Lactobacillus reuteri for Infants with Colic: A Double-Blind, Placebo-Controlled, Randomized Clinical Trial

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Objective To assess the safety of probiotic *Lactobacillus reuteri* strain Deutsche Sammlung von Mikroorganismen (DSM) 17938 with daily administration to healthy infants with colic and to determine the effect of *L reuteri* strain DSM 17938 on crying, fussing, inflammatory, immune, and microbiome variables.

Study design We performed a controlled, double-blinded, phase 1 safety and tolerability trial in healthy breast-fed infants with colic, aged 3 weeks to 3 months, randomly assigned to *L reuteri* strain DSM 17938 (5×10^8 colony-forming units daily) or placebo for 42 days and followed for 134 days.

Results Of 117 screened infants, 20 were randomized to *L reuteri* strain DSM 17938 or placebo (sunflower oil) (in a 2:1 ratio) with 80% retention. Eleven of the 20 (55%) presented with low absolute neutrophil counts (<1500/mm^3), which resolved in all subjects by day 176. *L reuteri* strain DSM 17938 produced no severe adverse events and did not significantly change crying time, plasma bicarbonate, or inflammatory biomarkers. Fecal calprotectin decreased rapidly in both groups. In the infants with dominant fecal gram negatives (*Klebsiella*, *Proteus*, and *Veillonella)*, resolution of colic was associated with marked decreases in these organisms.

Conclusions Daily administration of *L reuteri* strain DSM 17938 appears to be safe in newborn infants with colic, including those with neutropenia, which frequently coexists. A placebo response of 66% suggests that many infants with colic will have resolution within 3 weeks. *(J Pediatr 2017;191:170-8).*

Trial registration ClinicalTrials.gov: NCT01849991.

See editorial, p 6

Colic is defined as inexplicable and severe crying in an otherwise-healthy newborn. Despite 40 years of research, little is known about its pathogenesis. Colic appears to represent abdominal pain, as manifested by abdominal distension and tenderness. In the original review by Wessel et al,[1] colonic hyperperistalsis was emphasized, and the use of enemas was suggested. Of babies with colic, 92% were reported to cry mainly after feedings,[2] also consistent with a problem in the gastrointestinal tract. Two meta-analyses have suggested that *Lactobacillus reuteri* strain Deutsche Sammlung von Mikroorganismen (DSM) 17938 significantly reduces infant crying and fussing time in breast-fed infants with colic.[3,4]

An abnormal fecal microbial community in babies with colic was first postulated by Savino et al, who showed increased *Escherichia coli* and reduced *Lactobacilli*.[5] Our previous study suggested increased *Klebsiella* and reduced microbial diversity in these infants.[6] Therefore, we postulated that children with colic may have an abnormal gut microbiome; the intestine may be inflamed in colicky babies, based on a high fecal calprotectin,[6] and *L reuteri* strain DSM 17938 may reduce gut inflammation associated with this dysbiosis.[7-9] During the review of our proposal, the Food and Drug Administration (FDA) asked whether an immunosuppresive

AE	Adverse event
ANC	Absolute neutrophil count
FDA	Food and Drug Administration
IL	Interleukin
NCCIH	National Center for Complementary and Integrative Health
RR	Rate ratio

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effect of *L reuteri* strain DSM 17938, potentially produced by increased regulatory T cells,[7] could predispose newborn infants to more infections, lactic acidosis, or even lactobacillus bacteremia.

The aim of this study was therefore to demonstrate the safety of a liquid probiotic *L reuteri* strain DSM 17938, given over a 42-day period in infants with colic. In addition, we sought to investigate biomarkers that might give insight into the mechanism of action of *L reuteri* strain DSM 17938 related to infant colic.

Methods

This trial was a single center, randomized, double-blind, placebo-controlled trial, ClinicalTrials.gov: NCT01849991. Protocol and amendments were approved by the institutional review board at the University of Texas Health Science Center at Houston (HSC-MS-11-0203) and the FDA (investigational new drug: 13561); reviewed by the National Center for Complementary and Integrative Health (NCCIH) (5R34AT006727) and overseen by the Office of Clinical Research Affairs.

Screening included parent/guardians signing of the informed consent, a physical examination by one of the research clinicians, and a clinical blood draw. Barr diaries[10] required for eligibility had to show greater than 2 of 3 days of 3 hours' daily of crying and fussing (nonconsecutive) at age 21-90 days, with a checked box stating that on the days with >3 h/d "this was a typical day." Subjects were required to have no previous or continuous probiotic use; no history of antibiotic exposure; and to be otherwise healthy and exclusively breast-fed. Five of the children in the study were on acid blockers (4 on lansoprazole, 1 on ranitidine); they were not disqualified.

