Letter to the Editor

Effect of *Lactobacillus* GG on tolerance acquisition in infants with cow's milk allergy: A randomized trial

To the Editor:

The possible effect of probiotics on tolerance acquisition in patients with cow's milk allergy (CMA) is a largely unexplored research area. The only previous study of the effect of probiotic strains (not including *Lactobacillus* GG [LGG]) on tolerance acquisition in children with CMA yielded negative results.[1] Despite this finding and earlier conflicting results on probiotic use in patients with allergic disorders,[2] we asked, given the well-documented link between LGG and the immune system,[3-5] whether supplementation of an extensively hydrolyzed casein formula (EHCF) with LGG could affect tolerance acquisition to cow's milk protein (CMP).

Infants (age, 1-12 months) consecutively referred for strongly suspected CMA but still receiving CMP were invited to participate in the study. Subjects were randomly allocated to one of the 2 groups of dietary interventions: group 1 received EHCF (Nutramigen; Mead Johnson, Rome, Italy), and group 2 received EHCF containing LGG (at least 1.4×10^7 colony-forming units [CFU]/100 mL; Nutramigen LGG, Mead Johnson). When complete and stable remission of CMA was achieved (ie, after 3-4 weeks of exclusion diet), a double-blind, placebo-controlled food challenge (DBPCFC) was planned. Only infants with DBPCFC-proved CMA continued the investigation. After 6 and 12 months, full clinical evaluation, skin prick tests (SPTs), atopy patch tests (APTs), and DBPCFCs were planned, as described in the Methods section and Figs E1 and E2 in this article's Online Repository at www.jacionline.org. Occurrence of adverse events elicited by study formulas was monitored throughout the study. On the first visit, 153 infants were evaluated. Sixty-nine were excluded because of the presence of at least 1 exclusion criterion. CMA was highly suspected in 84 infants; all were invited to participate in the study, 4 refused, and 80 were enrolled and randomly assigned to either group 1 (EHCF) or group 2 (EHCF plus LGG). At the second visit, DBPCFCs were performed in 73 patients (36 in group 1 and 37 in group 2), and a diagnosis of CMA was confirmed in 55 patients. The patients (28 in group 1 and and 27 in group 2) were invited to continue the investigation, and all agreed. The demographic and clinical characteristics of the 2 groups were similar (Table I).

After 6 months of an exclusion diet, a DBPCFC was performed in 55 patients: 22 of 28 patients of group 1 and 11 of 27 patients of group 2 resulted positive. At this time, the rate of full clinical tolerance acquisition was higher in group 2 than in group 1 (Fig 1, *A*). Infants with persisting CMA (22 in group 1 and and 11 in group 2) were rechallenged at 12 months: 13 of 22 patients in group 1 and 5 of 11 patients in group 2 had positive DBPCFC results. All these patients remained on an exclusion diet. Again, the rate of tolerance acquisition was higher in group 2 than in group 1 (Fig 1, *A*). Patients with a negative DBPCFC result at 6 and 12 months were reassessed after 6 months to check the persistence of clinical tolerance to CMP. All subjects consumed regular doses of cow's milk (at least 1 full cup daily) without signs and symptoms related to CMA. Infants accepted the study formulas without problems, and no adverse events were observed.

TABLE I. Baseline main demographic and clinical characteristics of the study population

	Group 1	Group 2	*P* value
No.	28	27	
Male sex, no. (%)	21 (75.0)	16 (59.3)	.214
Age, mo (95% CI)	3.2 (2.1-4.3)	3.9 (2.5-5.2)	.421
Body weight, kg (95% CI)	5.7 (5.1-6.4)	5.8 (4.9-6.7)	.899
IgE-mediated CMA, no. (%)*	12 (42.9)	9 (33.3)	.467
Breast-feeding, no. (%)	23 (82.1)	22 (81.5)	1.0
<2 mo	20 (71.4)	21 (77.7)	.608
Gastrointestinal symptoms, no. (%)	17 (60.7)	19 (70.4)	.452
Vomiting, no. (%)	12 (42.9)	7 (25.9)	.187
Diarrhea, no. (%)	5 (17.9)	11 (40.7)	.062
Cutaneous symptoms, no. (%)	12 (42.9)	12 (44.4)	.906
Atopic dermatitis, no. (%)	12 (42.9)	8 (29.6)	.308
Urticaria, no. (%)	1 (3.6)	4 (14.8)	.193
Respiratory symptoms, no. (%)	6 (21.4)	4 (14.8)	.729

