ORIGINAL ARTICLES

Crying Time and RORγ/FOXP3 Expression in *Lactobacillus reuteri DSM17938*-Treated Infants with Colic: A Randomized Trial

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Objectives To evaluate crying time, retinoid-related orphan receptor-γ (RORγ) and forkhead box P3 (FOXP3) messenger RNA levels (transcription factors that can modulate T cell responses to gut microbes), and to investigate gut microbiota and fecal calprotectin in infants treated with *Lactobacillus reuteri* for infantile colic.

Study design A double-blind, placebo-controlled randomized trial was conducted in primary care in Torino from August 1, 2015 to September 30, 2016. Patients suffering from infantile colic were randomly assigned to receive daily oral *L reuteri* (1×10^8 colony forming unit) or placebo for 1 month. Daily crying times were recorded in a structured diary. FOXP3 and RORγ messenger RNA in the peripheral blood was assessed with real-time TaqMan reverse transcription polymerase chain reaction. Gut microbiota and fecal calprotectin were evaluated.

Results After infants with colic were supplemented with *L reuteri* DSM 17938 for 30 days, crying times were significantly shorter among infants with colic in the probiotic group compared with infants in the placebo group (74.67 ± 25.04 [IQR = 79] minutes /day vs 147.85 [IQR = 135] minutes /day [P = .001]). The FOXP3 concentration increased significantly (P = .009), resulting in decreased RORγ/FOXP3 ratios: 0.61 (IQR = 0.60) at day 0 and 0.48 (IQR = 0.28) at day 30 (P = .028). Furthermore, the probiotic increased the percentage of *Lactobacillus* (P = .049) and decreased fecal calprotectin (P = .0001).

Conclusions Infants with colic treated with *L reuteri* for 30 days had a significantly decreased crying time and an increased FOXP3 concentration, resulting in a decreased RORγ/FOXP3 ratio. The treatment reduced fecal calprotectin. *(J Pediatr 2018;192:171-7).*

Trial registration ClinicalTrials.gov: NCT00893711.

Infantile colic is defined as crying or fussing for more than 3 hours per day, more than 3 days per week, without obvious cause, and cannot be prevented or resolved by caregivers.[1] Although colic is considered a self-limiting and benign disorder, it represents 1 of the most common problems in the first 4 months of life.[2]

The etiology of colic is still not completely defined but is likely multifactorial.[2] One theory hypothesizes that an important factor is the immaturity of the infant's nervous or digestive system. Other proposed factors include allergy to cow's milk proteins, intestinal hypermotility secondary to an imbalance of the autonomic nervous system, and hormonal changes, particularly in the levels of motilin and ghrelin.[3,4] A new hypothesis proposed that immature hepatic synthesis leads to a reduction in the intraluminal bile acids levels and malabsorption with potential effects on gut microflora.[5]

Growing evidence in the literature has linked the gut microbiota and colic in infancy.[6,7] In infants with colic, high levels of *Escherichia coli*,[8] *Clostridium difficile*, and *Klebsiella*[9] and low levels of *Lactobacillus*[10] and *Bifidobacterium* have been observed.[11] This dysbiosis could be a possible cause of abnormal gut motility and increased gas production.

How the microbiota influence the immune system and the development of immune-mediated diseases late in life is not yet clear, but studies based on murine models showed that transcription factors such as retinoid-related orphan receptor-γ (RORγ) and forkhead box P3 (FOXP3) induce T helper (Th) 17 and regulatory T cell (Treg) populations that resided in colon and modulated responses to gut microbes.[12] The relationship between the gut microbiota and the immune system has been investigated, particularly in inflammatory bowel disease.[13] Pärtty et al[14] reported increased serum concentrations of various cytokines (such as interleukin [IL]-8, monocyte chemoattracant protein-a, tumor necrosis factor, macrophage inflammatory protein-1, and tumor necrosis factor-α) in infantile colic. In this context, we must consider that Th17 and regulatory T (Treg) cells have mutually antagonistic functions. Th17

FOXP3	Forkhead box P3
IL	Interleukin
mRNA	Messenger RNA
PCR	Polymerase chain reaction
RORγ	Retinoid-related orphan receptor-γ
TaqMan RT-PCR	TaqMan reverse-transcription PCR
Th	T helper cell
Treg	Regulatory T cell

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lymphocytes are involved in inflammatory and autoimmune processes, whereas Treg lymphocytes play critical roles in the maintenance of self-tolerance and the prevention of allergic and inflammatory reactions.[15]

Fecal calprotectin, which is a protein present in neutrophils and monocytes, has bactericidal, bacteriostatic, and fungicidal activities. The fecal calprotectin concentration is primarily used in gastroenterology as a marker for inflammatory bowel conditions.[16] Rhoads et al[9] observed increased fecal calprotectin levels in infants with colic.

