ORIGINAL ARTICLE Food allergy

Synbiotics-supplemented amino acid-based formula supports adequate growth in cow's milk allergic infants

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Abstract

Background: Children with cow's milk allergy (CMA) are at risk for inadequate nutritional intake and growth. Dietary management of CMA, therefore, requires diets that are not only hypoallergenic but also support adequate growth in this population. This study assessed growth of CMA infants when using a new amino acid-based formula (AAF) with prebiotics and probiotics (synbiotics) and evaluated its safety in the intended population.

Methods: In a prospective, randomized, double-blind controlled study, full-term infants with diagnosed CMA received either an AAF (control; n = 56) or AAF with synbiotics (oligofructose, long-chain inulin, acidic oligosaccharides, *Bifidobacterium breve* M-16V) (test; n = 54) for 16 wk. Primary outcome was growth, measured as weight, length and head circumference. Secondary outcomes included allergic symptoms and stool characteristics.

Results: Average age (±SD) of infants at inclusion was 4.5 ± 2.4 months. Both formulas equally supported growth according to WHO 2006 growth charts and resulted in similar increases of weight, length and head circumference. At week 16, differences (90% CI) in *Z*-scores (test–control) were as follows: weight 0.147 (−0.10; 0.39, p = 0.32), length −0.299 (−0.69; 0.09, p = 0.21) and head circumference 0.152 (−0.15; 0.45, p = 0.40). Weight-for-age and length-for-age *Z*-scores were not significantly different between the test and control groups. Both formulas were well tolerated and reduced allergic symptoms; the number of adverse events was not different between the groups.

Conclusions: This is the first study that shows that an AAF with a specific synbiotic blend, suitable for CMA infants, supports normal growth and growth similar to the AAF without synbiotics.This clinical trial is registered as NCT00664768.

Food allergies, including cow's milk allergy (CMA), affect up to 5% of infants and young children (1, 2). The long-term prognosis for CMA is good with the majority of children outgrowing this allergy over time (3). However, recent studies demonstrate that CMA and other food allergies persist into later life (4–6). Children with CMA or multiple food allergies are at risk of inadequate nutritional intake and subsequent inadequate growth (7). Dietary management of CMA requires avoidance of the offending cow's milk proteins (CMP) until immunological tolerance is acquired. Current guidelines advise the use of extensively hydrolysed formulas (eHF) as first line dietary management of CMA in infants and an amino acid-based formula (AAF) for management of complex CMA, multiple food allergies or when an eHF is not tolerated (8, 9). AAF has shown to be effective in allergy management while supporting adequate growth of CMA infants (10).

The gastrointestinal (GI) tract and its microbial population play a pivotal role in immune responses (11, 12). The developing gut microbiota in early life has been shown to be different in CMA infants compared to healthy controls (13–15) and is suggested to play a role in CMA and long-term development of allergies (16, 17). Studies have demonstrated possible benefits of modulating the gut microbiota in allergic infants or children using prebiotics and/or probiotics (18–25). An AAF with synbiotics (prebiotics and probiotics), which can modulate the gut microbiota, could therefore have beneficial effects in allergic infants. A recently developed AAF with specific synbiotics was proven to be safe and well tolerated and to promote normal growth in healthy term infants (26). As more evidence is emerging that CMA infants are at risk of developing faltering growth (7, 27, 28), effects of this new AAF on growth were evaluated in CMA infants. Additional benefits related to gut microbiota and the management of CMA were explored.

Methods

Infants aged 0–8 months were considered eligible for the study if they had confirmed IgE or non-IgE-mediated CMA by either positive double-blind placebo-controlled food challenge with cow's milk, history of acute severe reaction after cow's milk ingestion combined with positive IgE test, confirmed history of reaction to CMP with either cow's milk-specific IgE > 5 kU/l or with SPT wheal diameter ≥ 3 mm, no confirmed history of CMP reaction but with either SPT wheal diameter ≥ 6 mm, or cow's milk associated allergic eosinophilic gastroenteritis or non-IgE-mediated CMA. Subjects with birthweight <2.5 kg or born at <37 wk of gestation or suffering from severe concurrent illness, major congenital malformations, systemic or congenital infections were excluded. Subjects that received systemic antibiotics, prebiotics or probiotics 2 wk prior to enrolment were ineligible. Prebiotics or probiotics use was not permitted during the study. Subjects were recruited between April 2008 and March 2012 within 29 participating sites in the USA. Written informed consent was obtained from parents. Medical ethical approval was obtained by the Chesapeake IRB (Columbia, MD, USA).