Clinical and basic science laboratory assessments were conducted at screening, baseline, and follow-up visits (days 21, 42, 92, and 176). Clinical and basic science laboratory blood draws were collected at screening and end of treatment (day 42). Safety laboratory assessments included complete blood count, comprehensive metabolic panel (consisting of electrolytes, aspartate and alanine aminotransferases, urea nitrogen, creatinine, calcium, glucose, total protein, albumin, and C-reactive protein). Clinical laboratory results generally were considered abnormal if they were 2 times the upper limit in the Memorial Hermann Laboratory Directory of Services for aspartate aminotransferase and alanine aminotransferase; >20% for complete blood count and electrolytes; or >30% for glucose or kidney tests based on healthy infants.[11]

At the baseline visit, eligible subjects randomly were assigned to probiotic (*L reuteri* strain DSM 17938) or placebo (sunflower oil). Vials of sunflower oil and sunflower oil (placebo) with probiotic looked identical. Dose administration was explained to parents (5 drops once daily for 42 days). Physical examinations and laboratory values were completed at each visit. Stool also was collected for microbiota analysis and fecal calprotectin at baseline (day 1), at the end of treatment (day 42), and during observation period (day 92). Crying and fussing times were graded via the Barr diary, 2 diaries per week until day 92.[2] Case report forms were completed during each clinic visit. Weekly communications were completed through telephone calls or via email. Clinical visits were performed at Memorial Hermann Hospital/University of Texas Health Clinical Research Unit, Houston. Adverse events (AEs) were monitored strictly based on the FDA Adverse Events Response System and a clinical severity index.[12]

The biostatistician developed a block randomization with block size of 6 for allocation to each group. Randomization was implemented by research pharmacists. To detect potential differences in safety, subjects were randomized via a ratio of 2:1 (treatment to placebo).

The dose of *L reuteri* strain DSM 17938 was approximately 5×10^8 colony-forming units (given as 5 drops) or placebo (sunflower oil) (provided by BioGaia AB, Stockholm, Sweden). All *L reuteri* strain DSM 17938 vials contained $\sim 5 \times 10^8$ colony-forming units per day during treatment, documented by anaerobic cultures of every fifth returned vial.

Safety (primary outcome) was defined by strict monitoring of AEs and severe AEs throughout the study. A daily diary card was completed by the each study subject's parent, 2 days per week until the fifth visit. Secondary outcomes allowed us to estimate the effect sizes of biomarkers for future studies, which included crying and fussing time, immunologic, microbiologic, and hematologic findings.

The independent medical monitor and data safety monitoring board examined progress throughout the trial, convening after enrollment and follow up every 12 subjects. Study data were collected and managed with REDCap (Research Electronic Data Capture).[13] Our data management system allowed logic checks to ensure data quality. All errors or discrepancies were corrected with a Web-based query program.

Peripheral blood mononuclear cells were isolated from whole blood and processed by flow cytometry.[14]

Plasma levels of interleukin (IL)-1β, IL-2, IL-10, and tumor necrosis factor-α, tissue inhibitor of metalloproteinase-1, and osteoprotegerin were assessed by the use of human single or multiplex panel kits from Meso Scale Discovery (Meso Scale Diagnostics LLC, Rockville, Maryland); plasma tumor necrosis factor–like weak inducer of apoptosis was assessed by using a human enzyme-linked immunosorbent assay kit from eBioscience (a division of Thermo Fisher Scientific, Waltham, Massachusetts).

Stool samples were prepared and analyzed per manufacturer's instructions by fecal calprotectin enzyme-linked immunosorbent assay kit (Eagle Biosciences, Nashua, New Hampshire) as described.[14]

Parents were instructed to collect a stool sample within 48 hours of the visit; stool samples were subdivided and stored at –80°C until analyzed. DNA extraction, polymerase chain reaction amplification, pyrosequencing, and taxonomic identification of 16S rRNA gene sequences in stool specimens were performed as previously described[15] with QIIME[16] and the R statistical package R (R version 3.3.1, R Foundation for Sta-

tistical Computing, Vienna, Austria)[17] to analyze the microbial communities.

Statistical Analyses

Categorical variables were reported as frequency and percentages and compared with the Fisher exact test or χ^2 test. Normally distributed variables were summarized by means and SD and compared with the 2-sample *t* test, and variables that were not normally distributed were summarized by medians with IQRs and compared with the Wilcoxon rank sum test. For the primary analysis of AEs, we compared the percentage of subjects who experienced at least 1 AE between the 2 study arms by the Fisher exact test and estimated the rate ratio (RR) and 95% CI through Poisson regression. For the comparison of Barr diary crying time and fecal calprotectin, logarithmic transformation was applied to normalize distribution, and longitudinal models were used to compare the 2 groups. The adjusted geometric means and 95% CIs by groups were calculated. All the aforementioned analyses were conducted via the statistical software SAS 9.4 (SAS Institute, Cary, North Carolina; R version 3.3.1, ggplot 2.1.0 and gridExtra2.2.1; R Foundation for Statistical Computing).[17]

Results

Of 117 screened infants, 70 were eligible. Consent was obtained from parent/guardians between August 2013 until February 2016 of 21 infants, of whom 20 were randomized (**Figure 1**). During screening of infants, 54 were excluded because of formula feeding or previous probiotic use; 16 others were too old or had other medical conditions that led to

