled if the infant had symptoms of ongoing infection.

Venous blood samples were obtained at baseline, 4 and 6 months of age at least 2 h postprandially. Anesthetic topical cream was used before sampling. Blood was centrifuged for 10 min at 1300 × *g*, and serum then separated into microtubes and stored at –80°C. When all infants had completed the intervention, the tubes were transported frozen on dry ice to the Pediatric Research Laboratory at Umeå University for analyses. High-sensitivity C-reactive protein (hs-CRP) was determined by enzyme-linked immunosorbent assay (ELISA) (human CRP Quantikine® ELISA, R&D Systems Inc.). Before cytokine analyses, serum samples were thawed, shaken, and centrifuged at 10,000 × *g* for 10 min. Cytokines were assayed in duplicate, according to the manufacturer's instructions. Concentrations of interleukin (IL)-6, IL-10, IL-12, interferon gamma (INF-γ), and tumor necrosis factor alfa (TNF-α) were measured using Milliplex Human High Sensitivity T Cell Magnetic Beads (HSTCMAG-28SK; EMD Millipore) and TGF-β1 and -β2 using a TGF-β Magnetic Bead Kit (TGFBMAG-64K; EMD Millipore) from Merck KGaA. Cytokine measurements were performed using a Bio-Plex 200 instrument (Bio-Rad Laboratories). Concentrations of IL-6, IL-10, IL-12, IFN-γ, and TNF-α were read from a 7-point calibration curve and TGF-β1 and -β2 from a 6-point calibration curve, and calculations were made with Bio-Plex Manager 6.2 (Bio-Rad Laboratories). Samples with coefficient variation above 10% were re-assayed.

2.5 | Sample size and power calculation

The overall aims of the ALFoNS study were to evaluate growth and metabolic profile of the study population. As previously reported,[35] sample size was calculated to include 80 infants in each study group, allowing a drop-out rate of 20%, to detect a difference in weight (primary outcome) of 400 g (0.5 SD) at the end of intervention, with 80% power using a significance level of 0.05. Due to a short period of a higher drop-out rate than expected, eight additional infants were included to the study, resulting in 328 infants included. Based on previous pre-study calculations in a recent RCT on cytokines in infants a minimum sample size of 64 infants per randomized group was required to detect a difference of 0.5 SD in TNF-α concentrations between groups, with a power of 80% and a significance level of 0.05.[37]

2.6 | Statistical analyses

SPSS IBM Statistics version 28 was used for statistical analyses. Cytokine concentrations were assessed in all infants, whereas morbidity and treatment data were evaluated in infants who completed the entire intervention period, and during the follow-up period in those who completed their 12-month visit. For group comparison of continuous data Kruskal–Wallis test was used and for categorical data chi-square or Fishers exact test. Results are presented as mean ± SD as median with 25th and 75th percentiles, or as frequencies.

3 | RESULTS

A total of 328 infants were included in the study groups (Figure 1). Thirty-three FF (13%) and 10 BF (12%) infants were lost to follow-up during the intervention period (2–6 months), with no differences among formula groups in drop-out frequency due to gastrointestinal adverse event. During the follow-up period (6–12 months), there were few additional drop-outs (FF = 4, BF = 2), with no difference among the groups (Figure 1). Background and baseline characteristics of infants and parents are presented in Table 1.

3.1 | Morbidity

During the intervention period, the cumulative use of antipyretics was higher in all three FF groups than in the BF group (SF vs. BF $p < 0.001$, α-lac-EW vs. BF $p = 0.008$ and CGMP-RW vs. BF $p = 0.028$, respectively), however the longitudinal prevalence of fever was not significantly different among the study groups (Table 2). Few infants received systemic antibiotics during the intervention, but fewer in the α-lac-EW group