Subjects were randomized (week 0) to receive either Neocate Infant DHA and ARA (SHS International Ltd., Nutricia Advanced Medical Nutrition, Liverpool, UK) (control) or Neocate Infant DHA and ARA with synbiotics (test) for 16 wk. Details about test and control formula were published previously (26). Study staff and parents were all blinded to the treatment groups. At baseline (week -1), medical history, anthropometrics, scoring atopic dermatitis (29) (SCORAD), stool and blood samples were obtained. Formula intake, clinical allergy symptoms and stool characteristics were recorded in 7-day diaries. Anthropometrics were repeated at all visits (weeks 0, 2, 4, 8, 12, 16), SCORAD and stool samples were obtained at weeks 4 and 16, and blood samples were obtained at week 16.

The synbiotic blend used was described previously (26). Briefly, the prebiotics were chicory-derived neutral oligofructose, long-chain inulin (BENEO-Orafti SA, Oreye, Belgium) and a food-grade pectin-derived acidic oligosaccharide (pAOS) with a total amount of 8 g/l (6.8 g/l oligofructose:inulin 9:1 and 1.2 g/l pAOS). This was combined with the probiotic strain *Bifidobacterium breve* M-16V (Morinaga Milk Industry, Tokyo, Japan) at 1.47×10^9 colony-forming units (CFU)/100 ml formula.

The primary outcome parameter was growth, measured as weight, length and head circumference. Growth charts were plotted against the WHO growth standards using WHO ANTHRO software (version 3.2.2). Secondary outcomes parameters were as follows: (1) SCORAD (29), (2) allergic symptoms (dermatological, respiratory, gastrointestinal), (3) stool consistency, frequency and colour, and flatulence, (4) formula intake, (5) faecal microbiota (Bifidobacterium spp., *Clostridium histolyticum*, *Clostridium lituseburense*, *Eubacterium rectale*/*Clostridium coccoides* groups), and (6) faecal pH and short-chain fatty acids (SCFA). Allergic symptoms and flatulence were categorized as 'none', 'slight', 'moderate', 'severe' and 'very severe'. Stool frequency (per day) was categorized as 'no stools', '1–4 stools', '5–8 stools', '9–12 stools' and 'over 12 stools'. Stool consistency was recoded to 'preferred consistency' (from most preferred to least preferred) as (i) 'soft pudding like', (ii) 'soft formed', (iii) 'dry formed', (iv) 'dry hard pellets' and (v) 'watery'. Stool colour was categorized (from most preferred to least preferred) as (i) 'yellow', (ii) 'yellow/brown', (iii) 'green', (iv) 'dark brown' and (v) 'black'. The category most frequently reported during the 7 days was used.

Safety parameters included occurrence of (serious) adverse events ((S)AE), medication use and blood parameters (plasma albumin, plasma pre-albumin, ferritin, total iron binding capacity, haemoglobin, haematocrit, blood urea nitrogen, potassium, calcium, alkaline phosphatase, white blood cells, red blood cells (RBC), mean cell volume, platelets, sodium, creatinine, chloride).

Collected stool samples were immediately frozen, transported to the hospital and stored at −80°C until further analysis. Microbial measurement by fluorescent *in situ* hybridization (target population was expressed as percentage of total bacteria), faecal pH and SCFA content (expressed as percentages of the total amount of SCFA, which included acetic acid, propionic acid and butyric acid) were performed as described previously (25).

