PEDIATRICS®
OFFICIAL JOURNAL OF THE AMERICAN ACADEMY OF PEDIATRICS

Lactobacillus reuteri DSM 17938 in Infantile Colic: A Randomized, Double-Blind, Placebo-Controlled Trial

Francesco Savino, Lisa Cordisco, Valentina Tarasco, Elisabetta Palumeri, Roberto Calabrese, Roberto Oggero, Stefan Roos and Diego Matteuzzi

Pediatrics 2010;126;e526-e533; originally published online Aug 16, 2010;
DOI: 10.1542/peds.2010-0433

Lactobacillus reuteri DSM 17938 in Infantile Colic: A Randomized, Double-Blind, Placebo-Controlled Trial

WHAT'S KNOWN ON THIS SUBJECT: This article reports a clinical research on infantile colic and it's an eagerly awaited follow-up to an article published in *Pediatrics* in 2007. The earlier study generated interest among physicians but had the weakness of not being a double-blind, placebo-controlled study.

WHAT THIS STUDY ADDS: Data are presented from a prospective, randomized, double-blind, placebo-controlled study in breastfed colicky infants. The benefit of supplementation with *L reuteri* DSM 17 938 was clearly demonstrated, and microbiological analysis of the infants' feces revealed a modification in gut microbiota.

abstract

OBJECTIVE: To test the efficacy of *Lactobacillus reuteri* on infantile colic and to evaluate its relationship to the gut microbiota.

STUDY DESIGN: Fifty exclusively breastfed colicky infants, diagnosed according to modified Wessel's criteria, were randomly assigned to receive either *L reuteri* DSM 17 938 (10^8 colony-forming units) or placebo daily for 21 days. Parental questionnaires monitored daily crying time and adverse effects. Stool samples were collected for microbiologic analysis.

RESULTS: Forty-six infants (*L reuteri* group: 25; placebo group: 21) completed the trial. Daily crying times in minutes/day (median [interquartile range]) were 370 (120) vs 300 (150) ($P = .127$) on day 0 and 35.0 (85) vs 90.0 (148) ($P = .022$) on day 21, in the *L reuteri* and placebo groups, respectively. Responders (50% reduction in crying time from baseline) were significantly higher in the *L reuteri* group versus placebo group on days 7 (20 vs 8; $P = .006$), 14 (24 vs 13; $P = .007$), and 21 (24 vs 15; $P = .036$). During the study, there was a significant increase in fecal lactobacilli ($P = .002$) and a reduction in fecal *Escherichia coli* and ammonia in the *L reuteri* group only ($P = .001$). There were no differences in weight gain, stooling frequency, or incidence of constipation or regurgitation between groups, and no adverse events related to the supplementation were observed.

CONCLUSION: *L. reuteri* DSM 17 938 at a dose of 10^8 colony-forming units per day in early breastfed infants improved symptoms of infantile colic and was well tolerated and safe. Gut microbiota changes induced by the probiotic could be involved in the observed clinical improvement. *Pediatrics* 2010;126:e526–e533

AUTHORS: Francesco Savino, MD, PhD,[a] Lisa Cordisco, PhD,[b] Valentina Tarasco, MD,[a] Elisabetta Palumeri, MD,[a] Roberto Calabrese, BSc,[a] Roberto Oggero, MD,[a] Stefan Roos, PhD,[c] and Diego Matteuzzi, PhD[b]

[a]Department of Pediatrics, Regina Margherita Children Hospital, University of Turin, Turin, Italy; [b]Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy; and [c]Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden

KEY WORDS

Lactobacillus reuteri, infantile colic, FISH, gut microflora, *Escherichia coli*

ABBREVIATIONS

FISH—fluorescence in situ hybridization
IQR—interquartile range

This trial has been registered at www.clinicaltrials.gov (identifier 00893711).

Dr Savino had primary responsibility for protocol development as principal investigator, and he wrote the manuscript; Dr Cordisco performed the microbiological analysis, in particular the analysis of bacterial groups by FISH technique and of fecal ammonia; Dr Tarasco participated in the development of the protocol and in writing the manuscript and she was responsible for the screening of patients, enrollment, and outcome assessment; Dr Palumeri participated in the development of the protocol and in writing the manuscript; Mr Calabrese performed the final data analysis; Dr Oggero contributed to writing the manuscript; Dr Roos performed the microbiological analysis, in particular the analysis of fecal *L reuteri* DSM 17 938; and Dr Matteuzzi had primary responsibility for microbiological analysis and contributed to writing the manuscript.

www.pediatrics.org/cgi/doi/10.1542/peds.2010-0433

doi:10.1542/peds.2010-0433

Accepted for publication May 27, 2010

Address correspondence to Francesco Savino, MD, PhD, Department of Pediatrics, Regina Margherita Children Hospital, Piazza Polonia, 94 10126 Turin, Italy. E-mail: francesco.savino@unito.it

PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

FINANCIAL DISCLOSURE: *The authors have indicated they have no financial relationships relevant to this article to disclose.*