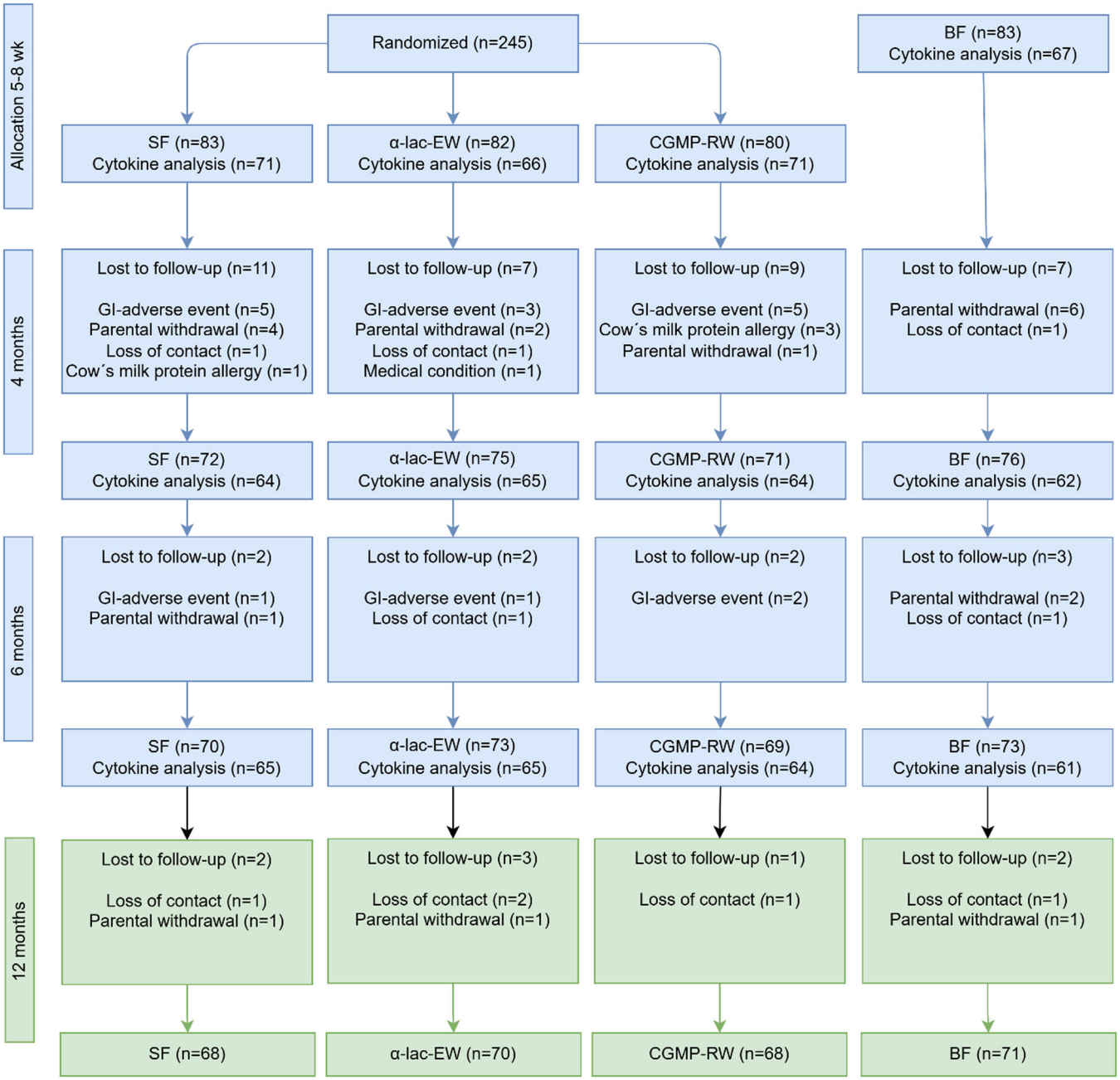

FIGURE 1 Study flowchart from inclusion to follow-up visit at 12 months of age. Reasons for loss to follow-up: GI adverse events; vomiting, stomach ache, and flatulence. α-lac-EW, α-lactalbumin-enriched whey; BF, breast-fed; CGMP-RW, casein glycomacropeptide-reduced whey; GI, gastrointestinal; SF, standard formula.

compared to the CGMP-RW group ($p = 0.008$). We also observed a trend toward a lower incidence of acute otitis media in the α-lac-EW compared to the CGMP-RW group ($p = 0.053$) (Table 2). During follow-up, the incidence of otitis media was higher in α-lac-EW than in BF infants ($p = 0.046$) as was the use of antibiotics ($p = 0.016$). No other significant differences in cumulative incidence or in longitudinal prevalence of infection-related morbidity or treatment outcomes, doctor's visits or skin problems were found among study groups during the intervention or follow-up (Table 2). The analyses from the total study population did not differ from the group of infants that had completed the intervention study with full compliance to the study protocol. Adverse events during the intervention period, that is, hospitalization, including noninfectious causes occurred in three infants in the SF group (perianal abscess, pylorus stenosis and apnea) and in three infants in the CGMP-RW group (bronchiolitis = 2, fever of unknown etiology = 1), as previously reported.[35] One infant was hospitalized during the follow-up period, diagnosed with febrile seizures (CGMP-RW group).

TABLE 1 Background and baseline characteristics of the study population.

	SF $n = 83$	α-lac-EW $n = 81$	CGMP-RW $n = 80$	BF $n = 83$
Infants				
Gestational age (week)	39.5 ± 1.2	39.7 ± 1.3	40.1 ± 1.2	40.0 ± 1.1
Boy, *n* (%)	43 (52)	40 (49)	40 (50)	40 (48)
Birth weight (kg)	3.47 ± 0.45	3.53 ± 0.44	3.61 ± 0.44	3.54 ± 0.42
Birth length (cm)	50.2 ± 2.2	50.1 ± 1.9	50.5 ± 1.7	50.4 ± 1.9
Birth head circumference (cm)	34.6 ± 1.2	35.0 ± 1.3	35.0 ± 1.2	35.0 ± 1.5
Age at baseline (days)	49.3 ± 5.0	49.4 ± 4.1	49.2 ± 5.8	50.5 ± 4.5
Ever breastfed before inclusion, *n* (%)	63 (76)	66 (78)	70 (88)	83 (100)
Days of breastfeeding (*n*)	15.5 ± 14.6	18.0 ± 15.2	17.5 ± 13.9	50.4 ± 4.6
Probiotics before inclusion,[a] *n* (%)	28 (34)	24 (30)	23 (29)	13 (16)
Mothers				
Age (years)	31.5 ± 4.8	31.1 ± 4.6	31.1 ± 4.6	32.6 ± 4.2
Non-Nordic origin, *n* (%)	6 (7)	11 (14)	6 (8)	8 (10)
Education at higher level,[b] *n* (%)	45 (54)	47 (58)	57 (71)	66 (80)
Smoking during pregnancy, *n* (%)	3 (4)	4 (5)	3 (4)	0 (0)
BMI (kg/m^2)	27.9 ± 5.2	27.9 ± 5.6	26.1 ± 4.2	25.2 ± 3.7

Note: Data presented as mean ± SD or as number (%).