Statistical power of the study had to be sufficient to allow detection of the smallest, clinically meaningful difference in growth increments between the groups should a difference exist. The American Academy of Pediatrics recommends as smallest meaningful difference in infant growth increments 3 g/d (30), which was used to set the equivalence margin for Z-score (0.429). To detect this difference at a 5% significance level with 80% power, 39 subjects were required and the study aimed for 40 completers in each group. Data analysis was carried out according to a pre-established statistical analysis plan.

Equivalence in weight gain, length gain and head circumference increase (Z-scores) between the groups was analysed using a mixed model repeated measurements with treatment,

visit and treatment by visit as fixed effects, subject intercept and slope as random effects, and week 0 Z-score as a covariate.

Baseline SCORAD and differences in change from baseline between the test and control group were analysed using a Wilcoxon–Mann–Whitney test. To test the association between test formula and distribution of number of subjects over categories of outcomes parameters, ordinal data were analysed using the Fisher's exact test. Fisher's Freeman–Halton exact test was used in situations of contingency table $> 2 \times 2$. *Post hoc* analyses were performed to determine differences in average stool colour and consistency between groups. Average stool consistency related to appearance of water content was tested in the following order: (i) 'watery', (ii) 'soft pudding like', (iii) 'soft formed', (iv) 'dry formed' and (v) 'dry hard pellets'. All analyses were performed on the intention-to-treat (ITT) population that included all subjects who provided informed consent and were randomized to one of the formula groups.

SAS® (SAS Enterprise Guide 4.3 or higher) for Windows was used for all analyses. Significant differences indicate statistically significant differences with $p < 0.05$.

Results

One hundred and ten subjects were enrolled and included in the ITT population. The subjects (median age: 4.4 months (range 0.6–8.9)) were randomized to the test (n = 54) or control (n = 56) formula. Baseline age, gender, ethnicity and SCORAD were well-divided over groups and are summarized in Table 1. Figure 1 shows the enrolment, dropouts and disposition of the subjects. Study formulas were well accepted, and intake levels were comparable in both groups (data not shown).

Baseline weight, length and head circumference were not significantly different between groups (Table 1). Use of both formulas resulted in similar weight gain for subjects after 16 wk of use; at week 16, difference (90% CI) in weight Z-scores (test–control) was 0.147 (−0.10; 0.39, p = 0.32). In both groups, comparable increase in length and head circumference was observed. At week 16, difference (90% CI) in length Z-scores (test–control) was −0.299 (−0.69; 0.09, p = 0.21) and difference (90% CI) in head circumference Z-scores (test–control) was 0.152 (−0.15; 0.45, p = 0.40). In addition, weight-for-age (Fig. 2a) and length-for-age (Fig. 2b) Z-scores were not significantly different between the groups.

To assess overall safety of the test formula, blood parameters were determined. Significant differences were found between the study groups regarding haemoglobin, haematocrit, RBC and alkaline phosphatase. However, these and all other values were within reference ranges. Evaluation of medication use showed a lower percentage of subjects in the test group that needed 'drugs for functional GI disorders' (test 4%, control 18%; p = 0.029) and 'antibacterials for systemic use' (test 17%, control 34%; p = 0.049). Within the latter category, it was specifically amoxicillin that was less often prescribed (test 9%, control 32%; p = 0.004).

A total of 81 subjects reported at least one AE during the study (Table 2), 43 subjects in the test group and 38 subjects in the control group (NS). Most subjects reported mild or moderate AEs.

Further subcategorization of the AE showed a significant difference between the groups in number of subjects that reported diarrhoea and infection (Table 2). More subjects in the test group experienced events categorized as diarrhoea compared to control (12 subjects (22%) and 2 subjects (4%), respectively; p = 0.004). In addition, fewer subjects experienced infection in the test group compared with control (1 subject (2%) and 10 subjects (18%), respectively; p = 0.008).

A total of 6 SAEs occurred during the study, 2 SAEs in the test and 4 SAEs in the control group. All SAEs were assessed by the investigators as being unrelated to the study formula.