Infantile colic, defined as paroxysmal, excessive, and inconsolable crying without an identifiable cause in an otherwise healthy newborn infant, is common in the first 3 months of life.[1,2] The classic definition of infantile colic is based on the rule of threes: fussy crying that lasts for ≥3 hours per day; for ≥3 days per week; and for a minimum of 3 weeks.[3] Colic affects 3% to 28% of infants, causing considerable stress and concern for parents,[4] and the pathogenesis of the condition remains elusive, although evidence suggests multiple independent causes.[2] The role of an aberrant intestinal microflora has recently been reproposed to affect gut motor function and gas production that lead to colicky behavior.[5] Increased presence of hydrogen gas produced by anaerobic Gram-negative bacteria and lower counts and specific colonization patterns of intestinal lactobacilli have been found in colicky infants.[6,7] Recently, coliform bacteria, particularly *Escherichia coli*, were found to be more abundant in the feces of colicky infants, suggesting a role for coliform colonic fermentation and consequent excessive intraintestinal air production, aerophagia, and pain, typical in crying infants.[8] Rhoads et al[9] demonstrated elevated levels of fecal calprotectin and a higher levels of *Klebsiella* in these patients, further supporting a role of the microbiota.

Previously, in a prospective randomized study, supplementation with the probiotic *Lactobacillus reuteri* ATCC 55 730 improved colicky symptoms in breastfed infants within 1 week of treatment compared with treatment with simethicone.[10] Recently, this strain was found to carry specific, unusual, potentially transferable resistance traits for tetracycline and lincomycin, which led to the development of a new daughter strain, *L reuteri* DSM 17 938 derived from *L reuteri* ATCC 55 730 by the natural removal of these unwanted plasmid-borne resistances. The daughter strain retained the probiotic properties and its safety and tolerance in adults.[11] The present randomized, double-blind, placebo-controlled study was designed to confirm this clinical finding and relate it to changes in fecal microbiologic profiles.

MATERIALS AND METHODS

Subjects

This randomized, controlled, double-blind study was performed on 50 breastfed infants (29 boys) who were consecutively recruited from general pediatricians and outpatients at the Department of Pediatrics, University of Turin (Regina Margherita Children Hospital) between March 2008 and August 2009. All infants were diagnosed with infantile colic according to the following modified Wessel's criteria: episodes of fussy crying that lasted ≥3 hours a day and episodes that lasted for ≥3 days in the 1 week before enrollment. All were born at term, adequate for gestational age (birth weight: 2500–4000 g), and aged 2 to 16 weeks at recruitment. Only exclusively breastfed infants were enrolled to prevent variability in the intestinal microbiota caused by diet. At enrollment, mothers were encouraged to avoid cow's milk in their diet, and adherence was confirmed during the follow-up visit. Exclusion criteria were clinical evidence of chronic illness or gastrointestinal disorders, any intake of probiotics and/or antibiotics in the week preceding recruitment, and any formula-feeding. Because gastroesophageal reflux was an exclusion criterion, no acid blockers were used in any of the infants who completed this study.

The study was approved by the local ethics committee (Comitato Interaziendale AA.SS.OO. O.I.R.M./S. Anna-Ordine Mauriziano di Torino) before the start, and written informed consent was obtained from parents before inclusion of the infants.

Study Objectives and Outcomes

Primary outcome was defined as a reduction of average crying time to <3 hours a day (the cutoff proposed by Wessel) on day 21. Secondary outcome was defined as the number of responders in each group on days 7, 14, and 21. Responders (defined in the protocol) were those who experienced a decrease in the daily average crying time of 50% from baseline. In addition, the intestinal microflora of the infants was analyzed to determine the effect of the probiotic on selected intestinal microbiota (*E coli*, *Clostridium butyricum*, *Lactobacillus*, and *Bifidobacterium*), by using fluorescence in situ hybridization (FISH).

Study Design and Sample Collection

Colicky infants were randomly assigned to receive *L reuteri* DSM 17 938 or placebo by using a computer-generated randomization list created by an independent departmental statistician (Mr Calabrese). Randomization was performed by the random-digit method, based on computer-generated numbers. We used a 2-treatment randomization scheme with random block of varying size (Stata 9 [Stata Corp, College Station, TX] Ralloc procedure). The pediatrician allocated the next available product on entry into the trial, and each patient received the study product directly from the department. Active study product consisted of a suspension of freeze-dried *L reuteri* DSM 17 938 in a mixture of sunflower oil and medium-chain triglyceride oil supplied in a 5-mL dark bottle fitted with a dropper cap. The placebo was identical in appearance and taste but without the live bacteria. Both formulations were administered in 5 drops, once a day, 30

minutes before the feed in the morning, for 21 days. During the study, parents were instructed to refrigerate the product when it was not in use. The bottles were coded and blinded by the study statistician for both the participants and for the physicians, and the code was revealed to the investigators once recruitment, data collection, and all laboratory and statistical analyses were complete.