*IgE-mediated CMA was defined by the presence of a clinical history suggestive of IgE-mediated mechanisms (acute onset of symptoms after the ingestion of CMPs), DBPCFC results (occurrence of typical symptoms within 2 hours after the administration of the last dose), occurrence of typical symptoms of IgE-mediated food allergy (vomiting, urticaria, asthma, and rhinitis) during the challenge, and positivity of SPT responses.

Fig 1, *B*, depicts the Kaplan-Meier curve showing the probability of acquiring tolerance at 6 and 12 months in the 2 groups. This Kaplan-Meier curve shows that the infants in group 2 had a higher probability of acquiring tolerance at 6 and 12 months compared with subjects in group 1. Binary regression logistic analysis revealed that the rate of patients acquiring tolerance at 12 months was positively influenced by the presence of gastrointestinal symptoms ($B = 3.34$; odds ratio, 28.4; 95% CI, 1.39-578.88; $P = .03$) and negatively influenced by the IgE-mediated mechanism of CMA ($B = -2.20$; odds ratio, 0.11; 95% CI, 0.01-0.68; $P = .01$).

The SPTs were performed in all study subjects at baseline and during follow-up in infants with IgE-mediated CMA. At the end of the study period, SPT responses were negative in all patients with tolerance acquisition. The total number of patients with positive SPT responses tended to decrease in both groups after 6 and 12 months, although the difference was not significant. Similarly, APTs were performed in all patients at baseline and during the follow-up in infants with non–IgE-mediated CMA. Again, at the end of the study period, APT responses were negative in all patients with tolerance acquisition, and the number of children with positive APT responses decreased after 6 and 12 months, but the difference between the 2 groups was not significant (see Table E1 in this article's Online Repository at www.jacionline.org).

We show that supplementation of EHCF with LGG accelerated the development of tolerance in infants to CMP. All patients consumed regular doses of cow's milk daily without showing any signs or symptoms related to CMA for 6 months after the negative DBPCFC result, which is compatible with the persistence of tolerance. Moreover, SPT and APT responses were negative in all patients who acquired tolerance to cow's milk. It is conceivable that the effect of LGG on acquisition of tolerance to CMP could be related to the immunoregulatory role played by LGG. LGG can balance the generation of cytokines possibly involved in IgE- or