Lactobacillus reuteri is a probiotic species with antimicrobial activity[16]; the DSM 17938 strain reduces pain perception via the transient receptor potential vanilloid 1 channel,[17] influences the activity of the potassium-dependent calcium channel intermediate konductance in enteric neurons, and serves as a tonic inhibitor of the contractility of the colon.[18,19] Moreover, some studies thus far conducted only in vitro or in animal models, have also demonstrated an anti-inflammatory effect of *L reuteri*, which could at least partly explain the clinical effect.[20-22] Despite growing data confirming the clinical efficacy of *L reuteri* on colic in breastfed infants,[23-25] the role of this bacteria remains under investigation.

In this double-blind, placebo-controlled clinical trial, we investigated whether supplementation with the probiotic *L reuteri* DSM 17938 for 1 month could reduce the crying time and modify the RORγ/FOXP3 expression gut microbiota, or fecal calprotectin level in a group of infants with colic.

Methods

This was a double-blind, randomized placebo-controlled trial (ClinicalTrials.gov: NCT00893711).

One hundred eighty infants were assessed for eligibility; 93 infants were excluded because they did not fulfill the inclusion criteria (n = 84) or the parents refused to participate (n = 9) (**Figure 1**; available at www.jpeds.com).

Infants younger than 2 months of age seen during outpatient control at the Newborn Unit at our Department of Pediatrics (Regina Margherita Children Hospital, Turin, Italy) were enrolled. The infants underwent blood tests during routine outpatient examinations.

We enrolled healthy term infants who were adequate for their gestational age (birth weight: 2500-4000 g), exclusively or predominantly fed breast milk, and less than 12 weeks of age. We excluded infants who weighed <2500 g at birth, had major medical problems, had an allergy to cow's milk protein, had gastroenteritis, or were taking antibiotics or any type of probiotics at the start of the trial. We also excluded breastfed infants whose mothers took probiotics. We used the modified Wessel criteria for the diagnosis of infantile colic.[1]

Infants without colic (control group, n = 27) were enrolled at our Department of Pediatrics (Regina Margherita Children Hospital, Turin, Italy) at the time of planned routine examinations at the outpatient ambulatory section of the Newborn Unit. Parents provided written consent for their infants to be included in the study.

The blood sample was collected only when the infant was subjected to blood sampling for other clinical reasons, and this was only possible in 16 of 27 subjects.

The study was conducted in accordance with the protocol, International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines, applicable regulations and guidelines governing clinical study conduct, and the ethical principles that have their origin in the Declaration of Helsinki. The study protocol was approved by the local ethics committee (Comitato Interaziendale AA.SS.OO. O.I.R.M./S. Anna-Ordine Mauriziano di Torino, n. 4698).

The parents of the enrolled infants were informed about the purpose, benefits, and possible risks of the study. Written informed consent was obtained.

The treatment included 5 drops of *L reuteri* DSM 17938 (1×10^8 colony-forming units per drop) in an oil suspension for 1 month. The placebo was based on maltodextrin in the same oil suspension with the same appearance, color, and taste as the treatment and was identically packaged and stored. The *L reuteri* dosage was the same dosage used in our previous trials, which showed effective management of colic.

Randomization, Allocation Concealment, Blinding

Infants with colic were randomly assigned to receive *L reuteri* DSM 17938 or placebo using a computer-generated randomization list created by an independent statistician. Randomization was stratified using the random-digit method based on computer-generated numbers. A statistician prepared the computer-generated randomization schedule using a 2-treatment randomization scheme with random blocks of varying size (Stata 9, Ralloc procedure; Stata Corp, College Station, Texas). The trial was double-blind, and treatment allocation was concealed from all study investigators and participants at every phase, including the measurement of outcomes. A hospital pharmacist assigned a study identification number to each enrolled infant and dispensed the study product according to the randomization schedule.

The primary outcome was evaluated based on changes in the crying time per day in a group of infants with colic at the time of allocation and after supplementation for 30 days with *L reuteri* DSM17938 or the placebo.