Over the course of the study, SCORAD decreased in both groups (Fig. 3). Neither the decrease from baseline, nor the

Table 1 Baseline characteristics, mean ± s.d. unless stated otherwise, of all randomized subjects (ITT population)

	Test (n = 54)	Control (n = 56)
Age (months)	4.72 ± 2.29	4.45 ± 2.61
Sex, male n (%)	33 (61)	35 (63)
Ethnicity, n (%)		
Asian	4 (7)	1 (2)
Black	11 (20)	5 (9)
Latino	5 (9)	9 (16)
Other	5 (9)	2 (4)
White	29 (54)	39 (70)
SCORAD (median [range])	0.0 [0.0–62.0]	4.5 [0.0–78.6]
Weight (g)	6559 ± 1415	6441 ± 1665
Length (cm)	63.8 ± 5.1	63.5 ± 6.2
Head circumference (cm)	41.7 ± 2.5	41.6 ± 3.0

None of the characteristics were statistically different ($p < 0.05$) between test and control tested as following: age by Student's *t*-test, sex by chi-square test, ethnicity by Fisher's exact test and SCORAD by Wilcoxon–Mann–Whitney test.

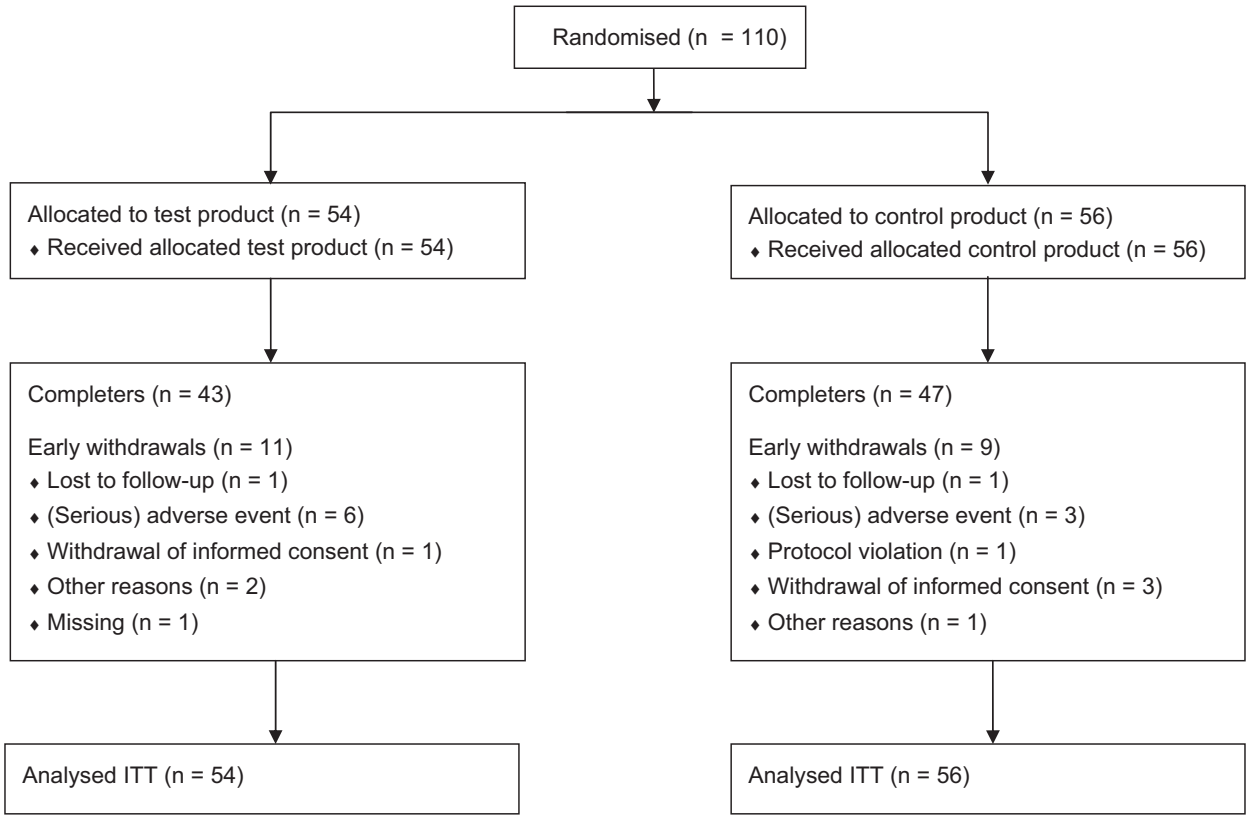

Figure 1 Flow chart of enrolled children according to entry criteria and adherence.