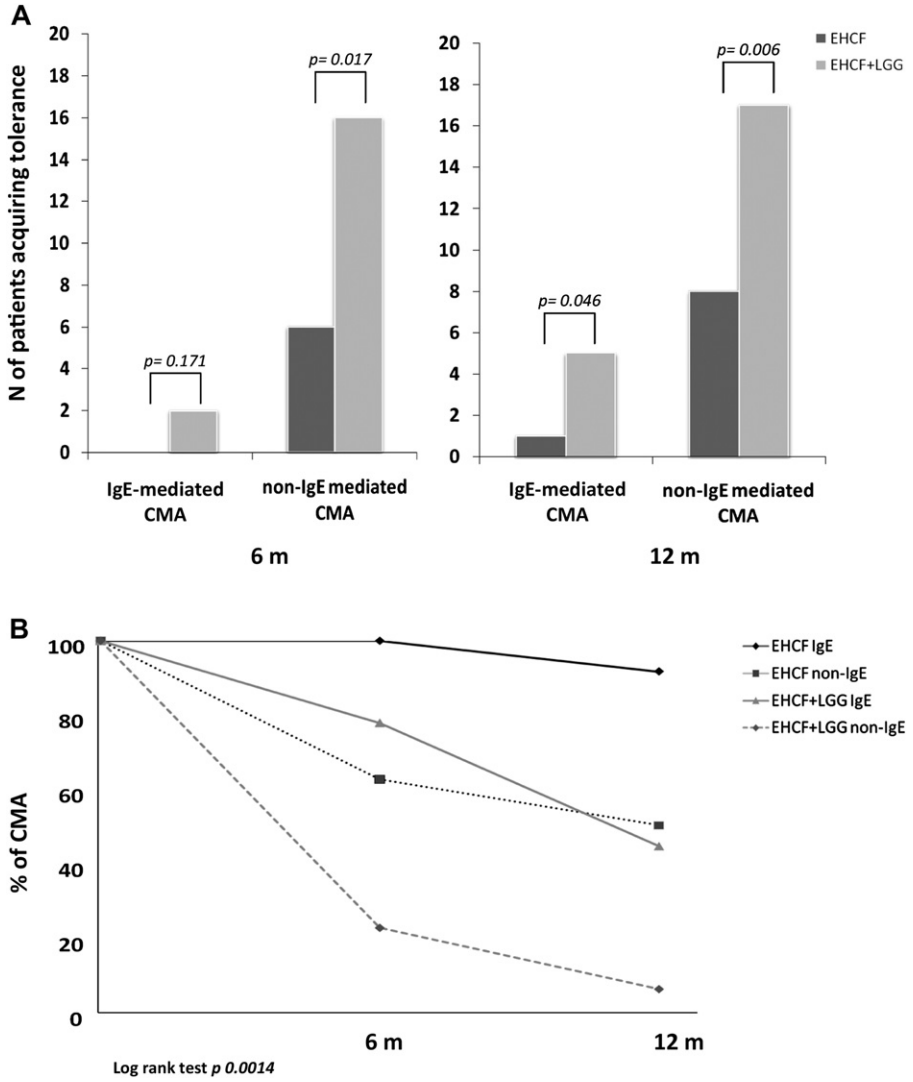

FIG 1. Effect of LGG on tolerance acquisition in infants with CMA. **A,** Number of patients acquiring tolerance at 6 and 12 months in the 2 study groups subdivided according to the CMA mechanism. **B,** Kaplan-Meier curve: probability of persistence of CMA in the study subjects subdivided according to the CMA mechanism and dietary intervention during follow-up.

non–IgE-mediated CMA (ie, IL-4, IL-5, IL-10, IFN-γ, TGF-β, and TNF-α), which can contribute to downregulation of inflammatory processes.[3-5] These effects were strain specific because studies conducted with other *Lactobacillus* species did not yield comparable results.[6] This specificity could be, at least in part, related to the fact that LGG contains 331 strain-specific proteins.[7] Finally, daily supplements of LGG given to infants were reported to induce an increase in a large number of taxa previously associated with a decreased risk for the development of allergy and atopy.[8] Our results suggest that an active exclusion diet (based on LGG supplementation of EHCF), which treats the symptoms of CMA and reduces the time of tolerance acquisition, could be one option to address the changing pattern of the disease characterized by an increasing persistence until later ages.[9]

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METHODS

Trial design

This randomized controlled open trial was conducted from October 2008 to June 2010. The trial was approved by the Ethics Committee of the University of Naples "Federico II" and was registered in the Australian New Zealand Clinical Trials Registry (ID no. ACTRN12610000566033). Infants (1-12 months of age) consecutively referred to the tertiary Pediatric Gastroenterology and Allergology Centre at the University of Naples "Federico II" for suspected CMA were considered eligible for the study. Exclusion criteria were concomitant chronic systemic diseases, congenital cardiac defects, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, celiac disease, cystic fibrosis, metabolic diseases, lactose intolerance, malignancy, chronic pulmonary diseases, malformations of the gastrointestinal tract, suspected eosinophilic esophagitis or eosinophilic enterocolitis, suspected food protein–induced enterocolitis syndrome, suspected CMP-induced anaphylaxis, and still on exclusion diet with one of the 2 study formulas or with another dietary regimen because of CMA.

At the first evaluation (visit 1), the clinical history and baseline clinical conditions were assessed by 3 experienced pediatric allergists. If CMA was strongly suspected by at least 2 of the 3 specialists, the child was invited to participate in the study, and at least 1 parent of each subject provided written informed consent. The parents were asked to avoid administering prebiotic-probiotic therapies to their child during the study.

