Probiotic *Lactobacillus rhamnosus* GG therapy and microbiological programming in infantile colic: a randomized, controlled trial

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BACKGROUND: Probiotic *Lactobacillus reuteri* and reduced allergen load may lessen the daily crying of colic infants, but the role of *Lactobacillus rhamnosus* GG (LGG) has remained obscure.

METHODS: Infants with colic ($n = 30$) were enrolled during the first 6 wk of life. All families received behavioral support and allergen avoidance diet: breastfeeding mothers followed cow's milk elimination diet and formula-fed infants received extensively hydrolyzed casein formula. The randomized, double-blind intervention employed of LGG 4.5×10^9 cfu/d or placebo for a 4-wk study period. Daily crying was recorded by diaries and parental interviews. Fecal calprotectin and gut microbiota composition by quantitative PCR were evaluated before and after the intervention.

RESULTS: Daily crying time was comparable between the probiotic (173 min) and the placebo group (174 min; $P = 0.99$) at the end of the intervention according to the parental diary. However, parents reported a decrease of 68% (95% confidence interval (CI): 58–78) in daily crying in the probiotic and 49% (95% CI: 32–66) in the placebo group ($P = 0.05$).

CONCLUSION: LGG in infants treated in tandem with behavioral support and a cow's milk elimination diet did not provide additional treatment effect for diary-verified colic crying although parental report of crying suggested the probiotic intervention effective.

One in five families encounter infant colic, defined as excessive and inconsolable crying exceeding 3 h a day on 3 d or more in 1 wk (1,2). The colic period peaks at 6 wk, disappearing gradually around 12 wk of age (3). Maternal emotional distress, anxiety, depression, and even child abuse may ensue, the entire family thus needing support during this stressful period (4–7). Such families should be recognized and ensured early referral for medical evaluation, as excessive crying is an unspecific symptom and might indicate some underlying medical problem (8). Further, with data accumulating to suggest that infant colic is not always benign and transient, but possible associated with subsequent problems such as functional

gastrointestinal disorders, allergic disease, migraine, and psychological disorders (9–11), early recognition and counseling might also prove beneficial.

There are three main approaches to the problem: behavioral support for the family, including professional follow-up of the infant (12); a cow's milk elimination diet either for the breastfeeding mother or as an extensively hydrolyzed casein formula for the infant (13–15); and administration of probiotics (16,17). To date, however, no consensus prevails on the last-mentioned strategy, and no study has combined these different approaches.

To test a comprehensive strategy, we evaluated a double-blind placebo-controlled intervention trial of *Lactobacillus rhamnosus* GG (ATCC 53103, LGG) in the treatment of colic infants.

RESULTS

A total of 30 out of 34 evaluated infants met the inclusion criteria and were equally distributed between the intervention groups (Figure 1). All except one were Caucasian, mean (range) gestational age and birth weight 40 wk (37 to 42) and 3,400 g (2,370–4,425), respectively. Baseline characteristics are shown in Table 1. The 4-wk follow-up was completed by all. There were no study product-related adverse effects.

Before the intervention, the mean (range) daily crying time was 310 min (180 to 720) according to diary data. Crying time was comparable between the probiotic and placebo groups by both parental interviews and diary data (Table 2). All crying data are shown in Table 2 and Figure 2.

The mean (SD) weight gain during the study was comparable between the two groups, 867 g (326) vs. 941 g (308), respectively; $P = 0.54$. Gastrointestinal function (regurgitation and stool frequency) were likewise comparable at enrolment. However, the number of daily regurgitations increased more often in the placebo than in the probiotic group during the follow-up (27 vs. 73% reported an increase, odds ratio (OR): 6.19; 95% confidence interval (CI): 1.36–38.04; $P = 0.04$). Stool frequencies remained comparable (data not shown). Three (10%) infants were admitted to hospital overnight due

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Figure 1. Flow chart of the study.

to parental exhaustion. All skin prick tests were negative. Challenge-proven cow's milk allergy was diagnosed after the study period in one (6.7%) child in the LGG and in three (20.0%) children in the placebo group (OR: 3.50; 95% CI: 0.32–38.23; $P = 0.70$). The intervention had no effect on fecal calprotectin levels (95% CI for group difference: –270 to 270; $P = 1.00$) (data not shown).