The secondary outcomes were to determine the RORγ (Th17 cell marker) and FOXP3 (Treg marker) messenger RNA (mRNA) expression levels and their ratio and to evaluate the gut microbiota and the value of fecal calprotectin after administration of the probiotic or the placebo. Daily crying and fussing times were recorded in a structured diary, and maternal questionnaires were completed to monitor the changes in infant colic symptoms and adverse events.

Finally, we compared the Th17/Treg balance, daily crying time, indicators of the microbiota and fecal calprotectin values in infants with colic and in a control group of healthy children.

At recruitment (day 0), each child was visited, and the parents were interviewed to complete a medical history card with personal data. After the visit, we collected 2 fecal samples and a tube of peripheral blood in a routine manner.

The children were divided into 2 groups based on the clinical examination and the Wessel criteria as follows: infants with colic (n = 60) and healthy control infants (n = 27). The parents were asked to complete a cry and fussing diary in which they recorded the frequency of colic episodes and the daily crying and fussing time (in minutes).[26]

In the group of infants with colic, 32 infants received supplementation with *L reuteri* DSM 17938, and the remaining 28 infants were given the placebo. At the follow-up visit at 1 month (day 30), 2 fecal samples and a blood sample (for routine analysis) were collected from each infant. We also collected the diaries and delivered a questionnaire to the parents on the degree of satisfaction.[27,28] Six children in the placebo group were lost to follow-up.

The control group (n = 27) was recruited among those hospitalized for episodes of apnea, apparent life-threatening event, congenital hypothyroidism, and mild infections of the high respiratory tract. There were no financial reimbursements or incentives. The blood sample was collected only when the baby was subjected to blood sampling for other clinical reasons, and this was only possible in 16 of 27 children (**Figure 1**).

Fecal samples were collected on the first day of hospitalization (before receiving any medication) and at the control visit after 30 days and stored in 2 sterile Eppendorf tubes (Eppendorf, Milan, Italy). One of these tubes was immediately subjected to a fecal calprotectin assay, and the second sample was stored in a freezer at -80°C until assessment of the gut microbiota. The same day, a tube of hemochrome, eventually recorded during hospitalization and during follow-up visit, was stored in a freezer at -80°C.

Total RNA was extracted from 200 µL of blood as previously described by Bergallo et al.[29]

Relative quantification of the mRNA expression levels of the selected genes was achieved using TaqMan amplification and normalization to the reference gene glyceraldehyde-3-phosphate dehydrogenase with the ABI PRISM 7500 real-time system (Life Technologies, Carlsbad, CA). The RoRγ, FOXP3, and glyceraldehyde-3-phosphate dehydrogenase expression levels were quantified by real-time polymerase chain reaction (PCR) as previously described by Mareschi.[30]

The Th17/Treg ratio was evaluated based on the RoRγ/FOXP3 mRNA levels.

Before DNA extraction, stools were diluted 1/10 and vortexed. Then, DNA was extracted as follows: 400 µL of supernatant was added to 400 µL of lysis buffer (400 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM EDTA, 1% sodium odecyl sulfate), incubated at room temperature for 90 minutes, and then centrifuged at 14 900 × g for 5 minutes. The supernatant was mixed with an equal amount of phenol–chloroform and centrifuged at 14 900 × g for 10 minutes. The supernatant was then mixed with an equal amount of isopropanol and incubated at -80°C for 30 min for precipitation. After centrifugation at 14 900 × g for 5 minutes, the pellet was washed with 70% ethanol, centrifuged at 14 900 × g for 5 minutes, dried, and resuspended in 20 µL of ultrapure H_2O.[29]

E coli ATCC 25922, *Lactobacillus plantarum* ATCC 14917, and *Bifidobacterium animalis spp lactis* BLC1 were provided by PBI International (Milan, Italy). All bacteria were grown in Luria Bertani broth overnight at 37°C in a shaking incubator.

Purified DNA from *E coli* strain ATCC 25922, L ATCC 14917, *B animalis* spp lactis BLC1, *Enterococcus faecalis* ATCC 29212, *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 14579, *Klebsiella pneumoniae* ATCC 43816, *Clostridium perfringens* ATCC 13124, and *Pseudomonas aeruginosa* ATCC 27853 (PBI International S.P.A., Milano, Italy) was used for assay optimization. DNA extractions were quantified using a NanoDrop Lite spectrophotometer Thermo Scientific (Paisley, United Kingdom) and were diluted to the desired concentrations prior to each experiment. Aliquots of a 100 ng/µL stock were diluted fresh before each experiment in PCR-grade water to minimize the chances of degradation at the attogram levels used. Based on Avogadro's number and genome size, we created tenfold dilutions from 10^9 to 10^{-1} genomes/µL.