Randomization and masking

Study subjects were randomly allocated to one of the 2 groups of dietary interventions: group 1 received EHCF (Nutramigen, Mead Johnson), and group 2 received EHCF containing LGG (Nutramigen LGG, Mead Johnson). Both products were commercially available as formula powder and widely used for dietetic treatment of patients with CMA in Italy. The composition of the 2 products was identical except for the presence of LGG; the price was the same, and the packaging was very similar. The physical and organoleptic properties of the 2 formulas were identical.

The computerized randomization allocation schedule was prepared by a biostatistician. The physicians responsible for enrollment of patients allocated the next available number on entry into the trial. At enrollment, the parent of the patient received a closed envelope containing a written prescription for an exclusion diet for CMA (commercial name, dosage, and how to replace the allergen in the diet with alternative food items equivalent in terms of nutrition according to specific daily reference intakes for age and sex), as described by others.[E1] In addition, to ensure the viability of the probiotic, parents were instructed to prepare the formula with water no hotter than 40°C (104°F); temperature was verified by the application of a temperature-sensitive tape provided by the investigators and applied to the exterior of the formula bottle. Because of the problems of conducting a double-blind study of commercially available products, we used the third-part blind observer method to assess the efficacy of LGG. The investigators performing the oral challenges were blinded to the patient's dietary intervention assignment to ensure unbiased efficacy assessment. When complete and stable remission of CMA was achieved (ie, after 3-4 weeks of exclusion diet), a DBPCFC was planned (visit 2). The results of the DBPCFC were assessed by 3 expert pediatric allergists. Infants found not to be affected by CMA at the DBPCFC were withdrawn from the study, whereas infants with DBPCFC-proved CMA continued the investigation. According to the results of the DBPCFC and the outcomes of the diagnostic work-up, subjects were classified as having IgE- or non–IgE-mediated CMA and invited to continue their dietary regimen. After 6 and 12 months of the study formula, subsequent visits (visits 3 and 4) were planned for a full clinical evaluation, SPTs, APTs, and DBPCFC. The results were recorded in a clinical chart. The possible occurrence of adverse events elicited by study formulas was also monitored throughout the study. The study design and the flow of patients throughout the study are depicted in Figs E1 and E2.

Effective probiotic supplementation

According to the manufacturer's specifications, the LGG concentration at release was 2.5×10^7 to 5×10^8 CFU/g, with a guaranteed level of 1.46×10^7 CFU/100 mL (approximately 1×10^6 CFU/g). The presence of LGG in the study formula was checked in 2 randomly selected batches in culture in a Petri dish, as previously described,[E2] and the results showed total counts of 4.5×10^7 CFU/g of powder and 8.5×10^7 CFU/g of powder, respectively. These total counts exceeded the guaranteed level of CFU/100 mL of study formula stated in the manufacturer's specifications.

Study tests

SPTs. Fresh cow's milk containing 3.5% fat was applied to the patient's volar forearm. SPTs were performed with a 1-mm single peak lancet (ALK-Abelló, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/mL) and isotonic saline solution (NaCl 0.9%) as positive and negative controls, respectively. Reactions were recorded on the basis of the largest diameter (in millimeters) of the wheal-and-flare response at 15 minutes. The SPT response was considered positive if the wheal was 3 mm or larger without reaction to the negative control.

APTs. The APTs were performed in study subjects, as previously described.[E3] Briefly, 1 drop (50 μL) of fresh cow's milk containing 3.5% fat was put on filter paper and applied with adhesive tape to the unaffected skin of the child's back with 12-mm aluminum cups (Finn Chambers on Scan pore; Epitest Ltd Oy, Tuusula, Finland). Isotonic saline solution was the negative control. The occlusion time was 48 hours, and results were read 20 minutes and 24 hours after removal of the cups. To exclude false-positive reactions, we also tested allergens in a 1:10 solution. Seventy-two hours after the start of the test, reactions were classified as follows: −, negative; +/−, doubtful, erythema only; +, weakly positive, erythema and slight infiltration; ++, strongly positive, erythema, infiltration, and papules; and +++, very strongly positive, erythema, infiltration, papules, and vesicles. Infants and their families were requested to report any delayed skin reaction that was noticed after this time. Irritant or doubtful reactions, including sharply demarcated confluent erythema, or reactions confined to margins without infiltration were deemed negative.