There was no statistical difference in gut microbiota composition between infants receiving LGG or placebo either at baseline or after intervention. However, an LGG effect was seen in an increase in different *Bifidobacterium* species. The number of *B. lactis* increased during the study in 47% of infants receiving LGG compared to 13% placebo (OR: 0.18; 95% CI: 0.04–0.77; $P = 0.02$). Again, *Bifidobacterium breve* increased in 40% of infants receiving LGG compared with 13% of those receiving placebo (OR: 0.18; 95% CI: 0.03–1.05; $P = 0.05$). A similar tendency was found in the number of *B. adolescentis* in stools (increase in 27% of probiotic recipients and in 7% in the placebo group; OR: 0.16; 95% CI: 0.02–1.59; $P = 0.10$). In general, *Bifidobacterium* brought out a distinction between colic infants and healthy controls, the median number of *Bifidobacterium*

genus in stools being lower in the former (1.1^9 vs. 4.6^{10} respectively; $P = 0.04$). Moreover, colic infants tended to be less frequently colonized with *B. breve* than healthy infants at baseline (13 vs. 50%, respectively; OR: 3.77; 95% CI: 1.04–13.65; $P = 0.07$), and at close of study despite the intervention (27 vs. 64%, respectively; OR: 1.52; 95% CI: 0.67–3.44; $P = 0.06$).

DISCUSSION

Based on parental diaries on the duration of crying, colic infants do not benefit from LGG in tandem with behavioral counseling and cow's milk elimination. However, parents reported in their interviews that daily crying decreased more in infants receiving LGG compared to placebo and, the number of cry days per week was lower and number of responders higher in the LGG group in comparison to placebo.

Two previous randomized double-blind placebo-controlled studies using *Lactobacillus reuteri* (DSM 17938) have shown a therapeutic effect (16,17), while a mixture of different probiotics (*L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99, *Propionibacterium freudenreichii* ssp, and *Shermanii* JS or *L. rhamnosus* and *Bifidobacterium infantis*) has not proved

successful (18,19). Of note, our design differed from earlier setups in several respects, in addition to the difference in probiotic strain. First, we enrolled only infants younger than 6 wk to coincide the maximum crying time in colic, while previous investigators enrolled infants up to 16 or 20 wk of age (16,17), a time span when crying might be of different etiology (12). Secondly, parents were referred to the study or independently sought participation if they perceived their infant's crying as colic. In previous studies, the colic diagnosis was based solely on daily crying amount (16,17). Thirdly, in addition to cow's milk elimination, behavioral counseling and lactation support were offered to all. This is thus the first study in which all potentially effective strategies against colic (13–16) were

equally in use in order to assess a specific benefit, if any, from LGG intervention.

Two different means of evaluating the effect of probiotics in infant crying were applied: a validated, prospective detailed cry diary and an interview on parental recall regarding crying during the previous week. The diary was assumed to be objective measuring the quantity of crying while parental recall may include some qualitative features of crying not captured by a diary affecting subjective experience and thus reflecting the real relevance of crying to the parents. The inclusion of this subjective perspective presumably adds a wider dimension to the potential effect of the intervention. Interestingly, the effect was not evident in diary data but only in parental interview. The discrepancy remains unexplained. In any case, parents' subjective perception of infant crying and of a potential effect of any colic intervention is clinically significant since it is parents' perception of colic which leads to referrals.

We regard good compliance as another strength of the study: all families attended the follow-up and returned stool samples as scheduled. In accord with earlier studies, we found the number of total bifidobacteria in stool samples to be lower in colic infants compared to the reference population (20,21). We chose as reference a population presumably representing a model of infant feeding and health: a vaginally delivered breastfed child free of signs and symptoms of disease in follow-up. In contrast to our earlier cohort, *B. breve* was not associated with excessive crying, this underlining the complexity of infant gut microbiota composition during its maturation process and the intricacy of any concept of normal colonization (21).