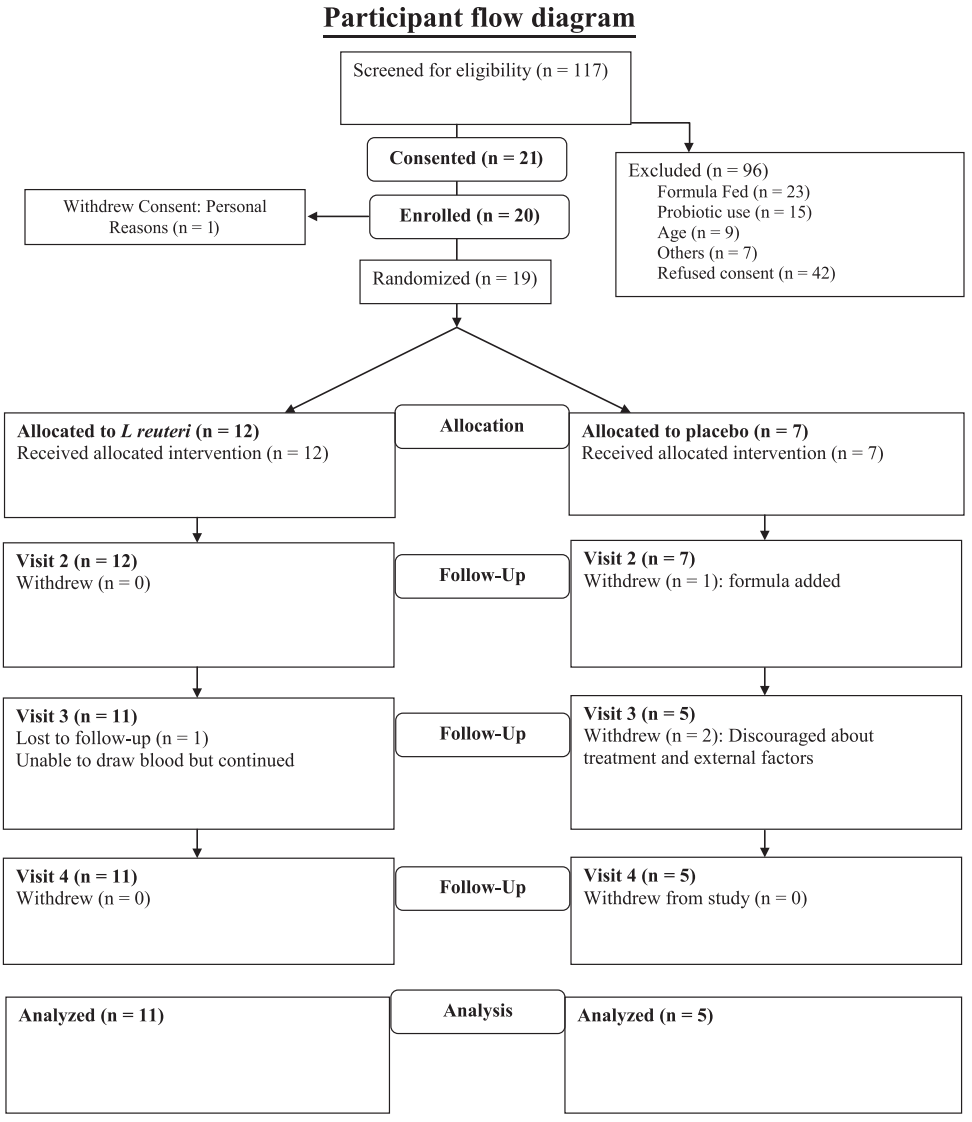

Figure 1. Participant consort diagram.

exclusion (**Figure 1**). Forty-three families either refused to participate or withdrew their initial consent, mostly because of blood drawing and the requirement of 5 subsequent visits to the clinic. Note that we originally aimed to screen 70 subjects to enroll 45 who met eligibility criteria. The proposed sample size (n = 45) was based on statistical analysis indicating that 30 treated patients would be enough to detect a significant increase in the number of expected cases of sepsis or fever (more than 2 cases) in children on treatment for 60 days at the $P < .01$ level. During the study, there were no cases of sepsis and only 1 patient developed a fever after a vaccination.

In total, 20 infants received study product (**Figure 1**). Two families were lost to follow-up, and 2 families left the study due to personal reasons. At baseline, there were no major differences between the treatment and placebo groups with respect to baseline characteristics including sex, ethnicity, weight, body mass index, vital signs, white blood cell count, glucose, blood urea nitrogen, or C-reactive protein, although age and length were greater in the *Lactobacillus reuteri* strain DSM 17938 group (**Tables I** and **II**; available at www.jpeds.com). All infants were healthy with normal growth indices.

There were no significant differences in major safety laboratory assessments by 42 days (**Table III**; available at www.jpeds.com). There was also no significant difference in crying plus fussing time (**Table IV**). The number of responders (defined as a 50% reduction in crying plus fussy time) at day 21 was 66% in both groups. At day 42, plasma IL-2 level was significantly lower in the *L reuteri* strain DSM 17938–treated group, Helios-positive (thymus-derived) Tregs were decreased by 10%-20%, and total Treg % remained the same in the *L reuteri* strain DSM 17938–treated group.