Abbreviations: α-lac-EW, α-lactalbumin-enriched whey; BF, breast-fed; BMI, body mass index; CGMP-RW, casein glycomacropeptide-reduced whey; SF, standard formula.

[a]Probiotics: *Limosilactobacillus reuteri* Protectis®, wash-out period of 7 days before inclusion.

[b]University- or higher professional education.

3.2 | Cytokines and hsCRP

The results from the cytokine analyses are presented in Table 3. During the intervention there were no significant differences in cytokine concentrations among the FF groups. The concentration of IL-6 was similar in all study groups at baseline, but then increased during the intervention period in all formula groups compared to the BF group. Concentration of hsCRP was low in all study groups and did not differ among the FF groups or between FF and BF groups at baseline or during the intervention (Table 3).

4 | DISCUSSION

In the present study, we evaluated possible effects of feeding α-lac-enriched low-protein infant formula on inflammatory response and infection-related morbidity and treatment compared to feeding standard infant formula or breast milk during infancy. We observed only minor possible impact on infection-related morbidity during and after the intervention period among the study groups. We found no differences in cytokine profile between the study groups during the intervention period, except for increased IL-6 in all FF groups compared to the BF group, where concentrations remained at pre-intervention level.

During the study period, there was a low consumption of antibiotics overall in the study population. However, we found a lower use of antibiotics in the α-lac-EW than in the CGMP-RW group during the intervention, and a higher use in α-lac-EW than in the BF group during follow-up, probably due to differences in the acute otitis media incidence between the groups during these periods. Acute otitis media is one of the most common reasons for antibiotic treatment during early childhood in developed countries.[38] In this study, 47% and 75% of all antibiotic treatment during and after intervention, respectively, were due to this diagnosis.

Despite similar incidence and prevalence of fever in the study groups, the use of antipyretics was found to be higher in all FF groups compared to the BF group during the intervention. However, antipyretics can also be used for treatment of pain and sometimes against "discomfort" during infection even if fever is not present. In the present study, we do not have enough detailed information to distinguish between the different reasons for giving antipyretics.

Our findings with no differences in the incidence of fever or respiratory tract infections between the study

TABLE 2 Morbidity and infection-related treatment between 2 and 6 months and 6 and 12 months in infants fed infant formula or breast milk during early infancy.

	2–6 months					6–12 months				
	SF $n = 70$	α-lac-EW $n = 73$	CGMP-RW $n = 69$	p[a]	BF $n = 73$	SF $n = 66$	α-lac-EW $n = 70$	CGMP-RW $n = 68$	p[a]	BF $n = 71$
Cumulative incidence, *n* (%)										
Fever	41 (58.6)	53 (72.6)	48 (69.6)	0.18	48 (65.8)	54 (81.8)	54 (77.1)	59 (86.8)	0.34	61 (85.9)
Respiratory tract infection	56 (80.0)	54 (74.0)	48 (69.6)	0.37	57 (78.1)	55 (83.3)	55 (79.7)	60 (89.6)	0.28	57 (81.4)
Otitis media	2 (2.9)	0 (0)	4 (5.8)	0.067	2 (2.7)	9 (13.6)	13 (18.6)[b]	6 (9.0)	0.26	5 (7.0)
Gastroenteritis	5 (7.1)	6 (8.2)	3 (4.3)	0.69	2 (2.7)	16 (24.2)	18 (25.7)	12 (17.9)	0.40	10 (14.1)
Rash/eczema	9 (12.9)	15 (20.5)	12 (17.4)	0.47	9 (12.3)	10 (15.2)	9 (13.0)	8 (11.9)	0.83	17 (24.0)
Doctor's visit	35 (50.0)	27 (37.0)	28 (40.6)	0.27	24 (32.9)	36 (54.5)	34 (48.6)	30 (44.8)	0.53	30 (42.3)
Infection-relatedhospitalization	1 (1.4)	0 (0)	3 (4.3)	0.80	0 (0)	0 (0)	1 (1.4)	0 (0)	1.0	0 (0)
Antipyretic treatment	49 (70.0)[b]	47 (64.4)[b]	42 (60.9)[b]	0.52	31 (42.5)	44 (68)	46 (69.7)	41 (61.2)	0.58	45 (63.4)
Antibiotictreatment	4 (5.7)	1 (1.4)[c]	9 (13.0)	0.049	3 (4.1)	12 (18.2)	15 (21.4)[b]	8 (11.8)	0.31	5 (7.0)
Treatment with bronchodilators	6 (8.6)	2 (2.7)	4 (5.8)	0.30	4 (5.5)	10 (15.0)	9 (13)	7 (11)	0.7	5 (7.0)
Longitudinal prevalence, % (IQR)										
Days with fever	0.8 (0.0; 2.4)	0.8 (0.0; 2.2)	0.8 (0.0; 1.7)	0.73	0.8 (0.0; 3.0)	1.7 (0.6; 3.9)	1.6 (0.5; 4.3)	1.9 (1.1; 3.7)	0.92	2.3 (1.0; 3.9)
Days withrespiratory tractinfection	8.1 (2.3; 18.2)	6.6 (1.0; 17.2)	10.2 (0.0; 21.7)	0.61	7.5 (2.6; 13.8)	7.8 (3.6; 19.5)	10.0 (1.7; 19.8)	8.0 (3.5; 18.1)	0.97	7.9 (3.7; 15.9)
Days withgastroenteritis	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.63	0.0 (0.0; 0.0)	0.0 (0.0; 0.3)	0.0 (0.0; 1.9)	0.0 (0.0; 0.0)	0.46	0.0 (0.0; 0.0)
Days with rash/eczema	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.53	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.95	0.0 (0.0; 0.0)
Days with antipyretics	0.8 (0.0; 3.3)	0.8 (0.0; 2.1)	0.8 (0; 1.6)	0.28	0.0 (0.0; 2.4)	1.4 (0.0; 3.1)	1.2 (0.0; 3.5)	1.1 (0.0; 2.7)	0.76	1.0 (0.0; 2.4)