Previous probiotic intervention studies in colic infants report increased lactobacilli and bifidobacteria, but a decrease in *Escherichia coli* (16,18). However, only one study could associate these alterations with a reduction in crying (16). The present intervention promoted an increase in *B. lactis* numbers. One possible mechanism in the impact of probiotics on infant colic is regulation of gut inflammation possibly induced by aberrant microbiota or sensitization to specific food antigens. Probiotics may degrade antigens to more tolerogenic form and thus balance the generation of pro- and anti-inflammatory cytokines (22). Preliminary data suggest that specific probiotics may promote oral tolerance induction (23). This might open up new insights into the long-term sequelae of infant colic as well as other gut-associated disorders in which a microbial etiology is beginning to emerge.

Table 1. Baseline characteristics of the participants

	LGG (n = 15)	Placebo (n = 15)
Mother		
University education	8 (53)	7 (47)
Depression	1 (7)	1 (7)
Smoking	3 (20)	0 (0)
Probiotic use during pregnancy	3 (20)	7 (47)
Infant		
Siblings	4 (27)	5 (33)
Family history of atopy	12 (80)	12 (80)
Perinatal antibiotic exposure	9 (60)	10 (67)
Vaginal delivery	13 (87)	12 (80)
Male	7 (47)	5 (33)
Birth weight (g)	3490 (430)	3320 (520)
Birth length (cm)	51.2 (2.0)	50.3 (2.2)
Age at entry (days)	38.0 (10.8)	34.8 (9.9)
Exclusive breastfeeding	6 (40)	5 (33)
Partly breastfeeding	7 (47)	8 (53)
Exclusively formula feeding	2 (13)	2 (13)
Earlier exposure to probiotics	11 (73)	8 (53)
LGG	4 (27)	3 (20)
Lactobacillus reuteri	7 (47)	5 (33)

Results are given as mean (SD) or as number (%) of subjects. All the characteristics were comparable between the study groups ($P > 0.05$). Comparisons were made using chi-square test, Fisher's exact test, or independent samples *t*-test as appropriate. LGG, *Lactobacillus rhamnosus* GG.

Table 2. The crying characteristics of the infants in the placebo and probiotic groups

		Mean daily crying time (SD)		Mean daily crying time decrease % (95% CI)	Number of responders (%)
		Day 0	Day 28		
Placebo	Interview (n = 15)	262 (82)	138 (108)*	49 (32–66)**	7 (47) [†]
	Diary ^a	332 (126)	174 (94)	32 (12–53)	4 (31) [‡]
LGG	Interview (n = 15)	236 (112)	70 (36)*	68 (58–78)**	13 (87) [†]
	Diary ^b	295 (116)	173 (64)	17 (2–33)	0 (0) [‡]

^an = 15 on day 0; n = 13 on day 28. ^bn = 15 on day 0; n = 14 on day 28. * $P = 0.03$; ** $P = 0.0495$; [†] $P = 0.02$; [‡] $P = 0.04$. All the other characteristics were comparable between the study groups ($P > 0.05$). Comparisons were made using chi-square test, Fisher's exact test or independent samples *t*-test as appropriate. CI, confidence interval; LGG, *Lactobacillus rhamnosus* GG.

Figure 2. The number of days per week the infant cried for more than 3 h/d in the LGG ($n = 15$) and placebo groups ($n = 15$) as reported by the parental interview on day 0 and day 28. At day 28 the number of cry days was significantly higher in placebo group compared to LGG group ($p=0.02$). Every box represents an infant. Comparisons were made using independent samples t -test. LGG, *Lactobacillus rhamnosus* GG.

In conclusion, we demonstrated that LGG supplementation in tandem with behavioral and nutritional counsel did not provide additional treatment effect for diary-verified colic crying.