At enrollment (day 0), parents were interviewed about gestational age, type of delivery, birth weight, presence of gastrointestinal disease, and crying time. Medical examinations were performed, and growth parameters were recorded at baseline and day 21. Parents were asked to fill in a structured diary to record the daily crying time (minutes), stool characteristics and frequency, and any adverse effects observed (constipation, vomiting, and cutaneous reactions) on each day of the study. To assess tolerance, growth parameters (weight gain per day), stooling characteristics (frequency and consistency), symptoms of digestive intolerance (constipation, regurgitation, or vomiting), and frequency of adverse events during the treatment period were evaluated. Adverse events, defined as illnesses, signs, or symptoms that occurred or got worse during the course of the study, were assessed through parental interview in their daily records. A general linear model for repeated measures was used to assess differences in growth parameters between groups at the different time points (day 0–21). Follow-up visits were performed by the same pediatrician on day 7 and 21.

Feces samples (10–15 g) for microbiologic analysis were collected by the investigators from each subject, directly from the diaper or anus, at enrollment and on day 21. Samples (blinded) were immediately placed at −20°C and stored until analyzed.

Analysis of Bacterial Groups by FISH

Intestinal bacterial groups were enumerated using specific FISH commercial kits (Microscreen B.V.; Microbial Diagnostics, Groningen, Netherlands) for the *Lactobacillus* group (*Lactobacillus* 10-ME-H006), the *Bifidobacterium* genus (*Bifidobacterium* 10-ME-H001), *E coli* group (*Escherichia* 1 coli 10-ME-H004), and *C butyricum* (*C butyricum* 10-ME-H009). Slides were enumerated in triplicate using a Nikon Eclipse E-600 epifluorescence microscope equipped with a mercury arc lamp (HBO, 100 W [Nikon, Tokyo, Japan]) and the fluorescein isothiocyanate–specific filter Nikon BA 520. Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged in each slide.

Analysis of Fecal *L reuteri* DSM 17 938

Fecal samples were thawed at room temperature, and 0.3 to 0.5 g was suspended in a peptone water (10% wt/vol) solution. This suspension was further diluted 1:10 in peptone water and plated on Rogosa agar (Oxoid, Cambridge, United Kingdom]) supplemented with 2 μg/mL ampicillin (Sigma-Aldrich, Stockholm, Sweden]) to selectively grow *L reuteri* strains with ampicillin resistance. Plates were incubated at 37°C for 48 hours anaerobically (AnaeroGen, Cambrigde, United Kingdom]). Colonies (5 per plate) were analyzed using strain-specific polymerase chain reaction assays as described by Egervärn et al,[12] with the modification that the DreamTaq green PCR master mix kit (Fermentas, Burlington, Ontario, Canada) was used.

Analysis of Fecal Ammonia

Fecal ammonia was determined colorimetrically according to Searcy et al[13] by using an enzymatic colorimetric test (Urea/BUN—Color [BioSystems S.A., Barcelona, Spain]).[14]

Statistical Analysis

The sample size was calculated to find a clinically relevant difference in the reduction in daily average crying time of 50 minutes between groups. With $\alpha = .05$, $\beta = 0.20$, and an estimated SD within groups of 55 minutes, 20 patients were needed per group. Twenty-five subjects per group were enrolled to allow for a 20% dropout rate.

Data are shown as mean ± SD or median and interquartile range (IQR) for continuous variables as appropriate and as number and percentage for categorical variables. Differences between groups were evaluated by Student's *t* test for independent samples or by Mann-Whitney test as appropriate, and associations between categorical variables were evaluated by Fisher's exact test. The Wilcoxon test and Friedman test were used to evaluate differences between paired samples for continuous variables when appropriate. Differences in the bacterial concentrations between day 21 and day 0 were analyzed by using the Mann-Whitney test. Responder data also were analyzed on an intention-to-treat basis, in which the infants who dropped out all were regarded as responders because all belonged to the placebo group. All reported *P* values are 2-sided, and differences were considered to be significant when $P < .05$. Data were analyzed by using SPSS 16 (SPSS Inc, Chicago, IL), and sample size calculation was performed by NCSS-PASS 2000 (Number Cruncher Statistical Systems, Kaysville, UT).

RESULTS

Efficacy

A total of 126 infants were eligible for inclusion. Subjects whose parents did not give informed consent ($n = 30$) and those who did not meet the inclusion criteria ($n = 46$) were excluded. The remaining 50 breastfed colicky infants were equally distributed be-

tween the *L reuteri* and placebo groups by the randomization procedure (Fig 1). Four patients (all in placebo group) were excluded from the analysis for the following reasons: fever (1 patient); failure to complete the diary (2 patients); and gastroesophageal reflux (1 patient). Forty-six infants completed the study (25 in the *L reuteri* group and 21 in the placebo group). There were no withdrawals attributable to any adverse effect related to the trial.