Abbreviations: α-lac-EW, α-lactalbumin-enriched whey; BF, breast-fed; BMI, body mass index; CGMP-RW, casein glycomacropeptide-reduced whey; IQR, interquartile range; SF, standard formula.

[a]p-Values: for differences between FF groups using chi-square test or Fishers exact test or Kruskal–Wallis test. Each outcome presented as cumulative incidence; *n* (%) or as longitudinal prevalence, median percentage (25th; 75th percentile) of days with an outcome.

[b]Significantly different versus BF ($p < 0.05$).

[c]α-lac-EW versus CGMP-RW ($p < 0.05$).

TABLE 3 Serum cytokine and hsCRP concentrations and at 2, 4, and 6 months of age in infants fed infant formula or breast milk.

	n	SF	*n*	α-lac-EW	*n*	CGMP-RW	*p*[a]	*n*	BF
TNF-α (pg/mL)									
2 months	71	26.1 (21.7; 31.0)	66	29.1 (24.8; 35.4)	71	26.1 (22.4; 33.0)	0.065	67	26.7 (23.4; 33.5)
4 months	64	25.8 (19.7; 31.9)	65	25.6 (21.2; 30.6)	64	25.9 (21.1; 35.0)	0.52	62	26.6 (21.7; 33.4)
6 months	65	26.5 (21.4; 31.1)	65	24.3 (19.0; 29.6)	64	23.9 (18.7; 29.2)	0.17	61	25.6 (20.9; 31.3)
INFγ (pg/ml)									
2 months	71	4.1 (1.6; 6.1)	66	4.5 (2.3; 6.1)	71	4.8 (2.9; 8.3)	0.06	67	4.4 (2.9; 6.6)
4 months	64	7.5 (5.3; 10.3)	65	6.4 (3.6; 9.6)	64	8.1 (4.4; 11.5)	0.14	62	6.5 (4.6; 8.4)
6 months	65	7.2 (5.3; 10.6)	65	8.4 (5.2; 10.7)	64	7.0 (4.5; 10.1)	0.68	61	6.8 (4.7; 11.0)
IL-6 (pg/ml)									
2 months	71	0.4 (0.1; 6.1)	66	0.1 (0.1; 3.4)	71	0.5 (0.1; 4.8)	0.53	67	0.1 (0.1; 4.0)
4 months	64	10.5 (2.1; 24.5)[b]	65	4.3 (0.1; 21.2)[b]	64	7.7 (2.1; 20.1)[b]	0.28	62	0.1 (0.1; 2.1)
6 months	65	13.4 (2.6; 37.0)[b]	65	20.2 (2.2; 66.5)[b]	64	9.0 (2.8; 31.2)[b]	0.19	61	0.2 (0.1; 8.4)
IL-10 (pg/ml)									
2 months	71	8.4 (4.0; 11.9)	66	8.0 (5.1; 11.3)	71	9.8 (5.5; 14.6)[b]	0.12	67	6.7 (4.0; 10.5)
4 months	64	9.3 (5.6; 14.2)	65	8.5 (4.8; 12.9)	64	9.3 (5.3; 16.4)	0.28	62	7.6 (4.5; 12.7)
6 months	65	9.2 (6.1; 15.0)	65	8.3 (5.0; 15.7)	64	8.9 (5.9; 12.8)	0.54	61	8.3 (5.3; 13.2)
IL-12 (pg/ml)									
2 months	71	1.1 (0.5; 1.6)	66	1.1 (0.6; 1.7)	71	1.3 (0.7; 1.8)	0.09	67	1.0 (0.6; 1.5)
4 months	64	2.2 (1.4; 2.9)	65	1.7 (0.9; 2.7)	64	2.4 (1.3; 3.2)	0.15	62	2.1 (1.1; 3.1)
6 months	65	2.2 (1.4; 3.5)	65	2.2 (1.2; 3.3)	64	2.0 (1.4; 2.9)	0.63	61	2.2 (1.6; 3.2)
TGF-β1 (ng/mL)									
2 months	71	59.8 (52.7; 65.0)	66	60.3 (53.7; 65.3)	71	58.5 (48.2; 67.4)	0.63	67	55.3 (50.8; 66.7)
4 months	64	57.2 (50.0; 67.6)	65	59.3 (53.1; 72.1)	64	61.3 (56.1; 69.1)	0.13	62	57.2 (51.5; 62.1)
6 months	65	54.7 (47.5; 61.6)	65	57.7 (49.5; 67.2)	64	57.3 (52.1; 65.7)	0.13	61	52.3 (46.0; 62.2)
TGF-β2 (ng/mL)									
2 months	71	1.2 (1.0; 1.4)	66	1.2 (1.0; 1.4)	71	1.2 (1.0; 1.3)	0.13	67	1.1 (1.0; 1.3)
4 months	64	1.3 (1.1; 1.7)	65	1.3 (1.1; 1.8)	64	1.4 (1.1; 1.8)	0.70	62	1.2 (1.1; 1.5)
6 months	65	1.3 (1.1; 1.6)	65	1.3 (1.1; 1.8)	64	1.3 (1.0; 1.5)	0.91	61	1.2 (1.0; 1.4)
hsCRP mg/L									
2 months	78	0.1 (0.1; 0.4)	71	0.1 (0.0; 0.2)	74	0.1 (0.1; 0.2)	0.58	72	0.1 (0.1; 0.3)
4 months	66	0.2 (0.1; 0.6)	68	0.1 (0.9; 0.3)	66	0.2 (0.1; 0.9)	0.56	67	0.2 (0.1; 0.6)
6 months	66	0.2 (0.1; 2.0)	69	0.2 (0.1; 0.9)	64	0.2 (0.1; 0.7)	0.70	66	0.2 (0.1; 0.5)