The primers and probe for TaqMan PCR were designed for *E coli* based on the alignment of 4 GenBank uidA sequences (accession numbers U00096, AE005385, AE016761, and AF305917). The primers and probe for *Bifidobacterium spp* were previously described by Penders et al.[31] Designed primers coliF (5′-GCAAGGTGCACGGGAATATT-3′), coliR (5′-CAGGTGATCGGACGCGT-3′) and coliProbe (5′-FAM-CGCCACTGGCGGAAGCAACG-TAMRA-3′), and the primers bifidoF (5′-GCGTGCTTAACACATGCAAGTC-TAMRA-3′), bifidoR (5′-CACCCGTTTCCAGGAGCTATT-3′) and bifidoProbe (5′-FAM-TCACGCATTACTCACCCGTTCGCC TAMRA-3) were used at 500 nM with 12.5 µL of 2 × TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, California), 250 nM of probe, and 5 µL of DNA template in real-time PCR mixtures (25 µL). Thermal cycling was performed using the ABI PRISM 7500 detection system (Applied Biosystems) as follows: 2 minutes at 50°C for the uracil-N-glycosylase reaction, 10 minutes at 95°C for denaturation, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

The primers and probe for TaqMan PCR were previously described by Mareschi et al.[30] Designed primers lattoF (5′-TGGAAACAGATGCTAATACCG-3′), lattoR (5′- CGTCCATTG TGGTAGATTCCCT-3′), and lattoProbe (5′-FAM- CTG AGACACGGCCCAWACTCCTACGG-TAMRA-3′) were used at 900 nM with 12.5 µL of 2 × TaqMan goTAQ qPCR Mastermix (Promega; Milano Italy), 200 nM of probe, and 5 µL of DNA template in real-time PCR mixtures (25 µL). Thermal cycling was performed using the ABI PRISM 7500 detection system (Applied Biosystems) as follows: 2 minutes at 50°C for the uracil-N-glycosylase reaction, 2 minutes at 95°C for denaturation, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

The specificity of each TaqMan PCR set was verified in silico with a basic local alignment search tool (BLAST) homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and using DNA from different fecal bacteria. The BLAST analysis revealed that in each TaqMan PCR, only the DNA of the respective species that was homologous was amplified and that the testing of the DNA templates of fecal bacteria confirmed the specificity.

For the construction of the quantification standards, 10-fold dilutions from 10^9 to 10^{-1} genomes/μL of purified DNA from *E coli, L plantarum*, and *B animalis* were used. All values were measured in duplicate, and linearity was reproduced in a second run.

The primer pairs and the TaqMan probe for conserved bacterial 16S ribosomal RNA genes and PCR conditions were described elsewhere.[29] Designed primers (Bac349F) GGCAGCAGTDRGGAAT and (Bac806R) GGACTACYV GGGTATCTAAT and the TaqMan probe FAM-TGCCAGC AGCCGCGGTAATACRDAG-TAMRA were used at 400 nM with 12.5 μL of 2 × TaqMan goTAQ qPCR Mastermix (Promega), 200 nM of probe, and 5 μL of DNA template in real-time PCR mixtures (25 μL). Thermal cycling was performed using the ABI PRISM 7500 detection system (Applied Biosystems) as follows: 2 minutes at 50°C for the uracil-N-glycosylase reaction, 2 minutes at 95°C for denaturation, followed by 10 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, followed by 35 cycles of 15 seconds at 95°C and 1 minute at 60°C. For the construction of the quantification standards, tenfold dilutions from 10^{10} to 10^2 genomes/μL of the purified DNA from the *E coli* strain, *L plantarum, B animalis, E faecalis, H influenzae, S aureus, B cereus, K pneumoniae, C perfringens,* and *P aeruginosa* were used. The percentage of a single bacterial species was estimated based on the ratio between a specific PCR count and a broad range count.

To determine the fecal calprotectin concentration, we used our department's rapid test (the Bühlmann Quantum Blue Calprotectin High Range; Bühlmann Laboratories, Schönenbuch, Switzerland). This test allows the quantitative determination of the fecal calprotectin level through a sandwich immunoassay with a measurement range between 100 and 1800 μg/g.

Statistical Analyses

The sample size was calculated to determine a clinically relevant difference in the reduction in the average daily crying time of 70 minutes between the groups. With $\alpha = 0.05$, $\beta = 0.80$, and an estimated SDs within groups of 80 minutes, 22 patients were needed per group; 26 subjects per group were enrolled to allow for a 20% dropout rate.

