

The acquisition of tolerance toward cow's milk through probiotic supplementation: A randomized controlled trial

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Background: Cow's milk allergy (CMA) is the most frequently diagnosed food allergy in infancy. In general, patients have a good prognosis because the majority acquire tolerance within the first years. Interventions have been proposed to accelerate tolerance and reduce morbidity. Probiotic supplementation could be effective through modulation of the immune system. **Objective:** We sought to determine whether supplementation with a combination of probiotics (*Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12) accelerates tolerance to cow's milk (CM) in infants with CMA.

Methods: We performed a double-blind, randomized, placebo-controlled trial in 119 infants with CMA. Infants received CRL431 and Bb-12 supplemented to their standard treatment of extensively hydrolyzed formula for 12 months. Primary outcome was clinical tolerance to CM at 6 and 12 months of treatment. Furthermore, we analyzed T- and B-lymphocyte subsets (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, and CD20⁺) in peripheral blood at randomization and at 12 months with flow cytometry and examined the presence of viable probiotic strains in fecal samples.

Results: The cumulative percentage of tolerance to CM at 6 and 12 months was similar in both groups: 56 (77%) in the probiotics group versus 54 (81%) in the placebo group. Infants in the placebo group had higher percentages of CD3⁺ and CD3⁺CD4⁺ lymphocytes compared with those seen in

probiotic-treated infants. Probiotic intake was confirmed because probiotics were isolated from feces more often in treated infants than in the placebo group.

Conclusion: Supplementation of CRL431 and Bb-12 to extensively hydrolyzed formula does not accelerate CM tolerance in infants with CMA. (J Allergy Clin Immunol 2008;122:1000-1006.)

Key words: Cow's milk allergy, probiotics, tolerance, infants, atopic eczema/dermatitis syndrome, flow cytometry

Cow's milk allergy (CMA) is the most common food allergy in early childhood.¹ From prospective studies, the estimated incidence is 2% to 5%.² The majority of infants become tolerant before the age of 3 years.³ However, children with CMA have an increased risk of atopic diseases in later life.⁴ It has been hypothesized that CMA is due to immaturity of local and systemic immune responses⁵ and is associated with an altered composition of the intestinal microbiota in Western society.⁶ Indeed, in the last 25 years, the prevalence of allergic diseases has doubled in the industrialized parts of the world.^{7,8} The increase in allergic disorders appears to correspond with the implementation of public health measures in the prevention of infectious diseases and increased availability of antibiotics. Based on epidemiologic studies and observational data, Strachan⁹ formulated the so-called hygiene hypothesis, stating that reduced microbial stimulation in early life increases susceptibility to allergic diseases. Specifically, alterations in the gut flora composition could be relevant to explain the increased prevalence of allergy. An association has been described between gut colonization with *Clostridium difficile* and allergy in children,¹⁰ whereas lactobacilli and eubacteria were more frequent in nonallergic children.⁶ Based on these findings, it has been proposed that specific bacteria, denoted as probiotics, could potentially restore intestinal homeostasis and prevent allergy through interaction with the intestinal immune cells.^{11,12} Probiotics have been extensively studied in the treatment of atopic eczema/dermatitis syndrome (AEDS)¹³⁻¹⁸ and allergic rhinitis.¹⁹ In infants with CMA, the use of *Lactobacillus* GG reduced the severity of AEDS and diminished intestinal inflammation.^{20,21} To date, however, no studies have been performed to assess whether probiotics can induce CM tolerance in established CMA.

The main purpose of our study was to determine whether supplementation of extensively hydrolyzed formula with a combination of 2 probiotics (*Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12) would affect tolerance acquisition to CM.

Because it was shown that infants with CMA have increased percentages of B lymphocytes (CD19⁺) and decreased CD8⁺ T

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Abbreviations used

AEDS:	Atopic eczema/dermatitis syndrome
CAMEL:	Cow's Milk Allergy Modified by Elimination and Lactobacilli
CMA:	Cow's milk allergy
CM:	Cow's milk
DBPCFC:	Double-blind, placebo-controlled food challenge
OR:	Odds ratio
SPT:	Skin prick test

lymphocytes in peripheral blood compared with levels seen in infants without CMA,²² we also studied changes in B- and T-lymphocyte populations on tolerance acquisition and in relation to probiotic treatment by means of a secondary end point.