Abbreviations: BF, breast-fed; CGMP-RW, casein glycomacropeptide-reduced whey; hsCRP; high-sensitivity CRP; IL, interleukin; SF, standard formula; TGF, transforming growth factor; TNF, tumor necrosis factor; α-lac-EW, α-lactalbumin-enriched whey.

[a]*p*-Values; differences between FF groups using Kruskal–Wallis test, post hoc Bonferroni. Data presented as median (25th; 75th percentiles).

[b]Significantly different versus BF ($p < 0.05$).

groups are supported by another study,[39] where infants were fed infant formulas supplemented with α-lac (25%) but with various concentrations of CGMP (15% or 10% of protein content, respectively), standard formula or breast milk. Furthermore, no effect on infection-related morbidity was seen in infants fed lactoferrin-enriched infant formula.[37]

The similar cytokine concentrations as well as clinical presentation of morbidity outcomes in all study groups in the present study, except for IL-6 in the BF

group, could indicate that other bioactive components than α-lac do influence the immune physiology to be more similar to BF infants. For example, the addition of human milk oligosaccharides (HMOs) to infant formula has been shown to reduce the incidence of respiratory tract infections and result in lower use of antipyretic and antibiotics compared to feeding standard formula, with incidences closer to those of BF infants.[40] The reasons for these findings are probably related to the ability of HMOs to affect gut microbiota composition[41] and the cytokine profile[31] to be more similar to BF infants. The addition of milk fat globule membrane (MFGM) to infant formula has also been found to have protective effects in infants against infections, resulting in lower incidence of otitis media,[7] respiratory tract infections, diarrhea[42] and fever, as well as lower usage of antibiotics[32] compared to feeding standard infant formula. Enrichment with MFGM also resulted in a cytokine pattern closer to BF infants when compared to infants fed standard formula.[32] MFGM has been shown to have modulatory effects on humoral immunity[7] and the gut microbiota.[43] Furthermore, addition of osteopontin to infant formula has been found to lower the pro-inflammatory cytokine TNF-α levels compared to standard formula,[33] and to increase the levels of circulating immune cells.[44] However, the addition of lactoferrin to infant formula did not result in any anti-inflammatory cytokine profile.[37] Our finding of similar cytokine profiles among the study groups in the present study also differ from a previous case-control study,[30] where FF infants were found to have higher concentrations of the pro-inflammatory cytokines TNF-α, IL-2, and lower concentrations of the anti-inflammatory cytokine TGF-β2 compared to BF infants. However, their study population was recruited from a selective sample of infants with high genetic risk for atopic disease which might have influenced their results. Furthermore, formula composition in that study was not described, which makes it difficult to compare the results. Previous data on possible effects of α-lac enriched formula on infection-related parameters and immune modulation are from in vitro or animal studies.[22,23,26,28] Only modest findings are reported in human studies, such as a minor impact on gut microbiota[45] and in a previous study,[46] no differences in distribution of white blood cells was found in infants fed α-lac-enriched formula up to 6 months of age compared to those fed standard infant formula.