The data were analyzed with GraphPad Prism 7.01 (Graphpad Software, La Jolla, CA). The quantitative variables were described in terms of the means and SD or the medians and variation ranges/IQRs. The qualitative variables were described through absolute frequencies and percentages. Depending on their distribution, comparisons of quantitative data samples were analyzed with the Student *t* test or the Mann-Whitney test. All tests were 2-tailed and considered significant at values of $P < .05$.

Results

Baseline characteristics of the study groups of infants recruited from August 1, 2015 to September 30, 2016, are reported in **Table I**.

Table I. Baseline characteristics of the study population

	L reuteri (n = 32)	Placebo (n = 28)	*P* value
Mean age (d ± SD)	47.90 ± 25.78	46.11 ± 20.30	.681*
Sex			
Female, n (%)	19 (59.4)	15 (53.6)	.795†
Male n (%)	13 (40.6)	13 (46.4)	.795†
Birth weight (g ± SD)	3211 ± 382.56	3085 ± 354.63	.521*
Gestational age (wk ± SD)	38.21 ± 1.73	38.60 ± 1.92	.453*
Nationality			
Italian, n (%)	25 (78.13)	24 (85.71)	.529†
Foreign, n (%)	5 (15.63)	4 (14.29)	1.000†
Mixed, n (%)	2 (6.25)	0	.494†
Feeding			
Exclusive breast milk, n (%)	28 (87.50)	22 (78.50)	.301†
Predominant breast milk, n (%)	4 (12.50)	6 (21.50)	.301†

*Mann-Whitney test.
†Fisher test.

All statistical analyses were completed on the intention-to-treat principle when possible. By 1 month, 75% (n = 24) of the treatment group and 36.4% (n = 8) of the placebo group showed at least a 50% reduction in the crying duration (responders). The OR was 0.19, the relative risk was 0.39, and the relative risk reduction was 0.61. The number needed to treat was 2.56.

We evaluated the crying time per day at the allocation time and after 1 month of treatment with *L reuteri* (n = 32) or the placebo (n = 28). The 2 groups were similar in all outcomes at baseline. At enrollment (day 0), the average crying time per day (minutes/day) was similar in the 2 groups (299 minutes/day [IQR = 115] in the first group treated with the probiotic vs 305.4 minutes/day [IQR = 133] in the second group treated with the placebo [$P = .191$]). Infants treated with *L reuteri* showed a significant reduction in the daily crying time at the end of the study (day 30) (74.67 ± 25.04 [IQR = 79] minutes/day vs 147.85 [IQR = 135] minutes/day in infants administered the placebo [$P = .001$]). Although the daily duration of crying decreased over the study period in both groups, the decline at 1 month was greater in the probiotic group than in the placebo group (**Table II**). Infant responders were significantly higher in the *L reuteri*-treated group than in the placebo group ($P = .004$). At day 30, we asked the parents to complete a questionnaire concerning their degree of satisfaction (Assessment of Quality of Life scale; Melbourne, Australia[27]), where the minimum score was 1 (not satisfied) and the maximum was 10 (very satisfied). The parents of the children who received the probiotic all recorded an average score of 8.34, whereas the parents of the placebo group reported a low score, with an average of 4.14 ($P = .00001$).

After supplementation with *L reuteri* DSM 17938 for 30 days in the infants with colic, the FOXP3 concentration increased significantly ($P = .009$), resulting in decreased a RoRγ/FOXP3 mRNA ratio (0.61 [IQR = 0.60] at day 0 and 0.48 [IQR = 0.28] at day 30 [$P = .028$]) (**Figure 2**; available at www.jpeds.com). Conversely, we did not observe a significant change in the RoRγ/FOXP3 mRNA ratio after 30 days in the placebo group (0.8995 [IQR = 1.146] at day 0 and 0.6887 [IQR = 0.276] at day 30 [$P = .19$]) (**Table III**).