TABLE 1 Baseline Characteristics of Participants in the Study Groups

Variable	Placebo ($N = 25$)	*L reuteri* ($N = 25$)	*P*
Type of delivery (cesarean), *n* (%)	6 (25)	13 (52)	.079[a]
Male, *n* (%)	14 (56)	15 (60)	.999[a]
Age at entry, median (IQR), d	28.5 (21)	32.5 (21)	.382[b]
Family history of gastrointestinal diseases (yes), *n* (%)	8 (32)	6 (24)	.754[a]
Family history of atopy (yes), *n* (%)	9 (36)	12 (48)	.567[a]
Entry weight, mean ± SD, g	4378.5 ± 795.0	4418.4 ± 723.6	.854[c]
Entry length, mean ± SD, cm	54.4 ± 2.4	54.4 ± 2.6	.999[c]
Entry head circumference, mean ± SD, cm	37.3 ± 1.5	36.9 ± 1.4	.286[c]
Entry stool frequency, median (IQR)	4.8 (2.4)	3.5 (3.1)	.146[b]

[a] Fisher's exact test.
[b] Mann-Whitney test.
[c] Student's *t* test.

There were no significant differences between the groups regarding type of delivery, gender, age on entry, family history of gastrointestinal diseases or atopy, or growth parameters (Table 1).

At enrollment, there was no difference in median crying time (minutes/day) between the groups: 370 (IQR: 120) vs 300 (IQR: 150) in the *L reuteri* and placebo groups, respectively ($P = .127$). Among colicky infants who received the probiotic there was a significant reduction in daily crying time at the end of the study (day 21) compared with placebo: 35 (IQR: 85) vs 90.0 (IQR: 148) minutes/day, respectively ($P = .022$) (Table 2). At enrollment, all infants had >180 minutes crying per day in both groups as defined by the inclusion criteria. By day 21, the number of subjects that had crying times > 180 minutes was significantly lower in the *L reuteri* group compared with the placebo group (4 vs 12, respectively; $P = .009$).

There was a significantly higher number of responders in the probiotic group compared with placebo on days 7 (20 vs 8; $P = .006$), 14 (24 vs 13; $P = .007$), and 21 (24 vs 15; $P = .036$) (Fig 2). Intention-to-treat analysis revealed that the number of responders in the *L reuteri* group was always significantly higher than in the placebo group (day 7: 21 vs 11; $P = .007$; day 14: 24 vs 16; $P = .011$; day 21: 24 vs 18; $P = .049$).

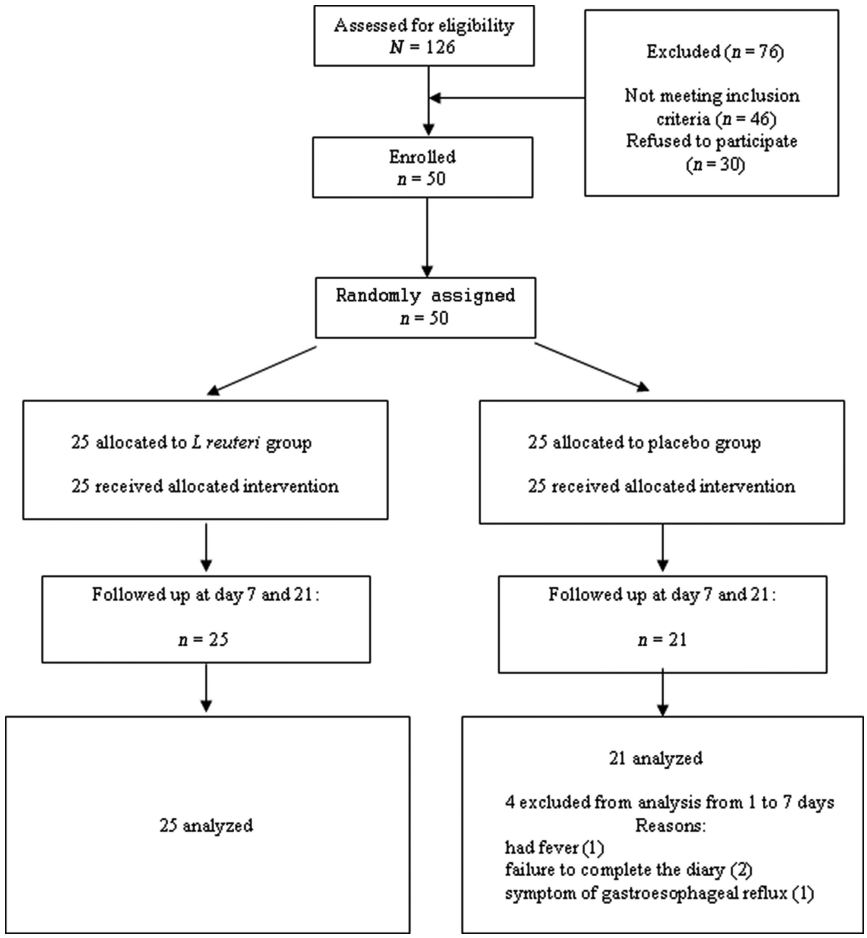

FIGURE 1
Patient enrollment and study progress.