IL-6 is produced in response to infection or injury and promotes recruitment and activation of immune cells and helps regulating the acute phase response. The lower IL-6 concentrations in BF than in FF groups is supported by a previous study,[33] evaluating enrichment of osteopontin to infant formula. The lower IL-6 in BF infants may be part of the protective effects of breast milk leading to reduced inflammatory activity. In the present study, we did find reduced use of antipyretics in BF compared to FF groups, but no differences in incidence or prevalence of infections between BF and FF infants during intervention, as was found in the study by Lönnerdal et al.[33]

Despite the higher IL-6 concentrations in FF infants, there was no difference in hsCRP compared to the BF group during intervention in the present study (Table 2). This could indicate that the higher IL-6 in all FF groups throughout the intervention period is related to other underlying mechanisms than infections, such as low-grade inflammation. Elevated IL-6, as a potential indicator of low-grade inflammation, has also been associated with overweight in older children.[47,48] Additional studies are needed to further investigate the cause of the higher IL-6 concentrations in the FF groups.

The lack of observed beneficial effects on morbidity and cytokine profile of α-lac-enriched formula in the present study may also have other explanations. In our study population, about 80% of FF infants had been BF to some extent before inclusion. Since type of feeding, breastfeeding or formula feeding, directly following birth will impact the composition of the gut microbiota,[49] this early exposure to breast milk may have altered the early development of the gut microbiota among FF infants. In addition, our study was conducted in a setting with a high socioeconomic status, a low burden of pediatric infectious diseases, good access to modern healthcare and with high coverage of childhood immunization, all factors contributing to favorable health outcomes in infants. Thus, to be able to detect any potential differences in infection-related morbidity between study groups under these circumstances, a larger sample size may have been needed.

A strength of this study is the RCT design with a large number of participants and a low drop-out rate. To our knowledge, no previous RCT has evaluated the cytokine profile in infants fed formula enriched in α-lac with concentrations more like that of breast milk, and with blood sampling both at baseline and during the intervention period. This study thus adds new information on the effects of α-lac on the inflammatory response. Although cytokines were analysed as secondary outcomes in the ALFoNS study, the required 64 samples or more were reached in all FF at all ages.

In summary, no specific effect was found on infection-related morbidity or immune response of an increased concentration of α-lac in low-protein infant formula to levels similar to that of breast milk when compared to the feeding of standard formula or breast milk. Reasons for the elevated serum IL-6 in FF compared to BF infants remain to be investigated in further studies.

ACKNOWLEDGMENTS

We are grateful to all families participated in the study and to our study nurses Daniella Hanmark, Louise Brisard, Gunilla Strinnholm and Carina Forslund for

their dedicated work. We thank Mona Svensson and Carina Lagerqvist for their assistance with laboratory analyses, and Elsa Nermark, medical student, Lund University, for her contribution to data collection. We also thank Sara Jesperson, Forum Söder, for statistical advice and support. Arla Foods Ingredients Group P/S, Viby J, Denmark funded this study and provided the infant formula. The study was also supported by a grant from Region Skåne Council Foundation for Research and Development.

CONFLICTS OF INTEREST STATEMENT

Lotte N. Jacobsen, Maria Nunez-Salces, and Anne Staudt Kvistgaard are employees of Arla Foods Ingredients and contributed to logistic work with the study and funding communication. Olle Hernell, Bo Lönnerdal, and Christina West have received research grants and honorarium from Arla Foods Ingredients and Pia Karlsland Åkeson has received a research honorarium. The remaining authors declare no conflict of interest.

ORCID

Ulrika T. Nilsson https://orcid.org/0009-0009-8911-703X
Olle Hernell https://orcid.org/0000-0001-8841-6200
Bo Lönnerdal https://orcid.org/0000-0002-3459-2484
Maria Nunez-Salces https://orcid.org/0000-0002-1879-7797
Christina West https://orcid.org/0000-0001-9599-2580
Pia K. Åkeson https://orcid.org/0000-0003-2010-3130