Table II. Crying and fussing time (mean minutes per day) ± SD in *L reuteri* and placebo groups

	Control group (n = 27)	Infant with colic *L reuteri* (n = 32)	Infant with colic Placebo (n = 22)	*P* value	95%CI
D 0	55.22 ± 32.41	299.66 ± 27.55	305.41 ± 30.09	.190	–9.85;12.20
D 14		118.32 ± 21.12	226.31 ± 19.77	.026	–25.02;–12,32
D 21		95.11 ± 16.34	187.68 ± 31.88	.009	–38.52;–46.12
D 30		74.67 ± 25.04	147.85 ± 37.99	.001	–87.32;–59.15

Furthermore, after *L reuteri* administration for 30 days in the infants with colic, we observed a significant increase in the percentage of *Lactobacillus*; the medians were 9.49% (IQR = 15.31) at day 0 and 23.06% (IQR = 33.19) after 30 days (*P* = .049). Placebo administration did not produce any significant change in the intestinal microbiota indicators (all *P* values were >.05) (**Table IV**).

In addition, we observed a decrease in the fecal calprotectin values (*P* = .0001); the medians were 541 µg/g (IQR = 429.5) at day 0 and 165 µg/g (IQR = 246.8) at day 30. Moreover, placebo administration did not significantly reduce the calprotectin values (*P* = .23); the medians were 361 µg/g (IQR = 376.5) at day 0 and 182 µg/g (IQR = 321.5) at day 30.

We also compared children with colic (n = 32) with a group of healthy children (n = 27). No significant difference in the RoRγ/FOXP3 mRNA ratio was observed between groups. Conversely, in terms of the gut microbiota, we observed that the absolute number and percentage of *E coli* had medians of 266.1 genome/mg feces (IQR = 18.28) and 2.91% (IQR = 15.54), respectively, in the colic group, whereas in the healthy control group, the median *E coli* concentration was 66.15 genome/mg feces (IQR = 1238.75), and the median percentage was 0.40% (IQR = 4.23). Among all analyzed indicators, there were significant differences in the absolute number (*P* = .0193) and the percentage (*P* = .0085) of *E coli*. The children with infantile colic had a higher median fecal calprotectin concentration (500 µg/g [IQR = 415] vs 145 µg/g [IQR = 106] in the healthy group [*P* = .0001]). Finally, the average crying time per day (minutes/day) was significantly lower in the healthy group than in the colic group (55.2 minutes/day [IQR = 71)] vs 299.66 minutes/day [IQR = 115], [*P* = .015]) (**Table II**).

Table III. Blood inflammatory markers (median, IQR), 2^{-DDCt}, in infant with colic at enrollment and after 30 days of supplementation with *L reuteri* or placebo

	Infant with colic *L reuteri* group (n = 32)	*P* value*	Infant with colic Placebo group (n = 22)	*P* value*
RORγ				
D 0	0.81 (0.66)	.648	1.53 (3.84)	.513
D 30	0.99 (0.28)		1.04 (0.73)	
FOXP3				
D 0	1.21 (1.44)	.009	1.79 (0.98)	.456
D 30	1.83 (1.29)		1.59 (0.62)	
Th17/Treg				
D 0	0.61 (0.60)	.028	0.90 (1.15)	.193
D 30	0.48 (0.28)		0.689 (0.28)	

*Mann-Whitney t test.

No adverse events were reported by parents during the study in either group of infants.

Discussion

Although infantile colic affects a large proportion of infants, its etiology has not yet been fully elucidated; therefore, treatment is not entirely clear. Moreover, a recent meta-analysis supported the efficacy of probiotics compared with traditional pharmacologic treatment with simethicone.[28,32,33] The rational use of probiotics is based on various studies that have recognized the root of colic as an alteration in the gut microbiota that causes increased intestinal inflammation.[9,34]

The first part of this study focused on the effects of *L reuteri* DSM17938 administration for 1 month in children with colic. Although how *L reuteri* improves infantile colic is not entirely unclear, the probiotic is reportedly capable of directly antagonizing enteric pathogens and can modulate the gut microbiota[24] and innate or adaptive immune responses.[35]

From a systems perspective, the degree of inflammation can be investigated through the analysis of various variables circulating in the peripheral blood, such as cytokines, or as was recently proposed, by the Th17/Treg ratio in the peripheral blood. This delicate balance has been proposed as a possible indicator of inflammation.[15] However, no studies are available on this marker in infants with colic.