TABLE 2 Crying Time in the *L reuteri* and Placebo Groups

Day of Study	*L reuteri* ($N = 25$), Median (IQR), min/d	Placebo ($N = 21$), Median (IQR), min/d	*P*[a]
0	370 (120)	300 (150)	.127
7	95 (85)	185 (149)	.082
14	60 (70)	150 (145)	.099
21	35 (85)	90 (148)	.022

Crying time was assessed by using daily parental reporting in structured diaries.
[a] Mann-Whitney test.

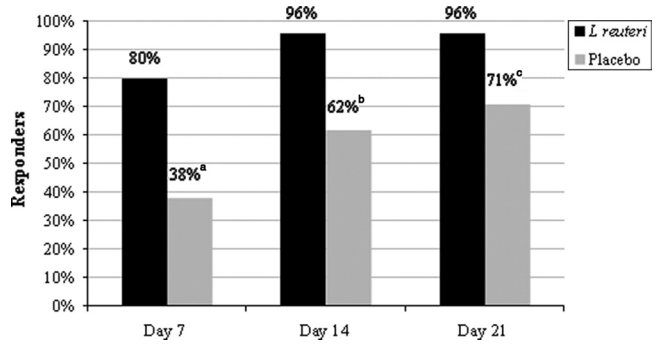

FIGURE 2

Effectiveness (number of responders) of *L reuteri* versus placebo during the study. Infants were classified as responders if they experienced a decrease in the daily average crying time (in minutes) of 50% from the baseline measurement. The bars show the percentage of responders in each group on days 7, 14, and 21 of supplementation. Statistical analysis was performed by using the Mann-Whitney test. [a] Day 7, $P = .006$; [b] day 14, $P = .007$; and [c] day 21, $P = .036$.

Microbiologic Analysis of Fecal Cultures

Microbial Groups in Fecal Cultures

Fecal counts of *Lactobacillus*, *bifidobacteria*, and *Clostridium butyricum* species were similar between the groups, but the levels of *E coli* were higher in the *L reuteri* group at the beginning of the study (Table 3). There was a significant reduction in *E coli* in the probiotic group compared with the placebo group (-6.55×10^7 [IQR: 4.87×10^8] vs 4.30×10^5 [IQR: 4.35×10^7], respectively; $P = .001$) during the course of the study. Lactobacilli were found to be significantly increased in the *L reuteri* versus placebo group (4.07×10^5 [IQR: 4.98×10^6] vs 0×10^0 [IQR: 3.27×10^4]; $P = .002$). There were no observed differences for *Bifidobacteria* ($P = .907$) or *C butyricum* ($P = .458$) (Tables 3 and 4).

Analysis of Fecal L reuteri *DSM 17 938*

Fecal samples were analyzed in 26 of the 50 infants (13 from each group, when samples were available). Twelve of the 13 infants who were given the probiotic were found to have fecal *L reuteri* DSM 17 938 on day 21 (Fig 3), whereas none of the infants who received placebo had detectable *L reuteri* DSM 17 938. The mean level of *L reuteri* DSM 17 938 in these infants was 2.8×10^4 colony-forming units per g of feces.

Fecal Ammonia Concentration

Fecal ammonia concentration (mg/L *reuteri*) on day 0 was 1.44 (IQR: 3.15) and 2.59 (IQR: 2.56) in the placebo and *L reuteri* groups, respectively ($P = .064$). There was a significant reduction in fecal ammonia in the probiotic group during the study compared with the placebo group (Tables 3 and 4).

Safety and Tolerance

Growth was significant in both groups as shown by weight, length, and head circumference ($P < .001$) (Table 5), with no significant differences between the groups, and average weight gain was similar between the *L reuteri* and placebo groups (median g/day were 34.3 (IQR: 16.1) and 29.9 (IQR: 19.4), respectively; $P = .140$). Gastrointestinal function was similar between the groups (Table 6). Adverse events reported during the study were rhinitis (*L reuteri* group, $n = 1$), eczema (placebo group, $n = 1$), fever (placebo group, $n = 1$), otalgy (placebo group, $n = 1$), and gastroesophageal reflux (placebo group, $n = 1$). All were deemed unrelated to study product.

DISCUSSION

Increasing evidence of efficacy from well-controlled clinical trials with certain probiotic lactic acid bacterial strains is leading to extended use of these dietary supplements in clinical practice. Recent demonstration that the gut microbiota and exogenous probiotics have a strong influence on gut function, through immunophysiological regulation in the intestinal mucosal barrier, opens new perspectives in nutrition.[15] For infants, the aim of probiotic supplementation is to provide a safe yet sufficient microbial stimulus for the immature immune system, which contributes to the anti-

TABLE 3 Bacterial Species per Gram of Feces and Ammonia Concentrations at the Start and End of the Study