METHODS**Study design**

The Cow's Milk Allergy Modified by Elimination and Lactobacilli (CAMEL) project was a randomized, double-blind, placebo-controlled study carried out between March 2004 and May 2007. Infants younger than 6 months with a diagnosis of CMA were included.^{23,24} One hundred ninety-three infants with suspected CMA who had shown improvement of food-related symptoms with an extensively hydrolyzed formula were referred from regional clinics and hospitals. Of the 193 infants, 119 met our inclusion criteria (mean age, 4.2 months; age range, 1.4–6.0 months; 55% boys). At least one parent of each infant provided written informed consent. The local ethics committee approved the study protocol. The study design is depicted in Fig 1.

Diagnosis of CMA

Infants referred to the CAMEL study were prescribed an extensively hydrolyzed casein-based formula (Friso 1 Allergy Care; Royal Friesland Foods, Meppel, The Netherlands) for at least 4 weeks. CMA was diagnosed with a food challenge according to the guidelines of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society of Pediatric Allergy And Clinical Immunology and criteria of Bock and Sampson.^{23,24} At inclusion (visit 1), an open CM challenge was conducted in one of the participating hospitals by the investigators (J.H. and B.E.S.). The test formula was Friso 1 Allergy Care mixed with additional cow's milk (CM) proteins (Protifar; Nutricia, Zoetermeer, The Netherlands) to achieve the CM protein concentration of standard infant formula. The challenge consisted of administration of 2 mL followed by 6, 20, 60, and 200 mL at 30-minute intervals. Infants were scored for 9 items divided into 4 main categories (general, skin, gastrointestinal, and respiratory) on a 0- to 3-point scale (0, none; 1, light; 2, moderate; and 3, severe). If both investigators independently scored any item at level 3 or 2 (or more) items at level 2, the test result was considered positive. Two hours after the final dose, infants were discharged and contacted by telephone the next morning to check for symptoms suggestive of a late response, such as vomiting or diarrhea. Patients returned to the outpatient clinic the next day only when they reported skin problems that needed to be evaluated by a physician. If infants did not show any symptoms within the initial 24 hours, parents were advised to reintroduce CM, starting 48 hours after the initial test at an increasing daily dosage. All of these presumed nonallergic children were followed up for at least 1 week. Late responders were rechallenged and included in our studies when test results were positive.

All infants with positive test results on challenge were randomized at visit 2. After 6 and 12 months of study formula, a double-blind, placebo-controlled food challenge (DBPCFC) was performed (visits 3 and 4).

All DBPCFCs took place in the outpatient clinic of the Erasmus MC–Sophia Children's Hospital on 2 separate days with a 1-week interval and were carried out by J.H. and B.E.S. The test formula was Friso 1 Allergy Care mixed with additional CM proteins (Protifar), and the placebo formula was standard

Friso 1 Allergy Care. After completion of the DBPCFC, the code of test formulas was broken.

Assignment

The groups were stratified and block randomized according to age at inclusion (<20 weeks or ≥20 weeks), birth weight (<2500 g or ≥2500 g), and (reported) atopic diseases in first-degree relatives (yes or no). A computerized randomization schedule was prepared by the biostatistician.

Probiotic supplements

Infants in the probiotic group received *Lactobacillus casei* CRL431 (*Lactobacillus paracasei* subspecies *paracasei*) and *Bifidobacterium lactis* Bb-12 (*B animalis* subspecies *lactis*; 10⁷ colony-forming units/g formula for each of the probiotic bacteria used) supplemented to Friso 1 Allergy Care, whereas the control group received Friso 1 Allergy Care alone. The colony-forming units per gram values of the formula were measured monthly and remained stable during the study. If infants had become CM tolerant after 6 months, they received either CM-containing follow-up milk (Friso 3) supplemented with CRL431 and Bb-12 or Friso 3 alone for 6 months.