We assessed safety by comparing the number of AEs in the 2 arms. Sixteen infants had treatment-unrelated AEs, which included thrush, diaper rash, vomiting, diarrhea, dermoid cyst, neutropenia, coryza, upper respiratory infection, and cradle cap. Five (71%) patients in the placebo group experienced at least 1 AE, and 10 (77%) patients in *L reuteri* strain DSM 17938 group experienced at least 1 AE ($P = 1.00$). The average number of AEs in *L reuteri* strain DSM 17938 group and placebo were 2.7 and 1.6, respectively; and the RR of experiencing AE in *L reuteri* strain DSM 17938 group compared with the placebo group was not significantly different, RR 1.71 (95% CI 0.63-4.67, $P = .292$).

At baseline, of the 20 infants that we randomized, 11 (55%) had neutropenia (absolute neutrophil count [ANC] < 1500/mm^3) (**Figure 2**). Most sources define mild neutropenia as counts ranging from 1000 to 1500.[17,18] Seven of the 14 infants with laboratory assessments, specifically complete blood counts at both the beginning and the end of treatment, had neutropenia during the study (**Figure 2**). In the 13 infants who were to receive *L reuteri* strain DSM 17938, the mean ± SD was 1400 ± 500/mm^3, and the mean ± SD in the 7 infants to receive placebo was 1700 ± 700/mm^3. In contrast, the mean ± SD for 1-month-old infants has been reported to be much greater (3800/mm^3, with a range of 1000-9800/mm^3).[18,19] Because neutropenia is associated with increased risk of infection, when some infants were noted at study completion to have neutropenia, our data safety monitoring board conferred with NCCIH

Table IV. Summary statistics of baseline Barr diary and fecal calprotectin by treatment group

Measurements	*L reuteri* n = 13	Placebo n = 7	*P* value
Barry diary			
Visit 2 (baseline), median (IQR)			
Crying and fussing time, min	275 (267, 368)	283.5 (255, 612)	.66
Crying time, min	109.5 (70, 185)	96.0 (38, 140)	.43
Fussing time, min	170.0 (147, 217)	231.0 (187, 502)	.09
Barr diary			
Follow-up visits, adjusted means (95% CI)			
Crying and fussing time, min			
Visit 3 (day 21)	99 (42, 234)	164 (110, 246)	.31
Visit 4 (day 42)	94 (61, 144)	29 (5, 162)	.19
Visit 5 (day 92)	36 (17, 78)	35 (14, 88)	.96
Crying time, min			
Visit 3 (day 21)	19 (6, 56)	17 (3, 81)	.92
Visit 4 (day 42)	14 (5, 34)	11 (2, 52)	.86
Visit 5 (day 92)	3 (1, 7)	5 (1, 22)	.55
Fussing time, min			
Visit 3 (day 21)	68 (28, 164)	111 (70, 177)	.38
Visit 4 (day 42)	58 (34, 100)	20 (5, 76)	.15
Visit 5 (day 92)	31 (14, 72)	22 (10, 49)	.56
Fecal calprotectin, μg/g, median (IQR)			
Visit 1 (baseline)	216 (132, 266)	148 (82, 192)	.19
Follow-up visits, adjusted means (95% CI)			
Visit 4 (day 42)	140 (78, 251)	103 (62, 172)	.50
Visit 5 (day 92)	75 (48, 118)	94 (58, 150)	.57

The adjusted geometric means and 95% CI of Barr diary data and fecal calprotectin data at follow-up visits are shown after we controlled for age and each individual baseline values. *P* values for baseline data are obtained by Wilcoxon rank sum test. For follow-up visits, longitudinal models were used as follows. Longitudinal model: (1). ln(Barr diary) = $\beta 0 + \beta 1$*visit3 + $\beta 2$*group + $\beta 3$*visit3*group + $\beta 4$*visit4 + $\beta 5$*group + $\beta 6$*visit4*group + $\beta 7$*(Age at baseline) + $\beta 8$*(Barr diary at baseline); Here, visit3and visit4 are dummy variables; visit3 = 1 if at visit 3, 0 otherwise; visit4 = 1 if at visit 4, 0 otherwise; group = 1 if in *L reuteri* strain DSM 17938 group, 0 otherwise; (2). ln(fecal calprotectin) = $\beta 0 + \beta 1$*visit4 + $\beta 2$*group + $\beta 3$*visit4*group + $\beta 4$*(age at baseline) + $\beta 5$*(fecal calprotectin at baseline); Here, visit4 is dummy variable; visit4 = 1 if at visit 4, 0 otherwise; group = 1 if in *L reuteri* strain DSM 17938 group, 0 otherwise.