Food challenge. All food challenges were performed in a DBPCFC manner and took place in the outpatient clinic of the department of pediatrics on 2 separate days with a 1-week interval. Parents of infants taking antihistamines were advised to withhold the treatment for 72 hours before and during the challenge. Randomization and preparation of the challenges were performed by a clinical dietician not directly involved in the procedures. Briefly, every 20 minutes, successive doses (0.1, 0.3, 1, 3, 10, 30, and 100 mL) of fresh pasteurized cow's milk containing 3.5% fat or a standard EHCF (Nutramigen) were administered. Full emergency equipment and drugs (epinephrine, antihistamines, and steroids) were at hand. The results were assessed simultaneously by 3 experienced pediatric allergists. Study subjects 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 at least 2 of the 3 specialists independently scored any item at level 3 or 2 (or more) items at level 2, the test result was considered positive. Clinical symptoms occurring within 2 hours of administering the highest dose were defined as immediate reactions, and those occurring more than 2 hours after the highest dose were defined as delayed reactions. The infants were observed for 2 hours after the final dose and then discharged. In the case of a positive DBPCFC result, at any testing dose, the patient remained under observation until after symptoms resolved. If patients did not show any symptoms within the first 24 hours, parents were advised to administer a single top dose of the tested formula (verum or placebo) to the infant every day at home for 7 days. If any symptoms occurred during this period, the patients returned to the outpatient clinic on the same day. After 7 days of verum or placebo administration, the patients were examined, and the parents were interviewed at the center. To rule out false-negative challenge results, parents were asked to contact the center if any symptoms occurred in the following 7 days after the DBPCFC procedures. The challenge result was considered negative if the patient tolerated the entire challenge, including the observation period. Clinical tolerance acquisition was defined by the presence of a negative DBPCFC result.

Children with negative DBPCFC results were re-evaluated after 6 months to check the persistence of tolerance acquisition.

Sample size

Twenty-five patients in each group were required to obtain a study power of 97% and a type 1 error of 0.05 (2-tailed test), estimating a difference of at least 50% (EHCF = 30% vs EHCF containing LGG = 80%) in the rate of patients acquiring tolerance within 12 months of the exclusion diet. This estimate was based on the results of our retrospective investigation (unpublished data). Considering that patients were enrolled before diagnostic challenge for CMA and a possible dropout, this number was increased to 40 per group.

Statistical methods

The SPSS software package (version 16.0 for Windows; SPSS, Inc, Chicago, Ill) was used for statistical analysis. The Pearson χ^2 test and the Fisher exact test were applied for categorical variables. Tests for equality of means were used to examine continuous variables. For all statistical tests, a 2-tailed *P* value of less than .05 was considered significant. The Kaplan-Meier curve with a log-rank test was used to calculate the probability of acquiring tolerance at 6 and 12 months in the 2 groups separately for patients with IgE-mediated CMA and patients with non—IgE-mediated CMA. Binary regression logistic analysis was conducted to assess the possible influence of the following variables on the primary outcome: sex, age, and body weight at randomization; breast-feeding; symptoms; and IgE-mediated mechanism.

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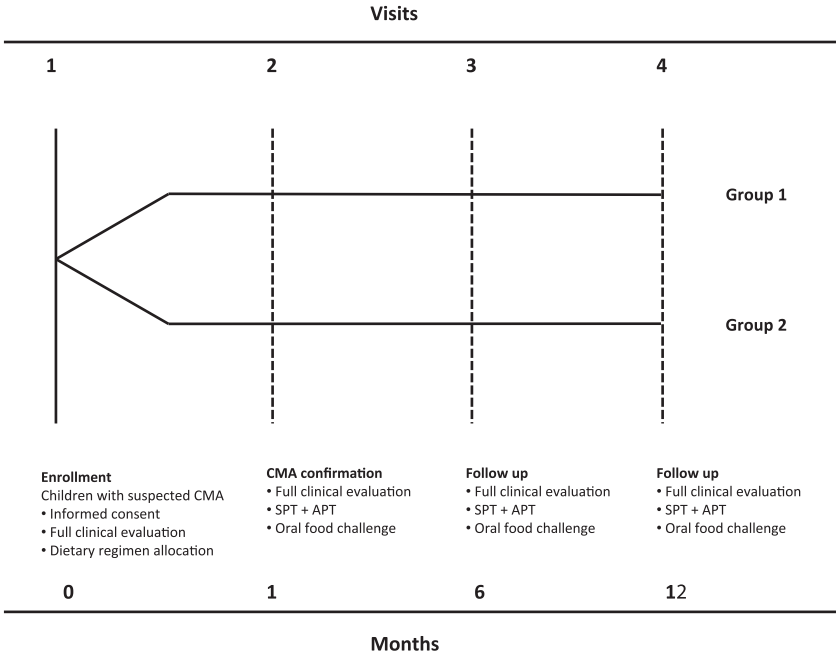