REFERENCES

1. Haschke F, Haiden N, Thakkar SK. Nutritive and bioactive proteins in breastmilk. *Ann Nutr Metab*. 2016;69(suppl 2):16-26.
2. Dib S, Fair FJ, McCann LJ, et al. Effects of exclusive breastfeeding promotion interventions on child outcomes: a systematic review and meta-analysis. *Ann Nutr Metab*. 2024;80(2):57-73.
3. Duijts L, Jaddoe VWV, Hofman A, Moll HA. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. *Pediatrics*. 2010;126(1):e18-e25.
4. Frank NM, Lynch KF, Uusitalo U, et al. The relationship between breastfeeding and reported respiratory and gastrointestinal infection rates in young children. *BMC Pediatr*. 2019;19(1):339.
5. Horta BL, Victora, Cesar G. *Short-Term Effects of Breastfeeding: A Systematic Review on the Benefits of Breastfeeding on Diarrhoea and Pneumonia Mortality*. World Health Organization; 2013. https://who.int/publications/i/item/9789241506120
6. Victora CG, Bahl R, Barros AJD, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet*. 2016;387(10017):475-490.
7. Timby N, Hernell O, Vaarala O, Melin M, Lönnerdal B, Domellöf M. Infections in infants fed formula supplemented with bovine milk fat globule membranes. *J Pediatr Gastroenterol Nutr*. 2015;60(3):384-389.
8. Camacho-Morales A, Caba M, García-Juárez M, Caba-Flores MD, Viveros-Contreras R, Martínez-Valenzuela C. Breastfeeding contributes to physiological immune programming in the newborn. *Front Pediatr*. 2021;9:744104.
9. Carr LE, Virmani MD, Rosa F, et al. Role of human milk bioactives on infants' gut and immune health. *Front Immunol*. 2021;12:604080.
10. World Health Organization. *Global Strategy for Infant and Young Child Feeding*. World Health Organization; 2003.
11. Davis EC, Wang M, Donovan SM. The role of early life nutrition in the establishment of gastrointestinal microbial composition and function. *Gut Microbes*. 2017;8(2):143-171.
12. Ho NT, Li F, Lee-Sarwar KA, et al. Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat Commun*. 2018;9(1):4169.
13. Milani C, Duranti S, Bottacini F, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev*. 2017;81(4):e00036-17.
14. Videhult FK, West CE. Nutrition, gut microbiota and child health outcomes. *Curr Opin Clin Nutr Metab Care*. 2016;19(3):208-213.
15. Wang M, Monaco MH, Donovan SM. Impact of early gut microbiota on immune and metabolic development and function. *Semin Fetal Neonatal Med*. 2016;21(6):380-387.
16. Simonyté Sjödin K, Hammarström ML, Rydén P, et al. Temporal and long-term gut microbiota variation in allergic disease: a prospective study from infancy to school age. *Allergy*. 2019;74(1):176-185.
17. West CE, Rydén P, Lundin D, Engstrand L, Tulic MK, Prescott SL. Gut microbiome and innate immune response patterns in IgE-associated eczema. *Clin Exp Allergy*. 2015;45(9):1419-1429.
18. Wiertsema SP, van Bergenhenegouwen J, Garssen J, Knippels LMJ. The interplay between the gut microbiome and the immune system in the context of infectious diseases throughout life and the role of nutrition in optimizing treatment strategies. *Nutrients*. 2021;13(3):886.
19. Davis EC, Dinsmoor AM, Wang M, Donovan SM. Microbiome composition in pediatric populations from birth to adolescence: impact of diet and prebiotic and probiotic interventions. *Dig Dis Sci*. 2020;65(3):706-722.
20. Brink LR, Mercer KE, Piccolo BD, et al. Neonatal diet alters fecal microbiota and metabolome profiles at different ages in infants fed breast milk or formula. *Am J Clin Nutr*. 2020;111(6):1190-1202.
21. O'Sullivan A, He X, McNiven EMS, Haggarty NW, Lönnerdal B, Slupsky CM. Early diet impacts infant rhesus gut microbiome, immunity, and metabolism. *J Proteome Res*. 2013;12(6):2833-2845.
22. Layman DK, Lönnerdal B, Fernstrom JD. Applications for α-lactalbumin in human nutrition. *Nutr Res*. 2018;76(6):444-460.
23. Brück WM, Kelleher SL, Gibson GR, Nielsen KE, Chatterton DEW, Lönnerdal B. rRNA probes used to quantify the effects of glycomacropeptide and α-lactalbumin supplementation on the predominant groups of intestinal bacteria of infant rhesus monkeys challenged with enteropathogenic *Escherichia coli*. *J Pediatr Gastroenterol Nutr*. 2003;37(3):273-280.
24. Gallo V, Arienzo A, Tomassetti F, Antonini G. Milk bioactive compounds and gut microbiota modulation: the role of whey proteins and milk oligosaccharides. *Foods*. 2024;13(6):907.
25. Nielsen CH, Hui Y, Nguyen DN, et al. Alpha-lactalbumin enriched whey protein concentrate to improve gut, immunity and brain development in preterm pigs. *Nutrients*. 2020;12(1):245.
26. Brück WM, Kelleher SL, Gibson GR, Graverholt G, Lönnerdal BL. The effects of alpha-lactalbumin and glycomacropeptide on the association of CaCo-2 cells by enteropathogenic *Escherichia coli*,