CRL431 and Bb-12 fulfill the requirements of the Food and Agriculture Organization/World Health Organization expert panel guidelines for probiotics.²⁵ Earlier clinical studies have shown CRL431^{26,27} and Bb-12^{14,28} to be well tolerated and safe in young infants.

Blinding

Probiotics and placebo formula were image and taste matched, and participants and researchers remained blinded to group assignment for the duration of the study. To optimize compliance, participants were supplied with study formula through the study team, and batches were delivered at home.

Clinical outcome

The primary end point was clinical tolerance to CM at 6 and 12 months after initial CMA diagnosis. J.H. and B.E.S. performed visits at randomization and at 6 and 12 months after the start of study formula. Structured interviews on symptoms of allergic disease, adverse events, use of antibiotics, daycare attendance, smoking in the house, and pet exposure, as well as an inspection of the skin, were performed. The SCORAD index was used to assess the severity of the eczema.²⁹ Skin tests were performed at randomization and at 12 months of study formula (visits 2 and 4). Fresh foods were applied to the volar side of the patient's forearm (ie, fresh CM, hen's egg, and soy). Skin prick tests (SPTs) were performed with a 1-mm single-peak lancet (ALK, Copenhagen, Denmark). Reactions were read at 15 minutes and considered positive if the wheal was 3 mm or larger, with NaCl 0.9% as a negative control and 10 mg/mL histamine dihydrochloride (ALK) as a positive control.

Fecal samples

Fecal samples were collected from 24 infants (11 on placebo and 13 on probiotics) at visit 3. All samples were frozen to –20°C within 15 minutes, brought to the hospital, and stored at –80°C until analyzed. Samples were analyzed with a microarray to detect and quantitate the small subunit ribosomal RNA of *B animalis*, *L casei*, and *L paracasei*, as adapted from Palmer et al.³⁰

Flow cytometry

Peripheral blood samples were collected by means of venipuncture at randomization and after 12 months of study formula (visits 2 and 4). Erythrocytes were lysed, and cells were washed twice with PBS with 1% newborn calf serum (denoted as FACS buffer). Next, samples were incubated with human serum for 10 minutes, washed with FACS buffer, and incubated with anti-CD3–fluorescein isothiocyanate (clone HIT3a), anti-CD4–phycoerythrin (clone RPA-T4), anti-CD8–peridinin-chlorophyll-protein (clone

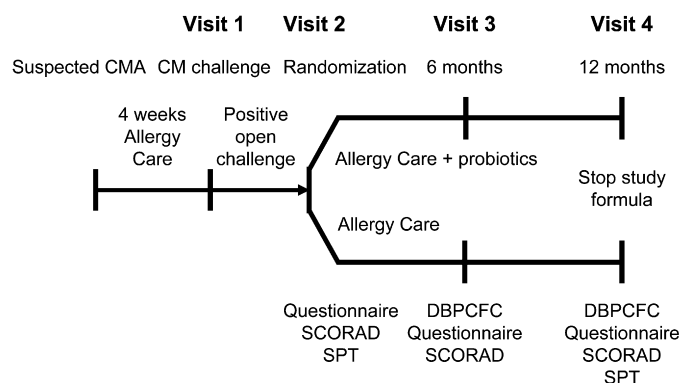


FIG 1. Design of the CAMEL study.

TABLE I. Baseline characteristics of the study population

Characteristics	Placebo (n = 60)	Probiotics (n = 59)
Boys, % (n)	60 (36)	51 (30)
Age at inclusion [mo (SD)]	4.1 (1.5)	4.3 (1.2)
Birth weight [g (SD)]	3445 (556)	3456 (504)
Gestational age [wk (SD)]	39.6 (1.7)	39.5 (1.6)
Caesarean delivery, % (n)	17 (10)	20 (12)
Breast-fed [ever % (n)]	75 (45)	73 (37)
Duration of breast-feeding, % (n)		
<2 mo	45 (27)	49 (25)
≥2 mo	30 (18)	23 (12)
Furred pet, % (n)	42 (25)	36 (21)
Daycare attendance, % (n)	53 (32)	46 (27)
Indoor smoking, % (n)	10 (6)	12 (7)
Antibiotic before intervention, % (n)	22 (13)	31 (18)
Positive SPT response, milk, % (n)	21 (12)	10 (6)
Positive SPT response, hen's egg, % (n)	22 (13)	17 (10)
Positive SPT response, soy, % (n)	3 (2)	2 (1)