METHODS

Study Objectives and Outcomes

Difference in daily average crying time between the LGG and placebo groups was selected as the primary outcome of the study. Daily crying time, number of responders (i.e., the infants whose crying time decreased at least the half), number of days the infant cried for more than 3 h (defined as cry days), and change in fecal calprotectin level were all selected as secondary outcomes. Comparison of gut microbiota both between the intervention groups and between colic infants and healthy reference population were other secondary outcomes. Mean weight gain, number of daily regurgitations, stool frequency, and allergic sensitization were also evaluated.

Study Population

Colic infants participating in this randomized, double-blind, placebo-controlled study were recruited in South-West Finland between February 2011 and January 2013. Advance information was given to families in maternity wards, well-baby clinics, and pediatric outpatient clinics in Turku University Hospital. Families interested contacted a research nurse. For enrolment, infants had to be full-term, under 6 wk of age exhibiting paroxysmal, unsoothable crying raising concern in the parents, crying at least 3 h per day on 3 d per week. Before the first study visit, parents were instructed to complete a diary for 3 d to assess the infant for eligibility (24). The infant was eligible if the diary recorded crying lasting least 180 min per day during the last 3 d. Infants with diagnosed chronic diseases were excluded. The reference population comprised 11 matched controls from an on-going clinical trial with accurate prospective monitoring of potential and relevant confounding factors concerning gut microbiota (feeding type, mode of delivery, and use of probiotics and antibiotics) and recording of the daily crying (25,26). The infants of the reference population were healthy, breastfed infants born in the same hospital by vaginal delivery at term and not exposed to antibiotics or probiotics during the neonatal period, cried less than 180 min per day, and provided fecal samples at 3 ($n = 6$) and/or 12 wk ($n = 11$) of age (25,26).

Study Design

All families received counseling comprising general information on infant colic, supportive discussions with study personnel, and free access to nurse and physician, together with the possibility of an overnight hospital stay in the case of parental exhaustion. During the study visit, breastfeeding and bottle feeding techniques were evaluated by a certified consultant (Ulla-Maija Eriksson), and proper feeding techniques inculcated on request.

At entry, skin prick tests were performed to study IgE-mediated sensitization to common food allergens (cow's milk, raw hen's egg white, and wheat) as previously described (27). To assess the impact of food allergy on colic as defined in Finnish guidelines, all breastfeeding mothers were asked to avoid products containing cow's milk and egg, and formula-fed infants received extensively hydrolyzed casein formula (Nutramigen; Mead-Johnson, Evansville, IN) during the study period. Dietary adherence was confirmed by interview during follow-up visits. At close of the study, breastfeeding mothers resumed their normal diet and standard formula was re-introduced for formula-fed infants. In the case of suspected cow's milk allergy, a formal challenge was implemented at the Department of Pediatrics, Turku University Hospital.

The infants received orally probiotic LGG (Mead-Johnson, Evansville, IN) 4.5×10^9 cfu/d or placebo (microcrystalline cellulose; Tamro, Vantaa, Finland) for 28 d (26,28,29). Breastfed infants received the product from closed sachets identical in appearance, taste, and smell. Parents were taught to open the sachets and mix the products immediately before administration in ~10 ml of breast milk given by spoon or bottle once a day. Formula-fed infants received LGG or placebo mixed with extensively hydrolyzed casein formula (Nutramigen-LGG or Nutramigen; Mead-Johnson). Nutramigen-LGG contains LGG 5.4×10^7 cfu/g of the product. In both cases, breastfed and formula-fed infants received a daily dose of 4.5×10^9 cfu of LGG, achieved by an appropriate daily amount of sachets, formula, or combination.

Parents were instructed to inspect the product for signs of degradation before use and keep the sachets refrigerated and formula at room temperature. The LGG and placebo sachets were prepared by the study nurses in the University laboratory, and the viability of the probiotic was confirmed by regular blinded analysis in the Functional Foods Forum, University of Turku.

Randomization

Participants were randomly assigned by an independent statistician (J.M.) to one or other study group using the random allocation rule. Study personnel, hospital staff, and parents were blinded to the randomization. To ensure allocation concealment, a study group member not involved in the conduct or reporting of the study was responsible for packaging and labeling the products. The study nurse allocated the next available product on entry into the trial, and each patient received the product directly from the study clinic. The code was revealed to the investigator once recruitment, data collection, and analysis were completed. Data were collected on printed case record forms, and group members entered the data. All data were kept confidential.