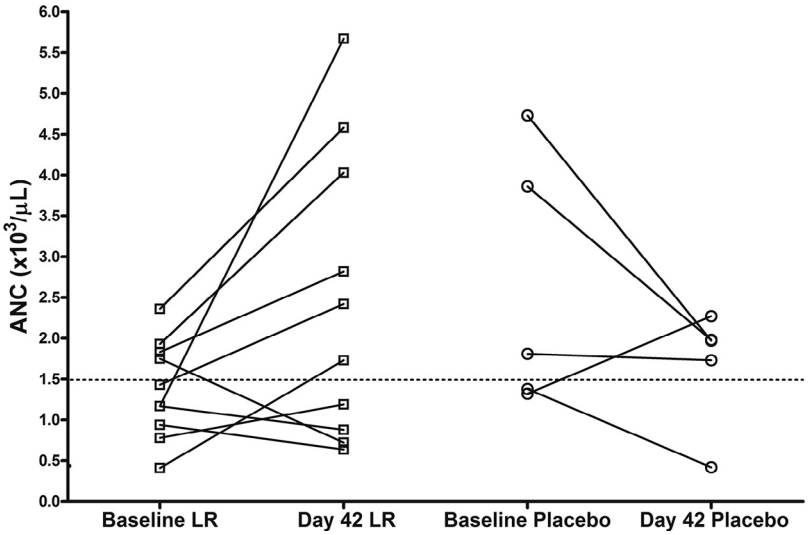

Figure 2. ANC from baseline to 42 days in the participants receiving *L reuteri* strain DSM 17938 or placebo (P). *Dotted line* indicates ANC = 1500, traditionally representing greater risk of infection in pediatric patients. LR, *L reuteri*.

and the FDA and called an interim investigator-blinded safety analysis. It was ruled that treatment with *L reuteri* strain DSM 17938 was not associated with neutropenia. We also found that in 7 of the 10 infants who received *L reuteri* strain DSM 17938 (and who had 2 blood draws), the ANC increased during treatment, whereas an increase in neutrophil count occurred in only 1 of 4 on placebo ($P = .065$). Crying and fussing time declined in both groups during treatment, as shown in **Table IV**, with no significant differences. Secondary analysis was showed no correlation between crying plus fussing time and ANC ($P = .38$).

Baseline value of fecal calprotectin was high in both groups, consistent with mild gastrointestinal inflammation.[6,20,21] During the course of the study, fecal calprotectin decreased (**Table IV**). When we controlled for age and used log transformation, the geometric mean of the comparison of fecal calprotectin at visit 4 vs baseline in *L reuteri* strain DSM 17938 group was 142.9 (95% CI 81.5-250.3), and the geometric mean of the comparison fecal calprotectin at visit 5 vs baseline in the control group was 77.2 (95% CI 51.1-116.6). For comparison, we and others have found that mean normal levels in adults are <50 μg/g.[14,22] Because all children in the current study were on breast milk throughout the study, we examined levels of calprotectin in breast milk. Fecal calprotectin in breast milk was 1475 ± 170.8 ng/mL, or 1.5 ± 0.17 μg/g, n = 3, which was much lower than that seen in feces (**Table IV**).

Fecal alpha-diversity, as assessed by the Shannon, Chao1, or Simpson diversity indices, showed no significant difference between the *L reuteri* strain DSM 17938–treated and placebo groups at days 1, 42, or 90. The stool specimens of all infants were dominated by a few (1-3) highly abundant species. These dominant species accounted for most (65%-87%) of the operational taxonomic units (or species) in the specimens. In fact, at baseline, the stools of 8 of 10 infants with colic at day 1 and day 42 had a single dominant species that accounted for >60% of the total species.

There were 8 *L reuteri* strain DSM 17938–treated infants with stools from the pretreatment and end-of-treatment visits with adequate stool DNA, and for those in the placebo group, there were 4 complete sets (visit 1, 3, and 4). Major operational taxonomic units at the family level (accounting for >60% of the total) are shown in **Table V** (available at www.jpeds.com). Of the 8 infants in the *L reuteri* strain DSM 17938 group, 4 were dominated by members of the family Gammaproteobacteria (*Escherichia* in 3 and *Klebsiell*a in 1); other dominant species included *Clostridia* (n = 2), *Bacteroides* (n = 1), and *Veillonella* (n = 1) (**Figure 3**). Of the 4 infants in the placebo group, the dominant family was *Bacteroides* in 3 and *Prevotella* in 1 (**Figure 3**). Thus, the infants in the 2 groups were not directly comparable with respect to community structure at entry.

During treatment, there were dramatic shifts in the percentage contributions of these organisms, without a major change in richness (alpha-diversity) in either group (**Figure 3**). In the *L reuteri* strain DSM 17938 group, we saw a major change (shift in percentage 5%-60%) of the major taxa in all patients.

Escherichia was the most consistently identified family in all infants participating in the study and was found in both study groups (**Figure 3**). At visit 4, when the colic had resolved, the percentage of *Escherichia* increased in 5 of 6 infants. A second family that is known to be an early colonizer of human infants, *Bacteroides*, was found to be prevalent in our population; its percentage abundance in stool also increased as the crying time decreased.