In our study, we showed that the administration of this probiotic induced a significant increase in FOXP3 mRNA levels (a Treg marker) and a decrease in the RoRγ/FOXP3 mRNA ratio. These results were consistent with a study by Kimura et al,[36] which tested the action of the probiotic in a mouse model of necrotizing enterocolitis. The effects on Treg lymphocytes could result from increased IL-10 secretion, which is a critical cytokine in determining the differentiation of lymphocytes into Treg cells; IL-10 secretion is reduced not only in colitis models but also in children with colic.[14]

Furthermore, we showed that the administration of *L reuteri* was associated with a significant decrease in the fecal calprotectin level, which is a clinical marker of intestinal inflammation in both adults and children.[11]

Regarding the gut microbiota, we showed that in children with colic, after a 1-month administration of *L reuteri*, a significantly increased *Lactobacillus* percentage was evident in the total bacterial count. This result is consistent with previous findings by Savino et al.[23] Concerning gut microbiota indicators, we observed significant increases in the absolute numbers and the percentages of *E coli* in children suffering from colic

Table IV. Gut microbiota indicators (median, IQR), *genome/mg feces*, in infant with colic at enrollment and after 30 days of supplementation with *L reuteri*

	Infant with colic *L reuteri* group (n = 32)	*P* value*	Infant with colic Placebo group (n = 22)	*P* value*
E coli				
D 0	266.1 (23.31)	.632	14863 (116907)	.546
D 30	1904 (14623.6)		1192 (33351.62)	
Lactobacillus				
D 0	27946 (165625)	.082	392660 (2002338)	.667
D 30	78460 (277772)		471345 (2360192)	
Bifidobacteria				
D 0	21260 (432389)	.383	1088422 (39003271)	.931
D 30	63040 (503326)		1113796 (2588638)	
Lactobacillus/E coli				
D 0	37.49 (237.21)	<.769	14.94 (382.39)	.605
D 30	13.02 (460.24)		53.74 (534.54)	
Bifidobacteria/E coli				
D 0	70.56 (863.99)	.529	378.32 (1515.84)	.605
D 30	36.65 (277.42)		408.75 (6246.08)	
Bifidobacteria/Lactobacillus				
D 0	2.44 (33.24)	.589	5.23 (73.99)	.730
D 30	1.61 (10.78)		11.36 (122.13)	
Total bacteria				
D 0	293448 (1006472)	.338	1737434 (5276032)	.730
D 30	419445 (719887)		1102853 (3238368)	
% of *E coli*				
D 0	1.70 (19.06)	.266	9.64 (13.28)	.605
D 30	7.26 (26.41)		0.91 (44.78)	
% of *Lactobacillus*				
D 0	9.49 (15.31)	.049	16.01 (30.17)	.999
D 30	23.06 (33.19)		16.52 (52.15)	
% of *Bifidobacteria*				
D 0	0.97 (12.95)	.836	17.33 (65.89)	.931
D 30	3.15 (8.14)		13.99 (50.07)	

*Mann-Whitney *t* test.

compared with subjects without colic, which was consistent with previous studies.[8,11] *E coli* is a well-known, gas-producing bacterium involved in meteorism and is the cause of abdominal bloating, which are both typical in children suffering from colic. Moreover, *E coli* is a potential producer of inflammatory lipopolysaccharide, which may be associated with the inflammation present in infants with colic.[34] Furthermore, we observed that infants with colic presented significantly higher levels of fecal calprotectin, which was in accordance with the data of Rhoads et al.[9] Therefore, we hypothesized that a local state of inflammation existed in children with colic.[14]

Walter et al[37] reported that the effects of *L reuteri* on immune cells contribute to tolerance in the gut. The modulation of dendritic cells by *L reuteri* is mediated through dendritic cell-specific icam-grabbing nonintegring (DC-SIGN) and promotes the development of regulatory T cells, which produce high amounts of IL-10 and (transforming growth factor-β. This suppression of the immune response is likely to underlie the ability of *L reuteri* to reduce intestinal inflammation in several murine colitis models.[37-39]

The trial has some limitations. Although the collection of feces for the determination of fecal calprotectin and the gut microbiota allowed us to obtain a good sample size, collecting a sufficient quantity of peripheral blood for the analysis of the RoRγ and FOXP3 mRNA levels was difficult. Considering the ages of the enrolled infants, the quantity of blood samples was also often poor. For confirmation of the observed data in peripheral blood, further studies using fluorescence-activated cell sorting to investigate Treg and T helper 17 cells and larger sample sizes will be necessary.

Because of the limited amount of blood samples from these children, the study was designed to investigate the transcription profiles of several mRNAs in the peripheral blood without detecting the corresponding translated proteins. Finally, development of improved methods to measure microbiota, such as 16S ribosomal RNA or pyrosequencing would be desirable. For example, analyzing the counts of *Klebsiella, Clostridium,* and *Enterococcus* could allow for the identification of new mechanisms of action of *L reuteri*.