Assessment of Infant Crying

To confirm eligibility, parents recorded their infants' behavior (sleeping, awake and content, fussing, crying, feeding) on three consecutive days prior to the intervention, using the validated Baby Day Diary (24). The 24-h day was divided into 5-min sections, and parents filled in the diary as convenient, usually during feeding. Instructions on record-keeping were given by the same research nurse (Ulla-Maija Eriksson). Recording was not completely successful in five cases by close of intervention: the diary not being returned in three cases and in two only 1 d being covered. Daily crying was estimated by parental interviews at entry and close of intervention. Crying was defined as "continuous crying" and fussing as "noncontinuous negative vocalization." Total distress included both. In interview, parents reported the infant's behavioral patterns, including sleeping patterns, crying (daily crying time in minutes, number of days per week on which crying extends more than 3 h), feeding, regurgitation (at enrolment: number of regurgitations per day; on days 7 and 28: an increase, no change, a decrease compared to study entry), vomits,

stools (consistency normal, loose, firm or hard, and frequency as 1/wk, 2–4/wk, 1–2/d, 3–4/d, >4/d), infections and other diseases, use of medications and parental coping. Specific items were administration and potential adverse effects of the study product. Family history of allergic and other diseases, parents' smoking, mother's education, mother's medication and use of probiotics during pregnancy and lactation, mother's diet, infant's gestational age, mode of delivery, birth characteristics, and earlier exposure to probiotics and medication were asked in interview at enrolment.

After the enrolment day (day 0), follow-up consultations were conducted by the same study nurse and physician on days 7 and 28, and when necessary during the study. A medical examination was made, and weight, length, and head circumference measured and growth charts drawn at each visit.

Fecal Sample Collection and Analysis

Fecal samples were collected from diapers on day 0 and 28, immediately frozen to -20°C and delivered within 24 h to the study clinic, where they were stored at -70°C until analyzed.

Calprotectin Assay

Calprotectin was analyzed using the CALPROLAB Calprotectin ELISA (ALP) kit (CALP0170, Calpro AS, Lysaker, Norway) according to manufacturer's instructions.

Sample preparation used the weighting method as instructed. Supernatants from the fecal sample extraction procedures were diluted 1:100 and analyzed using enzyme-linked immunosorbent assay.

DNA Isolation and Quantitative PCR Assay

Samples were pretreated and DNA extracted using the InviMag Stool DNA kit (Strattec Molecular, Berlin, Germany) and the automated KingFisher DNA extraction system (Thermo Fisher Scientific Oy, Vantaa, Finland), as previously described (30). DNA samples were stored at -20°C until analyzed. Quantitative PCRs were conducted as described elsewhere (31,32). For quantitative PCR, the standard DNA was prepared as previously (30). Detection and PCR amplification were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, CA).

Statistics

On the basis of a recent colic study with a probiotic intervention (16), sample size was calculated to find a difference of 55 min in daily average crying time between the groups. With 5% probability of type I error and 10% probability of type II error, and an estimated within-group SD of 45 min, 15 patients per group were needed.

Changes in the amount of microbiota between the two measurement points were categorized as any increase, no change, and any decrease, and the two groups compared using chi-square test for trend. Comparisons between two nominal variables were by chi-square or Fisher's exact test as appropriate. Microbiota values were compared using Mann–Whitney *U*-test, other continuous variables using independent samples *t*-test. The gut microbiota of the colic infants at enrolment and end of intervention was compared with controls at the age of 3 and 12 wk, respectively. Statistical analyses were made using SAS for Windows, version 9.2. *P* values below 0.05 were considered statistically significant.

Ethics Statement

The study was approved by the Ethics Committee of the Hospital District of South-West Finland. Written informed consent was obtained from the parents of the infants. The trial was registered at ClinicalTrials.gov (<http://www.clinicaltrials.gov/ct/gui/show/NCT00167700>).

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