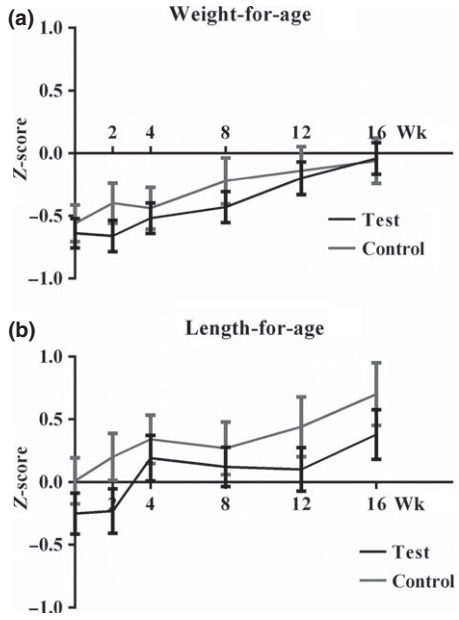

Figure 2 Weight-for-age (a) and length-for-age (b) Z-scores over time. Z-scores were calculated by using the WHO 2006 growth standards. Values are given as mean ± SEM.

number of subjects with a score of 0, differed significantly between the groups at weeks 4 or 16. In addition, severity of all other allergy symptoms assessed decreased with time and no significant differences were observed between groups.

Flatulence and stool frequency did not significantly differ between the two groups. *Post hoc* analyses indicated no significant differences between the groups on average appearance of water content and average preferred consistency per group. *Post hoc* analyses showed that in the test group, average colour was significantly different, that is more preferred stool colour, at week 0–2 ($p = 0.014$), week 2–4 ($p = 0.010$) and week 4–12 ($p = 0.008$) compared with control.

At baseline, levels of the evaluated bacterial groups were not significantly different between the study groups (Table 3). At weeks 4 and 16, faecal samples of the subjects in the test group had a significantly higher proportion of bifidobacteria ($p < 0.001$) compared with control. In contrast, the proportion of both *C. histolyticum* ($p = 0.009$ and $p < 0.001$) and *E. rectale*/*C. coccoides* ($p = 0.006$ and $p < 0.001$) were significantly lower in the test group compared with control. *C. lituseburense* proportions were not significantly different between groups. At weeks 4 and 16, faecal pH was significantly lower in the test group compared with control (Table 3; $p < 0.001$). At week 16, acetic acid levels were significantly higher and propionic acid levels were lower in the test group

Table 2 Overview of adverse events in the ITT population

	Test (N = 54)		Control (N = 56)	
Adverse Events, n (%)	43	(80)	38	(68)
Severity				
Mild	24*	(44)	14	(25)
Moderate	14	(26)	19	(34)
Severe	5	(9)	5	(9)
Preferred term description				
Body as a whole–general disorders	5	(9)	7	(13)
Foetal disorders	0	(0)	1	(2)
Gastrointestinal system disorders	23	(43)	18	(32)
Diarrhoea	12*	(22)	2	(4)
Hearing and vestibular disorders	1	(2)	2	(4)
Metabolic and nutritional disorders	2	(4)	1	(2)
Musculoskeletal system disorders	1	(2)	0	(0)
Neonatal and infancy disorders	0	(0)	1	(2)
Platelet, bleeding and clotting disorders	1	(2)	1	(2)
Psychiatric disorders	2	(4)	1	(2)
Resistance mechanism disorders	12	(22)	15	(27)
Infection	1*	(2)	10	(18)
Respiratory system disorders	29	(54)	23	(41)
Secondary terms	3	(6)	0	(0)
Skin and appendages disorders	20	(37)	14	(25)
Urinary system disorders	0	(0)	3	(5)
Vision disorders	1	(2)	3	(5)

n = number of subjects with at least one AE.
If a subject had more than one AE, the AE with the highest severity was counted.
*$p < 0.05$ by Fisher's exact test (difference in number of subjects between groups).