L reuteri strain DSM 17938 did not change any of the biomarkers of inflammation in plasma that we measured (**Table III**). *L reuteri* strain DSM 17938 did not change the total percentage of circulating Tregs, as measured by Foxp3 and CD25 positivity, but did reduce the number of thymus-derived

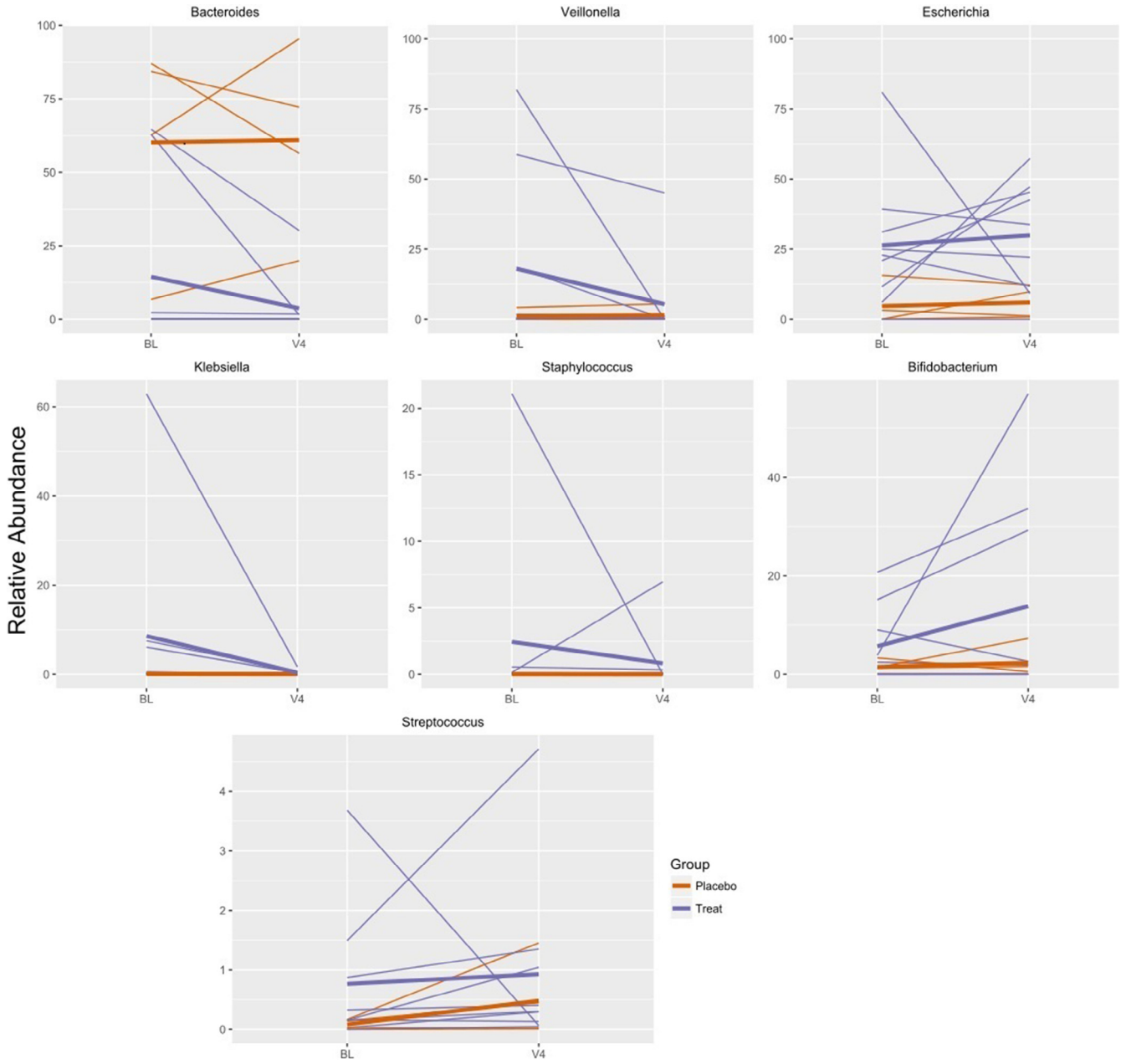

Figure 3. Changes in relative abundance of prominent genera over time in infants treated (*blue*) and infants give a placebo (*orange*). Each *thin line* represents a single infant. The mean for treated and placebo infants is represented by *bold lines* of the corresponding color. Only infants that had baseline (BL) and fourth-visit (V4) specimens are represented in the graph.

(Helios$^+$) Tregs (**Table III**), suggesting that there was a coordinate increase in peripheral inducible Tregs to balance the difference.

Discussion

In this pilot randomized clinical trial, we found no significant changes in important safety and immune markers in infants with colic treated with probiotics. A strength of this study was the very careful follow-up of population of breast-fed infants with colic. The current study was designed with scrutiny by FDA and the National Institutes of Health/NCCIH, due to previous reports of *Lactobacillus* bacteremia, meningitis, endocarditis, and D-lactic acidosis, mostly in adults.[23,24] Furthermore, newborn infants are considered a vulnerable population. In 2014, a fatal case of gastrointestinal mucormycosis caused by *Rhizopus oryzae* was reported in an infant born premature. The infant had been given a probiotic to prevent necrotizing enterocolitis, but testing of the same lot of unopened probiotic powder revealed contamination with *R oryzae*.[25]

None of the 20 studies in the meta-analysis of probiotics used to prevent necrotizing enterocolitis by Wang et al, and none of the *L reuteri* strain 17938 colic trials in the systematic reviews by Xu et al or by Harb et al were conducted in the US, most likely because of the requirement for US Food and Drug Administration approval.[4,26,27] Our study demonstrated that in a small but carefully selected sample of healthy newborns, with 42 days of daily treatment with *L reuteri* strain DSM 17938, there was no increase in the rate of infection, lactic acidosis, gastrointestinal symptoms, or other AEs. However, findings of safety in such a small sample size cannot be overstated.