SK-1), and anti-CD20-CyChrome (clone 2H7; all from Becton, Dickinson and Company, Franklin Lakes, NJ) or appropriate isotype control for 30 minutes on ice. After incubation, cells were washed and resuspended in 1% paraformaldehyde, and fluorescence was measured with a FACSCalibur (Beckton Dickinson and Company). For each sample, 50,000 events were analyzed. Cells that had been incubated with isotype control served as negative controls. The results were analyzed with FlowJo (Tree Star, Inc, Ashland, Ore).

Statistical analysis

The SPSS software package (version 12.01 for Windows; SPSS, Inc, Chicago, Ill) was used for statistical analysis. The Pearson χ^2 test, Fisher exact test, Student *t* test, analysis of covariance, Mann-Whitney test, and repeated-measures ANOVA were applied to examine differences between treatment groups. With multivariate stepwise logistic regression analysis, we assessed predictors for the persistence of CMA at 6 and 12 months after randomization. The model used data available at randomization: sex, delivery type, age at randomization, family history of atopy, presence of furred pets, SPTs to milk and hen's egg, duration of breast-feeding, daycare attendance, and antibiotic use. Outcomes are reported as odds ratios (ORs). For all statistical tests, a 2-tailed *P* value of less than .05 was considered significant.

RESULTS

Baseline characteristics

At visit 1, 119 (61%) of the recruited infants showed urticaria, worsening of AEDS, vomiting, diarrhea, physician-diagnosed

wheezing, or convincing behavioral symptoms on challenge with CM-containing formula. Forty percent of the included infants expressed symptoms in 2 or more organ systems: 32% expressed skin reactions, 18% expressed subjective reactions, and 10% expressed gastrointestinal symptoms. These 119 infants were randomized and enrolled in the intervention study. Seventy-four infants with negative CM challenge results were not included.

Baseline characteristics were comparable between the 2 intervention groups (Table I). There were no differences between the 2 intervention groups in onset and type of CM-related complaints and age of first formula use.

One hundred eleven (93%) infants (56 receiving placebo and 55 receiving probiotics) underwent DBPCFCs after 6 months of study formula. A total of 106 (89%) infants (55 receiving placebo and 51 receiving probiotics) completed the entire study (Fig 2).

Development of CM tolerance

The first DBPCFC at 6 months was carried out in 111 infants. In the probiotics group 31 (56%) of 55 infants had become tolerant compared with 30 (54%) of 56 in the placebo group (difference, 2%; 95% CI, -15.7 to 21.3; not significant, *P* = .92). At 6 months, 44% of the responsive infants expressed symptoms in 2 or more organ systems, 36% expressed skin reactions, 4% expressed subjective reactions, and 16% expressed gastrointestinal symptoms. Infants with persisting CMA were rechallenged at 12 months. In the probiotics group 11 (48%) of 23 of the infants were tolerant versus 15 (60%) of 25 in the placebo group (difference, -12%; 95% CI, -40.1 to 15.9; not significant, *P* = .58). At 12 months, the cumulative tolerance was 81% in the placebo group and 77% in the probiotics group (*P* = .95; OR, 1.1 [95% CI, 0.6-1.9]). At 12 months, the majority of the responsive infants expressed symptoms in 2 or more organ systems (27%) or skin reactions (41%). Less frequent were subjective reactions (18%), gastrointestinal symptoms (9%), and respiratory symptoms (5%). Data on tolerance to CM after 6 and 12 months of study formula are summarized in Fig 2. By using logistic regression analysis, none of the parameters used for block randomization was found to affect the outcomes.