FIG E1. Study design.

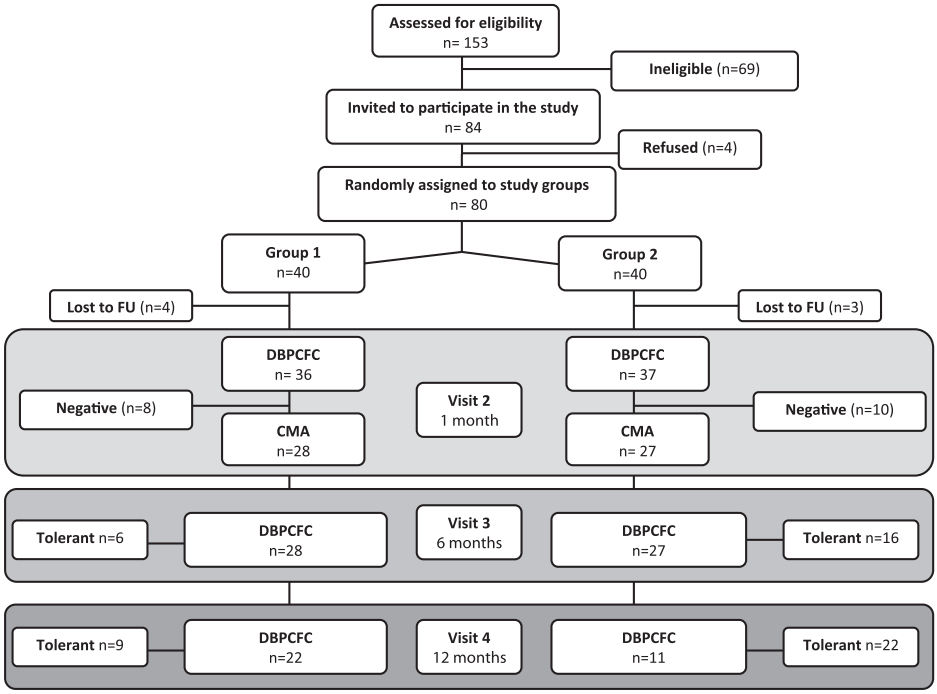

FIG E2. Flow of participants throughout the study. *FU*, Follow-up.

TABLE E1. Results of allergy screening tests during the study

	Group 1 (n = 28)	Group 2 (n = 27)	*P* value
IgE-mediated CMA, no. (%)	12 (42.9)	9 (33.3)	
Positive SPT response for CMP, no. (%)			
Visit 2	12/12 (100)	7/9 (77.8)	.171
Visit 3	12/12 (100)	6/7 (85.7)	.368
Visit 4	9/12 (75.0)	3/6 (50.0)	.344
Non–IgE-mediated CMA, no. (%)	16 (57.1)	18 (66.6)	
Positive APT response for CMP, no. (%)			
Visit 2	10/16 (62.5)	10/18 (55.6)	.681
Visit 3	6/10 (60.0)	2/10 (20.0)	.170
Visit 4	1/6 (16.7)	0/1 (0)	1.0

Allergy screening tests were first performed in all subjects at visit 2, when the diagnosis of CMA was defined, and then at visits 3 and 4 only in patients with positive results at the last follow-up visit. There are no intergroup differences at any time point.