	At Day 0				At Day 21			
	Placebo		*L reuteri*		Placebo		*L reuteri*	
	Median (IQR)	*P*	Median (IQR)	*P*	Median (IQR)	*P*	Median (IQR)	*P*
Bacteria/variable, species per g								
E coli	2.49×10^6 (4.53×10^7)	.006	1.54×10^8 (6.61×10^6)	.006	2.85×10^7 (1.78×10^8)	.181	2.70×10^7 (4.19×10^8)	.181
C butyricum	1.84×10^6 (6.79×10^7)	.562	4.26×10^6 (2.62×10^7)	.562	2.70×10^5 (7.79×10^6)	.377	1.96×10^6 (4.83×10^7)	.377
Lactobacillus	8.20×10^3 (2.10×10^5)	.988	8.40×10^3 (3.00×10^5)	.988	9.99×10^2 (7.00×10^5)	.015	4.15×10^5 (7.21×10^6)	.015
Bifidobacteria	2.28×10^9 (6.20×10^9)	.638	2.26×10^9 (4.60×10^9)	.638	2.85×10^9 (9.80×10^9)	.717	3.19×10^9 (4.70×10^9)	.717
Ammonia, mg/L	1.44 (3.15)	.064	2.59 (2.56)	.064	1.65 (3.12)	.665	1.05 (1.74)	.665

Fecal bacteria genus and species were analyzed by using FISH. The number of fecal samples for each analysis was 2. *P* was calculated by using the Mann-Whitney test.

TABLE 4 Bacterial Species per Gram of Feces and Ammonia Concentration Variation From 21 to 0 Day in the Study Groups

	Difference Between Days 21 and Day 0, Median (IQR)		*P*
	Placebo	*L reuteri*	
Bacteria/variable, species per g			
E coli	4.30×10^5 (4.35×10^7)	-6.55×10^7 (4.87×10^8)	.001
C butyricum	-1.00×10^0 (5.91×10^6)	0.00×10^0 (1.52×10^7)	.458
Lactobacillus	0.00×10^0 (3.27×10^4)	4.07×10^5 (4.98×10^6)	.002
Bifidobacteria	0.00×10^0 (3.09×10^9)	2.19×10^8 (2.52×10^9)	.907
Ammonia, mg/L	0.33 (0.81)	−1.10 (1.60)	<.001

The number of fecal samples for each analysis was 2. *P* was calculated by using the Mann-Whitney test.

FIGURE 3
L reuteri DSM 17 938 colonies recovered from feces of infant in the *L reuteri* DSM 17 938–supplemented group. Shown are colonies of *L reuteri* DSM 17 938 recovered from the feces sample from an infant in the *L reuteri* group on day 21. The colonies were grown on Rogosa agar supplemented with 2 μg/mL ampicillin to selectively grow *L reuteri* strains with ampicillin resistance. Colonies were confirmed as *L reuteri* DSM 17 938 by analysis using strain-specific polymerase chain reaction.

TABLE 5 Growth Parameters in the Study Groups

Variable	At Entry, Mean ± SD		After 1 wk, Mean ± SD		After 3 wk, Mean ± SD	
	Placebo	*L reuteri*	Placebo	*L reuteri*	Placebo	*L reuteri*
Weight	4379 ± 795	4418 ± 724	4619 ± 844	4678 ± 727	5079 ± 864	5177 ± 648
Length	54.4 ± 2.4	54.4 ± 2.6	NA	NA	55.3 ± 2.5	55.2 ± 2.6
Head circumference	37.3 ± 1.5	36.9 ± 1.4	NA	NA	38.1 ± 1.5	37.7 ± 1.3

There were no significant differences between the groups. NA indicates not available.

inflammatory tone of the intestinal milieu.[16]

In the present study, colicky infants who received *L reuteri* DSM 17 938 showed significant reduction in daily crying time, confirming the effects of this probiotic on colic symptoms in breastfed infants in a double-blind, randomized, placebo-controlled protocol. The primary outcome showed significant effect of the supplementation with *L reuteri* DSM 17 938 at day 21, but analysis of responders revealed that the effect was observed at least at day 7, although significance was not quite reached when compared with actual crying times before day 21, which also confirms earlier observations.[10]

Possible mechanisms of the action of *L reuteri* include an improvement in gut motility and function[17] and direct effects on visceral pain,[18,19] both of which may induce a calming effect and reduced crying in infants. IKunze et al[18] hypothesized that probiotic effects could be mediated by their action on colonic intrinsic sensory neurons. They demonstrated in animal models that *L reuteri* ingestion increased myenteric AH neuron (an enteric neuron with an afterhyperpolarizing potential following an action potential) excitability while reducing the size of slow afterhyperpolarization, mediated through intermediate conductance calcium-dependent K^+ channel opening. n addition, Wang et al[19] demonstrated that *L reuteri* ingestion could enhance tonic inhibition of rat colon contractile activity by acting via the intermediate conductance calcium-dependent K^+ channel current in myenteric AH cells. They speculated that modulation of motility via AH cell excitability could be a pathway through which probiotics influence extrinsic sensory neurons and thus central nervous system activity.