Effects of probiotics on SCORAD scores

Overall, the SCORAD score improved at 6 and 12 months (Fig 3). The probiotics group (*n* = 51) showed improvement at 6 and 12 months, and the placebo group (*n* = 54) showed improvement

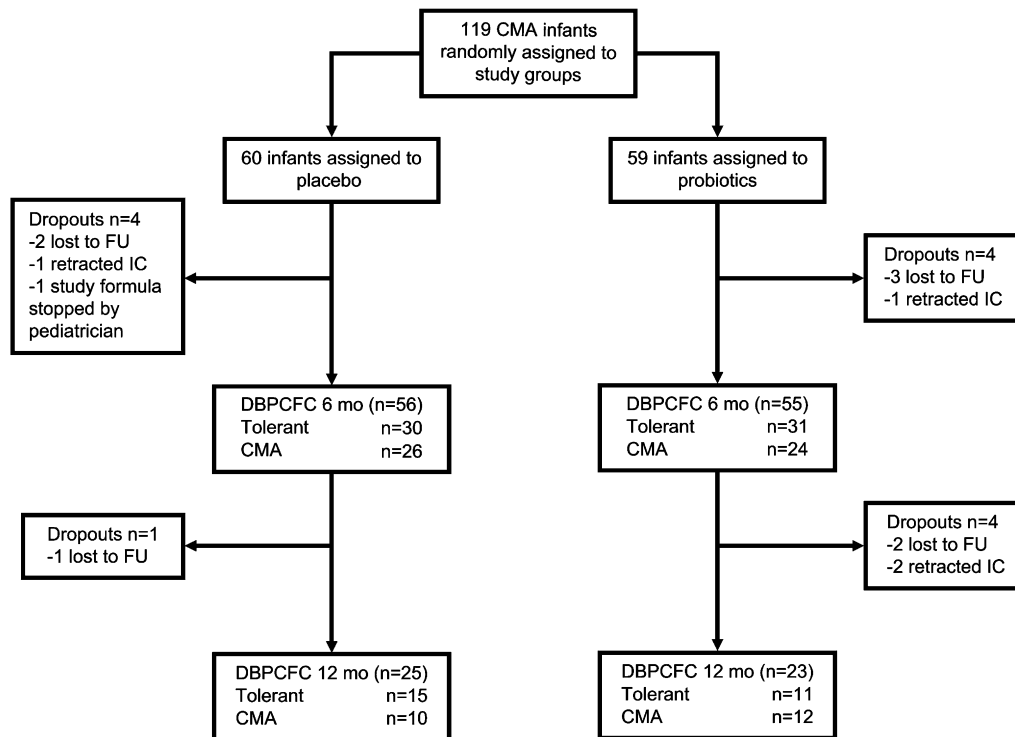


FIG 2. Flow chart of the study population. FU, Follow-up; IC, informed consent.

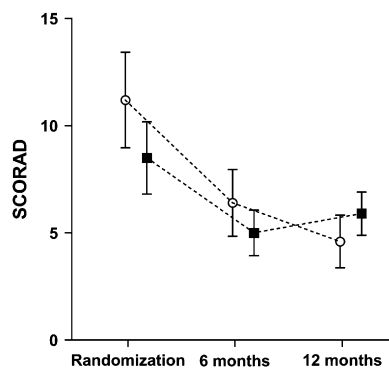


FIG 3. No additional effects of probiotics on clinical severity of AEDS is shown. Mean SCORAD scores ($\pm$ SEM) at randomization and 6 and 12 months of study formula are shown. Solid squares, placebo group ($n = 54$); open circles, probiotic group ($n = 51$). SCORAD scores significantly improved from baseline in the probiotics group at 6 and 12 months and in the placebo group at only 6 months.

at only at 6 months. However, after adjusting for the baseline values, there were no significant differences in the change from baseline between probiotic or placebo treatment at 6 months ($P = .92$) and 12 months ($P = .14$).