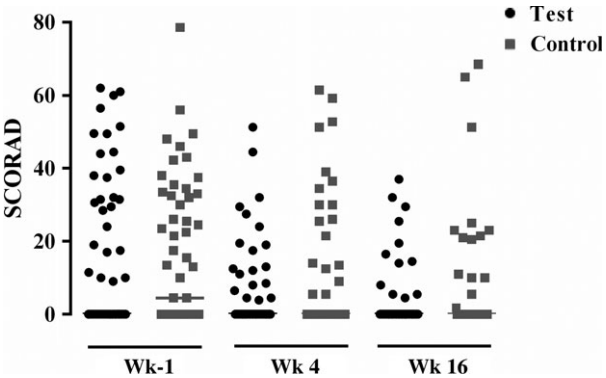

Figure 3 SCORAD over time (ITT). Week -1 indicates baseline prior to test and control formula consumption. Each subject is visible as a single dot and median as a thin line. A thick line represents accumulated dots of multiple individuals.

compared with control (Table 3; $p = 0.004$ and $p = 0.006$, respectively). The difference in propionic acid levels between the formula groups was already observed in week 4 ($p = 0.039$). No differences in levels of butyric acid and lactic acids were observed between groups.

Table 3 Faecal microbiota levels (% of total level of bacteria) and faecal pH and SCFA

	Week	Test	Control	p-value
Bifidobacteria (%)				
Mean	−1	14.3 (20.2), [46]	16.7 (23.2), [44]	0.60
(s.d.),	4	41.6 (26.6), [35]	9.4 (15.9), [38]	<0.001
[n]	16	51.2 (21.1), [39]	13.0 (18.6), [34]	<0.001
C. histolyticum (%)				
Mean	−1	9.3 (9.8), [46]	9.1 (8.6), [44]	0.93
(s.d.),	4	4.1 (4.3), [35]	8.4 (8.8), [38]	0.009
[n]	16	7.1 (5.3), [39]	17.6 (10.0), [34]	<0.001
E. rectale/C. coccoides (%)				
Mean	−1	23.8 (17.8), [46]	20.1 (16.5), [44]	0.30
(s.d.),	4	16.4 (15.9), [35]	27.7 (17.9), [38]	0.006
[n]	16	13.8 (10.4), [39]	35.5 (15.2), [34]	<0.001
pH				
Mean	−1	6.55 (0.67), [47]	6.51 (0.60), [45]	0.72
(s.d.),	4	5.96 (0.63), [35]	6.69 (0.46), [38]	<0.001
[n]	16	5.82 (0.72), [39]	6.72 (0.47), [35]	<0.001
Acetic acid (%)				
Mean	−1	76.6 (9.7), [41]	79.3 (8.5), [43]	0.17
(s.d.),	4	81.0 (10.4), [32]	77.3 (10.1), [37]	0.15
[n]	16	80.7 (10.7), [37]	68.6 (21.5), [35]	0.004
Propionic acid (%)				
Mean	−1	15.2 (8.4), [41]	14.7 (7.8), [43]	0.75
(s.d.),	4	12.0 (9.2), [32]	16.8 (9.6), [37]	0.039
[n]	16	11.4 (7.3), [37]	22.6 (21.4), [35]	0.006

p values were calculated by Student's *t*-test. Short-chain fatty acids (SCFA) are represented as % of the total amount of SCFA, which includes acetic acid, propionic acid and butyric acid.

Discussion

Dietary management of CMA may require the use of an adapted infant formula like eHF or AAF. A suitable formula should not only be hypoallergenic, but also needs to ensure adequate growth as CMA infants are known to be at risk of faltering growth (7, 27, 28). This prospective, randomized, double-blind controlled study is the first study that investigates growth of CMA infants when using a AAF supplemented with oligofructose, long-chain inulin, acidic oligosaccharides and *B. breve* M-16V and evaluates safety of this formula in this population. These synbiotics were carefully selected and not derived from cow's milk to ensure the safety of the formula for the intended population.