Despite the significantly different response of the infants supplemented with probiotics, those who received placebo thrived and had reduced crying time by the end of the study. Thus, placebo treatment elicited a considerable effect, which has been seen in previous studies on infantile colic. The placebo response could be elicited by the mother's cow's-milk-free diet, suggested by Hill et al,[20] or more likely by physiologic maturation that ultimately resolves colic during normal development.

FISH analysis demonstrated a significant increase in fecal lactobacilli after

TABLE 6 Parameters of Gastrointestinal Function in the Study Subjects

Variable	Placebo ($N = 25$)	*L reuteri* ($N = 25$)	*P*
Stool frequency at entry, median (IQR), d	4.8 (2.4)	3.5 (3.1)	.146[a]
Stool frequency at 1 wk, median (IQR), d	4.0 (1.4)	4.0 (2.6)	NS
Stool frequency at 3 wk, median (IQR), d	3.0 (1.9)	3.5 (2.1)	.906[a]
Constipation reported among infants, *n* (%)	11 (46)	8 (31)	.383[b]
Regurgitation reported among infants, *n* (%)	2 (8)	4 (15)	.669[b]

NS indicates not significant.
[a] Mann-Whitney test.
[b] Fisher's exact test.

supplementation with *L reuteri*, and importantly, by using strain selective analysis, the administered bacterial strain was recovered only in these infants. The ability of *L reuteri* DSM 17 938 to resist the harsh gastric acidic conditions and temporarily colonize the entire human gastrointestinal tract after oral ingestion has been demonstrated[11,21,22] and likely contributes to its ability to influence human physiology.

E coli is a member of the normal intestinal microflora of humans but has traditionally been viewed as a common cause of gastrointestinal disease[23,24] and extraintestinal infections, such as urinary tract infection[25] and neonatal sepsis.[26] We hypothesized an association between the abundance of coliform species and decreases in other beneficial bacteria and considered the role of colonic *E coli* fermentation in excessive intestinal air-load and consequent pain, typical of infantile colic. We recently reported the higher prevalence of coliform bacteria, in particular *E coli*, in colicky infants compared with healthy counterparts, implicating *E coli* in infantile colic.[8] This, together with the present finding that fecal *E coli* levels were significantly reduced during *L reuteri*, but not during placebo supplementation, suggests that *L reuteri* promotes gut health through a reduction of *E coli* colonization.

Proteolytic bacteria, such as fusobacteria, propionibacteria, and clostridia, mainly ferment amino acids, which results in the production of several toxic metabolic end-products, including ammonia and indole.[27] Thus, coliform growth and carbohydrate fermentation affect ammonia absorption and urea nitrogen recycling and excretion. Heavey et al[28] reported that fecal ammonia concentrations are significantly different in breastfed and formula-fed infants particularly in the preweaned period. The observed reduction in fecal ammonia concentrations in breastfed infants given *L reuteri* could be related to modification of bacterial enzyme activity through the reduction of *E coli* and other members of the gut microbiota and more rapid trapping of luminal NH_3 as NH_4^+.

L reuteri has been used widely in foods and supplements for children and infants with good tolerance in both clinical trials[10,29,30] and in clinical use with no adverse events reported in the literature. Our observations in healthy, breastfed infants younger than 2 months confirm the good tolerance of the new strain.

CONCLUSIONS

Administration of *L reuteri* DSM 17 938 to colicky infants is well tolerated and improves symptoms of infantile colic compared with placebo, and this effect may be related to induced changes in the fecal microbiota, particularly *E coli*. These findings provide important insights into the role of an aberrant bacterial flora in the pathogenesis of infantile colic and the potential to overcome this with probiotic supplementation.

ACKNOWLEDGMENTS

This study was funded by BioGaia AB (Stockholm, Sweden).

We thank Francesca Verna and Emanuela Locatelli for collecting fecal samples and Cecilia Petrén and Klara Båth for analyses of fecal *L reuteri*.

REFERENCES

1 Roberts DM, Ostapchuk M, O'Brien JG. Infantile colic. *Am Fam Physician*. 2004;70(4):735–740

2 Savino F. Focus on infantile colic. *Acta Paediatr*. 2007; 96(9):1259–1264

3 Wessel MA, Cobb JC, Jackson EB, Harris GS Jr, Detwiler AC. Paroxismal fussing in infancy, sometimes called "colic." *Pediatrics*. 1954;14(5):421–435

4 Keefe MR, Kajrlsen KA, Lobo ML, Kotzer AM, Dudley WN. Reducing parenting stress in families with irritable infants. *Nurs Res*. 2006;55(3):198–205

5 Gupta SK. Is colic a gastrointestinal disorder? *Curr Opin Pediatr*. 2002;14(5):588–592

6 Savino F, Cresi F, Pautasso S, et al. Intestinal microflora in breastfed colicky and non-colicky infants. *Acta Paediatr*. 2004;93(6):825–829