Because the SCORAD score in the overall population was low, we performed a subgroup analysis of infants with moderate-to-severe eczema (SCORAD score, ≥ 15) at randomization. The probiotics group ($n = 18$) had a mean SCORAD score of 27.6 (95% CI, 19.1-36.0) at randomization, improving to 14.7 (95% CI, 7.2-22.1) and 8.1 (95% CI, 1.9-14.3) after 6 and 12 months of study formula, respectively ($P < .01$). The mean SCORAD scores for the placebo ($n = 14$) group were 26.8 (95% CI, 21.2-32.4) at

randomization and 11.2 (95% CI, 5.0-17.4) and 8.5 (95% CI, 3.7-13.3) at 6 and 12 months, respectively ($P < .001$). In this subgroup the SCORAD score improved independent of probiotic treatment.

Effects of probiotics on symptoms and medication use

We found no difference in hospital admissions and reported wheezing between the study groups. The use of medication was comparable for both groups during the study period (Table II). Parents were asked to document whether their infants showed any symptoms as a result of the study formula. The study formula, with or without the probiotic supplementation, was well tolerated. We did not note any difference between the groups.

Predictors of CMA persistence

A positive SPT response to CM at randomization was a strong predictor of persisting CMA at 6 months (OR, 5.0; 95% CI, 1.5-16.4; $P = .009$). A positive SPT response to hen's egg also predicted the persistence of CMA at 12 months (OR, 4.0; 95% CI, 1.4-11.4; $P = .01$). Positive SPT responses to milk and hen's egg at 12 months were both predictive of a positive DBPCFC result at 12 months (milk, $P < .001$; hen's egg, $P = .007$).

Flow cytometry

We found similar $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD20^+$ percentages in both treatment groups at randomization (Table III). After 12 months of treatment, there was a significant change from baseline of the $CD3^+$ cells in the placebo group versus the probiotics group, with placebo values being 4.5% higher ($P = .016$, analysis of covariance, adjusted for baseline).

TABLE II. Clinical characteristics of infants in the study population during the formula study period

	Placebo	Probiotics	P value
Hospital admission			
Inclusion-6 mo	4/56 (7%)	7/55 (13%)	.36
6-12 mo	4/55 (7%)	6/51 (12%)	.52
Wheezing			
Inclusion-6 mo	20/56 (36%)	23/55 (42%)	.56
6-12 mo	14/55 (26%)	19/51 (31%)	.21
Use of asthma medication			
Inclusion-6 mo	15/56 (27%)	17/55 (31%)	.68
6-12 mo	11/55 (20%)	17/51 (33%)	.13
Use of topical steroids			
Inclusion-6 mo	18/56 (32%)	15/55 (27%)	.68
6-12 mo	14/55 (26%)	10/51 (20%)	.50
Use of antibiotics			
Inclusion-6 mo	17/56 (30%)	21/55 (28%)	.43
6-12 mo	18/55 (33%)	25/51 (49%)	.11
Positive SPT response, milk 12 mo	8/54 (15%)	10/49 (20%)	.60
Positive SPT response, hen's egg 12 mo	14/54 (26%)	16/49 (33%)	.52
Positive SPT response, soy 12 mo	6/54 (11%)	3/49 (6%)	.49

TABLE III. Lymphocyte subset percentages of lymphocyte gate at baseline (t = 0) and 12 months (t = 12)

Lymphocyte subpopulation	Probiotics		Placebo	
	t = 0	t = 12	t = 0	t = 12
CD3 ⁺	66.4 (9.2, n = 33)	64.4* (7.9)	66.5 (10.0, n = 44)	68.9* (9.6)
CD3 ⁺ CD4 ⁺	46.9 (8.9, n = 33)	41.4†‡ (6.2)	47.7 (10.7, n = 44)	45.2† (10.1)
CD3 ⁺ CD8 ⁺	16.9 (6.7, n = 30)	18.9‡ (6.6)	16.2 (5.2, n = 42)	18.2 (5.9)
CD20 ⁺	21.6 (7.2, n = 30)	20.8 (9.6)	20.9 (7.7, n = 46)	18.0 (8.9)

Data given are percentages of the lymphocyte gates of patients with paired data (mean [SD, n]).

**P* = .016 for the change from baseline (probiotics vs placebo).

†*P* = .038 for the change from baseline (probiotics vs placebo).

‡*P* < .05, t = 0 vs t = 12 months, paired-samples *t* test.