Salmonella typhimurium and *Shigella flexneri*. *FEMS Microbiol Lett*. 2006;259(1):158-162.
27. Pellegrini A. Isolation and identification of three bactericidal domains in the bovine alpha-lactalbumin molecule. *Biochimica et Biophysica Acta Gen Subj*. 1999;1426(3):439-448.
28. Ng TB, Cheung RCF, Wong JH, et al. Antiviral activities of whey proteins. *Appl Microbiol Biotechnol*. 2015;99(17): 6997-7008.
29. Oevermann A, Engels M, Thomas U, Pellegrini A. The antiviral activity of naturally occurring proteins and their peptide fragments after chemical modification. *Antiviral Res*. 2003;59(1): 23-33.
30. Kainonen E, Rautava S, Isolauri E. Immunological programming by breast milk creates an anti-inflammatory cytokine milieu in breast-fed infants compared to formula-fed infants. *Br J Nutr*. 2013;109(11):1962-1970.
31. Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG, Buck RH. Similar to those who are breastfed, infants fed a formula containing 2'-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. *J Nutr*. 2016;146(12): 2559-2566.
32. Li X, Peng Y, Li Z, et al. Serum cytokine patterns are modulated in infants fed formula with probiotics or milk fat globule membranes: a randomized controlled trial. *PLoS One*. 2021; 16(5):e0251293.
33. Lönnerdal B, Kvistgaard AS, Peerson JM, Donovan SM, Peng Y. Growth, nutrition, and cytokine response of breast-fed infants and infants fed formula with added bovine osteopontin. *J Pediatr Gastroenterol Nutr*. 2016;62(4):650-657.
34. Almeida CC, Mendonça Pereira BF, Leandro KC, Costa MP, Spisso BF, Conte-Junior CA. Bioactive compounds in infant formula and their effects on infant nutrition and health: a systematic literature review. *Int J Food Sci*. 2021;2021:8850080.
35. Tinghäll Nilsson UT, Hernell O, Lönnerdal B, et al. Low-protein formulas with alpha-lactalbumin-enriched or glycomacropeptide-reduced whey: effects on growth, nutrient intake and protein metabolism during early infancy: a randomized, double-blinded controlled trial. *Nutrients*. 2023;15(4):1010.
36. Tinghäll Nilsson U, Lönnerdal B, Hernell O, Kvistgaard AS, Jacobsen LN, Karlsland Åkeson P. Low-protein infant formula enriched with alpha-lactalbumin during early infancy may reduce insulin resistance at 12 months: a follow-up of a randomized controlled trial. *Nutrients*. 2024;16(7):1026.
37. Björmsjö M, Hernell O, Lönnerdal B, Berglund SK. Immunological effects of adding bovine lactoferrin and reducing iron in infant formula: a randomized controlled trial. *J Pediatr Gastroenterol Nutr*. 2022;74(3):e65-e72.
38. Suzuki HG, Dewez JE, Nijman RG, Yeung S. Clinical practice guidelines for acute otitis media in children: a systematic review and appraisal of European national guidelines. *BMJ Open*. 2020;10(5):e035343.
39. Sandström O, Lönnerdal B, Graverholt G, Hernell O. Effects of α-lactalbumin–enriched formula containing different concentrations of glycomacropeptide on infant nutrition. *Am J Clin Nutr*. 2008;87(4):921-928.
40. Puccio G, Alliet P, Cajozzo C, et al. Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. *J Pediatr Gastroenterol Nutr*. 2017; 64(4):624-631.
41. Berger B, Porta N, Foata F, et al. Linking human milk oligosaccharides, infant fecal community types, and later risk to require antibiotics. *mBio*. 2020;11(2):e03196-19.
42. Li X, Peng Y, Li Z, et al. Feeding infants formula with probiotics or milk fat globule membrane: a double-blind, randomized controlled trial. *Front Pediatr*. 2019;7:347.
43. He X, Parenti M, Grip T, et al. Publisher correction: metabolic phenotype of breast-fed infants, and infants fed standard formula or bovine MFGM supplemented formula: a randomized controlled trial. *Sci Rep*. 2019;9(1):11589.
44. West CE, Kvistgaard AS, Peerson JM, Donovan SM, Peng Y, Lönnerdal B. Effects of osteopontin-enriched formula on lymphocyte subsets in the first 6 months of life: a randomized controlled trial. *Pediatr Res*. 2017;82(1):63-71.
45. Brück WM, Redgrave M, Tuohy KM, et al. Effects of bovine α-lactalbumin and casein glycomacropeptide–enriched infant formulae on faecal microbiota in healthy term infants. *J Pediatr Gastroenterol Nutr*. 2006;43(5):673-679.
46. Andersson Y, Hammarström ML, Lönnerdal B, Graverholt G, Fält H, Hernell O. Formula feeding skews immune cell composition toward adaptive immunity compared to breastfeeding. *J Immunol*. 2009;183(7):4322-4328.
47. Aygun AD, Gungor S, Ustundag B, Gurgoze MK, Sen Y. Proinflammatory cytokines and leptin are increased in serum of prepubertal obese children. *Mediat Inflamm*. 2005;2005(3): 180-183.
48. Mărginean CO, Meliţ LE, Huţanu A, Ghiga DV, Săsăran MO. The adipokines and inflammatory status in the era of pediatric obesity. *Cytokine*. 2020;126:154925.
49. Aguilar-Lopez M, Dinsmoor AM, Ho TTB, Donovan SM. A systematic review of the factors influencing microbial colonization of the preterm infant gut. *Gut Microbes*. 2021;13(1):1-33.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nilsson UT, Hernell O, Lönnerdal B, et al. Immunological effects of alpha-lactalbumin-enriched low-protein infant formula: a randomized controlled trial. *J Pediatr Gastroenterol Nutr*. 2025;1-10. doi:10.1002/jpn3.70189