An unexpected finding encountered in more than one-half of the infants was neutropenia, an abnormality associated with increased risk of infection. Mild neutropenia generally has been defined as an ANC < 1500/mm^3 in children.[19] At baseline, 8 infants had peripheral blood neutropenia, 6 *L reuteri* strain DSM 17938–treated and 2 placebo-treated, a finding that improved in the *L reuteri* strain DSM 17938–treated group with time, whereas in the placebo group we found that 3 of 4 cases (who had blood samples available at study beginning and completion) had either no change or a decrease in ANC. Because of small numbers and this being a safety trial, we cannot make definitive statements about the correlation between colic and a low neutrophil count. However, our studies lead to 2 considerations, that the "old literature" normal range of neutrophil counts for infants in the 2- to 6-month-old age range should be lower or that colic may be a condition associated with neutropenia. One hypothesis consistent with our data is that in the infant with colic, mild intestinal inflammation could lead to neutrophil emigration into the gut lumen, resulting in an elevated fecal calprotectin (which constitutes 60% of neutrophil cytosolic protein). The blood neutrophil count is replenished by production, with release of neutrophils from the bone marrow. More than 90% of mature neutrophils are in the bone marrow. However, during early postnatal life, it is not unusual for infections to cause neutropenia, especially viral infections. We do not believe any of our patients had neonatal alloimmune neutropenia or congenital neutropenias.[28]

Our working hypothesis is that gut inflammation may be related to colic.[6] The current results are consistent with this hypothesis. At enrollment, ~50% of the infants presented with platelet counts >450 000, a condition not unusual at this age but generally thought to be suggestive of mild inflammation.[29] Furthermore, fecal calprotectin was elevated at enrollment and diminished over the course of the study in both groups of infants. Calprotectin is viewed as an innate antimicrobial peptide.[30] We chose to monitor this marker because levels of the often-used alternative biomarker, lactoferrin, are elevated in children on breast milk. Konikoff and Denson reviewed previously the finding that calprotectin is up to 10-fold greater in infants than in adults.[22] However, calprotectin may not be an optimal marker for measuring gut inflammation in breast-fed infants, because its level is reported to be increased in children on breast milk.[20,21] Our study has ruled out that the level of calprotectin in breast milk was high enough to explain the increased level in stool.

Although the gut may be inflamed, we did not find evidence of systemic inflammation in these babies, based on a group of cytokines that we assessed in serum, nor did we find evidence in a previous study of colic that used a different panel.[31] Recently, several chemokines were reported to be abnormal in infants with colic by Partty et al.[32] These included IL-8 (generally thought to be a chemokine), monocyte chemotactic peptide-1, and macrophage inhibitory protein-1-beta. Our cytokine levels are similar to those they reported, but we did not measure chemokines. Of note, their study also did not find alterations in cytokines or intestinal fatty acid-binding protein, a peptide released by damaged enterocytes. No group to date has performed endoscopy or colonoscopy on infants with colic to directly address the question of inflammation.

We found that *L reuteri* strain DSM 17938 treatment did not change the percentage of Foxp3$^+$Tregs in peripheral blood compared with placebo, but the percentages of CD25$^+$ and Helios$^+$ populations among Foxp3$^+$Tregs were both lower in *L reuteri* strain DSM 17938 groups compared with placebo group at day 42. In human CD4$^+$ cells, the level of Helios expression is positively associated with CD25 expression.[33] Helios expression in human and murine Tregs discriminates thymic-derived nTregs (Helios$^+$) from inducible Tregs (Helios$^-$, iTregs).[33,34] Our results, therefore, indicate that inducible Tregs in the gut may be "shaped by probiotic."

Previously, Moore et al found evidence of elevated breath hydrogen, suggesting an abnormal population of colonic microorganisms, in their children with colic.[35] In a previous report we found *Klebsiella* in 8 of 17 infants with colic compared with 1 of 18 without colic.[6] We are aware of several previous studies of the fecal microbial composition of infants with colic from other centers. Savino et al showed that there were decreased *Lactobacilli* and increased anaerobic gram-negative organisms in the stools of infants with colic and later found *Lactobacillus brevis* and *Lactococcus lactis* were present only in colicky infants and *Lactobacillus acidophilus* was found only in healthy infants.[36,37] However, Roos et al and Sung et al did not find changes in fecal microbiota or *Escherichia* numbers, respectively.[38,39] In the current study, definitive effects of *L reuteri* strain DSM 17938 on alpha- or beta-diversity or relative composition of the different species could not be addressed because of a poorly diverse microbial population, small numbers of observations, and differential community composition at the time of randomization, similar to Roos et al.[38]

Our study differed from some previously published *L reuteri* strain DSM 17938 trials in that we found 66% of infants in the placebo group had resolution of their colic by 3 weeks. In the previous trials, the placebo response at 3 weeks was low (5%-38%) in 3 studies[3,40,41] but was similar to ours (48%-71%) in the studies of Sung et al and Savino et al.[39,42]

Our study suggests safety and tolerability of probiotic *L reuteri* strain DSM 17938 in infants with colic. We frequently found laboratory abnormalities in these infants, such as neutropenia, thrombocytosis, and elevated fecal calprotectin, possibly consistent with mild gut inflammation. Future research will be needed to prove the concepts of dysbiosis, gut

inflammation, and probiotic efficacy in infants with this condition. ■

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50 Years Ago in *The Journal of Pediatrics*

Prevention and Management of Acute Hyperuricemia in Childhood Leukemia

Holland P, Holland NH. J Pediatr 1968;72:358-66.