This study demonstrates adequate growth in CMA infants measured by weight, length and head circumference. The growth is similar to the AAF without synbiotics and is adequate when compared to WHO growth standards (31). Comparable results were shown previously when this formula was consumed by healthy infants (26). Studies with other synbiotics-supplemented infant formulas also demonstrated adequate growth when comparing to the same formula without synbiotics (32, 33). A systematic review by ESPGHAN concluded that administration of currently evaluated probiotic- and/or prebiotic-supplemented formulas to infants does not

raise safety concerns (34). It was also concluded that safety and clinical effects of one product cannot be extrapolated to other products. This highlights the importance of the current study, providing evidence on growth and safety of the specific synbiotics-supplemented AAF.

The number of AEs, allergic symptoms, including spitting up and vomiting, and flatulence were not different between groups. Safety parameters were not different between the two study groups, except for haemoglobin, RBC, haematocrit and alkaline phosphatase; however, all values were within reference ranges and, therefore, not deemed clinically relevant. Evaluation of the type of AEs reported indicated that more subjects had diarrhoea in the test group compared with control. The events were described by the investigators as 'diarrhoea', 'watery stool' or 'stool change: runny'. In eight of twelve subjects, the events were reported by the investigator as not or unlikely related to study formula. In four subjects, the event started prior to formula intake and in a fifth subject within 2 days after influenza vaccination. All events were classified as mild or moderate, did not require further action and stopped, except for one event, with time prior to study end. Similar to the current study, in healthy infants receiving the same AAF with or without synbiotics, laboratory parameters reflecting water balance were not different between groups (26). The average consistency score in the present study was not different between groups.

Furthermore, fewer subjects were reported with AE classified as infection in the test group and fewer subjects needed 'antibacterials for systemic use', specifically amoxicillin in the test group. Also, less medication categorized as 'drugs for functional GI disorders', which were mostly prescribed in this study for gas relief, was prescribed in the test group. These effects may be related to the use of this specific synbiotic blend of oligofructose, long-chain inulin, acidic oligosaccharides and *B. breve* M-16V, but need further study. It could be speculated that the observed effects on gut physiology, such as lower faecal pH and increased levels of acetic acid, typically reflecting the presence of bifidobacteria, contribute to this beneficial effect.

To our knowledge, this is the first study which shows that the indigenous gut microbiota of CMA infants receiving an AAF can be influenced by synbiotics. As expected, synbiotics in the test formula increased Bifidobacterium, a genus typically predominant in the GI tract of breastfed infants (35).

The increase of bifidobacteria was associated with decreased presence of clostridial groups *C. hystoliticum* and *E. rectale*/*C. coccoides*, which are typically abundant in adults. Increased abundance of the latter group has been observed in infants with CMA aged 2–12 months, suggesting this group is part of a gut microbiota dysbiosis in CMA (14, 15, 17). It can therefore be hypothesized that abolishing this gut microbiota dysbiosis may decrease CMA risk or CMA persistence. The changed gut microbiota-associated metabolic activity, that is lower stool pH, increased acetic acid and lower propionic acid, are similar to previously published studies with hydrolysed or whole protein infant formula supplemented with pre- or synbiotics (25, 36, 37).

Overall, our data showed that SCORAD and allergy symptoms were not different between study groups, indicating the synbiotics-supplemented AAF is equally effective in CMA management as the AAF without synbiotics when used for 16 weeks in a heterogeneous CMA infant population. Further studies should focus on the potential benefits of the supplemented synbiotic blend in the dietary management of specific CMA subsets. These studies are ongoing and will aid optimization of dietary management of CMA in infants prone to inadequate growth and other food allergy-related concomitant conditions.

In conclusion, a specific synbiotics-supplemented AAF demonstrated normal growth and growth similar to an AAF without synbiotics when used in CMA infants. In addition, the formula was shown to be safe and suitable for dietary management of CMA in infants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of significantly different hematologic and blood chemistry parameters and MCV.