Additional analysis revealed that the CD3⁺CD4⁺ population mainly contributed to this change of CD3⁺ cells. The change from baseline within the CD3⁺CD4⁺ population was 3.5% higher in the placebo group (*P* = .038, adjusted for baseline). No difference in change from baseline was found in the CD3⁺CD8⁺ population, and the percentage of mature B-cell (CD20⁺) subpopulation (*P* = .85 and *P* = .22, respectively, adjusted for baseline).

Further analysis indicated that the CD3⁺ change from baseline for infants with persistent CMA compared with that seen in tolerant infants also was significantly different, being 4.7% higher in the group with persistent CMA (*P* = .032, adjusted for baseline; Fig 4). However, this difference was independent of the probiotic intervention.

Fecal sample analysis

After 6 months of study formula, *B. animalis* was detected in 58% and *L. casei* and *L. paracasei* in 22% of the infants receiving probiotics. In the placebo group the percentages were significantly lower (ie, 5% and 4% respectively; data not shown).

DISCUSSION

We show that supplementation of a combination of CRL431 and Bb-12 to hydrolyzed formula fails to induce additional or accelerated CM tolerance during 12 months of treatment in

infants with CMA. Tolerance for CM developed in the majority of infants in both groups already after 6 months. We did find minor effects of probiotic treatment on lymphocyte subpopulations in peripheral blood, and viable probiotic strains were often detectable in fecal samples of treated infants, confirming that the treatment had actually succeeded in modifying the intestinal microflora. To our knowledge, we are the first to study the effect of probiotic supplementation on established CMA.

There is convincing data that specific probiotic strains can influence immune function through different pathways, including effects on local immune cells and T and B cells. An *in vitro* study has shown that a strain of *L. paracasei* (subspecies *paracasei* B21060) inhibited the proliferation of CD3⁺CD4⁺ from both healthy donors and patients with inflammatory bowel disease.³¹ Although we were unable to detect a probiotic effect on the primary outcome, the flow cytometric data indicate that probiotics did modulate the immune system. The CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, and CD20⁺ lymphocyte percentages before the start of treatment were comparable between both treatment groups and those of earlier studies of healthy infants in the same age range.^{32,33} Twelve months of probiotic supplementation significantly decreased the CD3⁺ and CD3⁺CD4⁺ percentages compared with those seen after placebo. This probiotic effect was most prominent in infants with persistent CMA.

Treatment of allergies with probiotics focuses on early allergic manifestation, such as AEDS. The first studies of probiotic

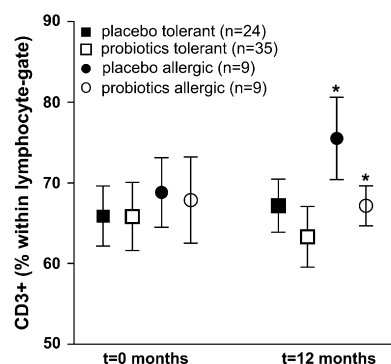


FIG 4. CD3⁺ percentage in lymphocyte gate at 0 and 12 months categorized into intervention and outcome at 12 months. CD3⁺ change from baseline for infants with persistent CMA compared with that seen in tolerant infants was significantly different, being 4.7% higher in the persistent CMA group (* $P = .032$, adjusted for baseline). This difference was independent of the probiotic intervention. Data are expressed as means (95% CIs).

treatment on AEDS^{14,20} have reported a positive effect on the severity of AEDS. Recent and larger trials could not confirm a probiotic effect on the severity of AEDS.^{13,16,34} The results are difficult to compare, mainly because of the different patient populations.

Advantageous effects of probiotics on AEDS and fecal α_1 -antitrypsin/TNF- α were reported by Majamaa and Isolauri,²⁰ whereas Viljanen et al^{21,34} found no effect on AEDS or fecal inflammatory markers in the infants with CMA by using the same probiotic strain (*Lactobacillus* GG). We observed a significant overall improvement in the severity of AEDS, as determined by the SCORAD score. The change from baseline was not different between infants receiving probiotics or placebo. Improvement of AEDS on use of an extensively hydrolyzed formula was earlier described by Brouwer et al.¹³ Hence our findings indicate that these probiotics did not improve AEDS symptoms in infants with established CMA.