In conclusion, a significantly higher degree of intestinal inflammation was observed in the children with colic, as demonstrated by the fecal calprotectin values. This finding supports the hypothesis that dysbiosis and an inflammatory state may contribute to the onset of colic. The data obtained after treatment with *L reuteri* are also interesting; in addition to the clinical response highlighted by the decrease in crying hours, the treatment modulated the FOXP3 concentration, resulting in a decreased RoRγ/FOXP3 mRNA ratio. Compared with the post-placebo results, it is likely that the probiotic induced both local and systemic reductions in inflammation. New studies

are warranted to better understand the efficacy of probiotics in infants with colic. ■

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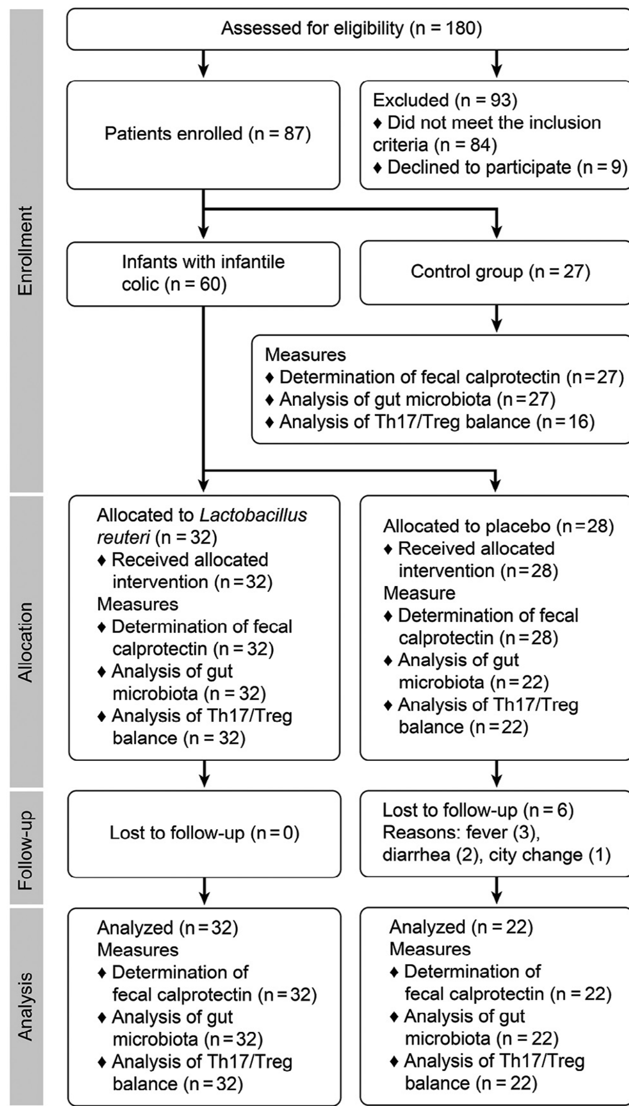

Figure 1. Flow diagram of the subjects' progression during the study.

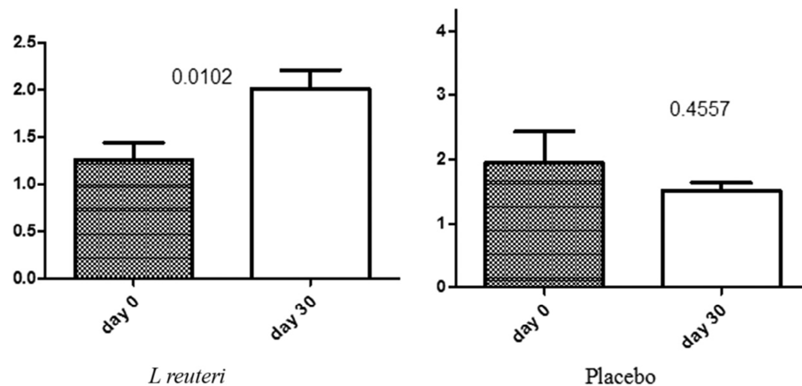

Figure 2. Expression of FoxP3 at day 0 and day 30 in infants treated with *L reuteri* or placebo. Real-time TaqMan RT-PCR was used to quantify the expression of FoxP3 mRNA in *L reuteri* (n = 32) and placebo (n = 22) in the peripheral blood. $2^{-\Delta\Delta ct}$ represented by vertical bars. The data were analyzed using Mann-Whitney U test. *P* values of ≤ .05 were considered significant.