7 Savino F, Bailo E, Oggero R, et al. Bacterial counts of intestinal *Lactobacillus* species in infants with colic. *Pediatr Allergy Immunol*. 2005;16(1):72–75

8 Savino F, Cordisco L, Tarasco V, Calabrese R, Palumeri E, Matteuzzi D. Molecular identification of coliform bacteria from colicky breastfed infants. *Acta Paediatr*. 2009;98(10):1582–1588

9 Rhoads JM, Fatheree NY, Norori J, et al. Altered fecal microflora and increased fecal calprotectin in infants with colic. *J Pediatr*. 2009;155(6):823.e1–828.e1

10 Savino F, Pelle E, Palumeri E, Oggero R, Miniero R. *Lactobacillus reuteri* (American type culture collection strain 55 730) versus simethicone in the treatment of infantile colic: a prospective randomized study. *Pediatrics*. 2007;119(1). Available at: www.pediatrics.org/cgi/content/full/119/1/e124

11 Rosander A, Connolly E, Roos S. Removal of antibiotic resistance plasmids from *Lactobacillus reuteri* ATCC 55 730 and characterization of the resulting daughter strain, *L reuteri* DSM 17 938. *Appl Environ Microbiol*. 2008;74(19):6032–6040

12 Egervärn M, Lindmar H, Olsson J, Roos S. Transferability of a tetracycline resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal tract of humans. *Antonie Van Leeuwenhoek*. 2010; 97(2):189–200

13 Searcy RL, Reardon JE, Foreman JA. A new photometric method for serum urea nitrogen determination. *Am J Med Technol*. 1967; 33(1):15–20

14 Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem*. 1962;8:130–132

15 Van Niel CW. Probiotics: not just for treatment anymore. *Pediatrics*. 2005;115(1): 174–177

16 Rautava S, Kalliömaki M, Isolauri E. New therapeutic strategies for combating the increasing burden of allergic diseases: probiotics—a Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. *J Allergy Clin Immunol*. 2005;116(1):31–37

17 Indrio F, Riezzo G, Raimondi F, et al. The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. *J Pediatr*. 2008; 152(6):801–806

18 Kunze WA, Mao YK, Wang B, et al. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium dependent potassium channel opening. *J Cell Mol Med*. 2009;13(8B):2261–2270

19 Wang B, Mao YK, Diorio C, et al. Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. *Neurogastroenterol Motil*. 2010;22(1):98–107, e33

20 Hill DJ, Roy N, Heine RG, et al. Effect of a low-allergen maternal diet on colic among breastfed infants: a randomized, controlled trial. *Pediatrics*. 2005;116(5). Available at: www.pediatrics.org/cgi/content/full/116/5/e709

21 Wall T, Båth K, Britton RA, Jonsson H, Versalovic J, Roos S. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. *Appl Environ Microbiol*. 2007;73(12):3924–3935

22 Abrahamsson TR, Sinkiewicz G, Jakobsson T, Fredrikson M, Björkstén B. Probiotic lactobacilli in breast milk and infant stool in relation to oral intake during the first year of life. *J Pediatr Gastroenterol Nutr*. 2009; 49(3):349–354

23 Nowrouzian F, Hesselmar B, Saalman R, et al. *Escherichia coli* in infants' intestinal microflora: colonization rate, strain turnover, and virulence gene carriage. *Pediatr Res*. 2003;54(1):8–14

24 Rhodes JM. The role of *Escherichia coli* in inflammatory bowel disease. *Gut*. 2007; 56(5):610–612

25 Yamamoto S. Molecular epidemiology of uropathogenic *Escherichia coli*. *J Infect Chemother*. 2007;13(2):68–73

26 Hammerman C, Kaplan M. Probiotics and neonatal intestinal infection. *Curr Opin Infect Dis*. 2006;19(3):277–282

27 Holdeman, LV, Cato EP, Moore WEC. Anaerobe Laboratory Manual. 4th ed. Blacksburg, VA: Virginia Polytechnic Institute and State University; 1977

28 Heavey PM, Savage SAH, Parrett A, Cecchini C, Edwards CA, Rowland IR. Protein-degradation products and bacterial enzyme activities in faeces of breast-fed and formula-fed infants. *Br J Nutr*. 2003;89(4): 509–515

29 Weizman Z, Alsheikh A. Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study. *J Am Coll Nutr*. 2006;25(5):415–419

30 Abrahamsson TR, Jakobsson T, Bottcher MF, et al. Probiotics in prevention of IgE-associated eczema: a double-bind, randomized, placebo-controlled trial. *J Allergy Clin Immunol*. 2007;119(5): 1174–1180

Lactobacillus reuteri DSM 17938 in Infantile Colic: A Randomized, Double-Blind, Placebo-Controlled Trial

Francesco Savino, Lisa Cordisco, Valentina Tarasco, Elisabetta Palumeri, Roberto Calabrese, Roberto Oggero, Stefan Roos and Diego Matteuzzi

Pediatrics 2010;126;e526-e533; originally published online Aug 16, 2010;
DOI: 10.1542/peds.2010-0433