Food allergy has been studied in several probiotic trials. Previous studies on probiotic supplementation of infants given a diagnosis of CMA²⁰ or suspected of having CMA^{17,21,34,35} have been reported. Brouwer et al¹³ have performed a CM challenge before intervention with probiotics. However, only 4 of 50 infants had CMA confirmed by DBPCFC. In the suspected CMA studies probiotic intervention took place before the CM challenge. None of these studies specifically addressed the question of whether probiotic supplementation affects the outcome of the CM challenge. These trials focused on AEDS, fecal inflammatory markers, and PBMC cytokine production as outcome measures.

There might be several explanations why probiotics failed in our study. First, the probiotics might have had an insufficient immunomodulatory effect. Indeed, the observed effects were small and unlikely to be clinically relevant. This is corroborated by other prevention studies failing to show clinically relevant effects besides prevention of AEDS.³⁶⁻³⁹ Next, the compliance with the study protocol could have been suboptimal. We think that this is not the case because the study formula was delivered at home on request of the parents. It was reasoned that this would enable us to actively monitor the compliance and facilitate good compliance. The intake of probiotics was actually confirmed by fecal analyses that identified the specific bifidobacteria and lactobacilli strains mainly in the probiotic treatment group.

Finally, lack of efficacy might have resulted from insufficient numbers of the probiotics reaching the mucosal immune cells. In

this respect, recently, a Dutch consumer organization assessed the concentration of viable lactobacilli and bifidobacteria in 28 commercially available probiotic products.⁴⁰ It was established that large differences exist between the tested preparations and that various products did not contain sufficient viable numbers of the probiotic strains to adequately colonize the intestine. In this trial the concentration of the supplemented probiotics was determined monthly. Furthermore, CRL431 and Bb-12 were used in earlier trials in infants and were shown to colonize the gut.^{41,42} The concentrations of used probiotics strains in this trial were estimated at 10^9 colony-forming units/d for both CRL431 and Bb-12. We demonstrated that these concentrations were sufficient for colonization of the gut.

We have found a high cumulative percentage of tolerance in our study group.³ It can be argued that not all the included infants had CMA because we applied an open food challenge and because of subsequent bias at inclusion. This could also explain the relatively low rate of IgE-sensitized infants in our studies compared with those in other published cohorts.⁴³⁻⁴⁵

Based on the complete absence of any effect of the probiotic intervention, we deduce that the major conclusion of our study (no efficacy of probiotic intervention in ongoing CMA) might not have been seriously influenced by this issue. Furthermore, our subgroup analysis in infants with IgE plus CMA suggests that the acquisition of tolerance in the probiotics group appears to be diminished. However, the subgroups are too small for adequate statistical analyses and subsequent conclusions.

In our model positive SPT responses to milk and hen's egg at randomization were predictive for persistent CMA at 6 and 12 months, respectively. These data confirm the report by Saarinen et al,⁴⁶ who established that a positive SPT response to hen's egg at randomization predicts persistent CMA at age 2 years. One could argue that in these children CM rechallenge should be postponed.

What are the clinical consequences of our findings? The results do not support the use of probiotic supplementation of extensively hydrolyzed casein formula with these specific probiotic strains for tertiary prevention in infants with CMA. It is unclear whether other probiotic strains might be more successful. The potential modulatory effect of probiotics should be weighed against the excellent prognosis of CMA with high likelihood of spontaneous tolerance within the first year. Consequently, the effects of any intervention next to the use of hydrolyzed formula are likely to be of limited effect. We conclude that probiotic treatment with CRL431 and Bb-12 to extensively hydrolyzed formula does not accelerate tolerance acquisition in infants with CMA, despite the evidence of intestinal colonization and support for immunomodulatory activity.

Clinical implications: The results do not support supplementation of these specific probiotics to extensively hydrolyzed formulas for tertiary prevention of CMA in infancy. Whether other probiotics might be more successful is unclear.

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APPENDIX

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