Tumor lysis syndrome is a frequent complication of childhood leukemia caused by rapid turnover of leukemic blasts. The most feared consequence of tumor lysis syndrome is oliguric renal failure secondary to formation of uric acid crystals in the kidneys. Holland and Holland presented the clinical course of 5 children with acute lymphoblastic leukemia and tumor lysis syndrome. These children were treated with hyperhydration, urinary alkalinization, diuresis, and dialysis. Two of the 5 patients were treated with allopurinol, making theirs the second report in the literature to describe the use of allopurinol in children.[1]

Allopurinol blocks the production of uric acid, which accumulates in the blood as DNA is broken down from leukemic blasts. More than 60 years ago, chemists working to synthesize analogs of DNA base pairs serendipitously discovered allopurinol. Their work also led to the discovery of 6-mercaptopurine (6-MP), one of the first chemotherapy drugs used to treat pediatric leukemia. Today, 6-MP remains part of the backbone chemotherapy regimen used to treat childhood acute lymphoblastic leukemia. For the discovery of 6-MP and allopurinol, in addition to other important drugs still in use today, the Nobel Prize in Medicine was awarded in 1988.[2]

Although allopurinol may prevent production of uric acid, it does not degrade existing uric acid. Enzyme therapy with urate oxidase or recombinant rasburicase may be used to eliminate existing uric acid. In clinical trials, rasburicase significantly reduced uric acid levels in patients with hyperleukocytosis and high risk of tumor lysis syndrome.[3,4] Fifty years later, allopurinol is still used prophylactically in patients with low risk of tumor lysis syndrome, whereas rasburicase is used prophylactically in patients with hyperleukocytosis and high risk of tumor lysis syndrome.

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Table I. Comparison of baseline characteristics of randomized patients in the 2 study groups

Variables	*Lactobacillus reuteri* strain DSM 17938 (n = 13)	Placebo (n = 7)	*P*
Age at the time randomized, d, median (Q1, Q3)	57 (39, 72)	40 (34, 51)	.053*
Gestational age, wk, mean ± SD	39.3 ± 1.3	39.1 ± 1.0	.72†
Birth weight, kg, mean ± SD	3.4 ± 0.5	3.3 ± 0.4	.74†
Birth height, cm, mean ± SD	52.2 ± 2.0	50.3 ± 2.5‡	.10†
Weight at the time randomized, kg, mean ± SD	5.1 ± 0.8	4.8 ± 1.0	.41†
Height at the time randomized, cm, mean ± SD	59.3 ± 2.6	55.7 ± 3.7	.02†
Male, n (%)	9 (69)	3 (43)	.35§
Race, n (%)			NR
White	11 (85)	3 (43)	
African American	1 (8)	0 (0)	
Asian or Pacific Islander	1 (7)	3 (43)	
Mix (white/Asian)	0 (0.0)	1 (14)	
Ethnicity, n (%)			.27§
Not Hispanic or Latino	12 (92)	5 (71)	
Hispanic or Latino	1 (8)	2 (29)	
Breast feed, n (%)			N/A
Yes	13 (100)	7 (100)	
Any formula, n (%)			1.00§
No	10 (77)	5 (71)	
Yes	3 (23)	2 (29)	
Formula type			
Earth's Best	1 (33)	0 (0)	
Gentls Good Start	1 (33)	0 (0)	
Similac Advance	1 (33)	1 (50)	
Similace Sensitive	0 (0)	1 (50)	

N/A, not applicable; *NR*, not reported due to zero cells; *Q1*, first quartile; *Q3*, third quartile.
*Denotes *P* values obtained by Wilcoxon rank sum test.
†Denotes *P* values obtained by 2-sample *t* test.
‡n = 5.
§Denotes *P* values obtained by Fisher exact test.

Table II. Comparison of clinical parameters by treatment group at baseline visit and day 42

Parameters (normal range for age)	Baseline: pretreatment, mean ± SD			Day 42: post-treatment, mean ± SD		
	L reuteri strain DSM 17938 (n = 13)	Placebo (n = 7)	*P* value	*L reuteri* strain DSM 17938 (n = 10)	Placebo (n = 5)	*P* value
Hgb	11.6 ± 1.5	11.7 ± 1.2	.92*	11.4 ± 1.0	11.0 ± 0.7	.42*
HCT	34.0 ± 5.0	34.5 ± 4.2	.83*	33.6 ± 3.4	31.8 ± 2.0	.30*
WBC	8.1 ± 1.8	10.4 ± 2.9	.03*	9.7 ± 2.5	10.6 ± 2.5	.47*
ANC	1.4 ± 0.5	2.4 ± 1.4	.10*	2.5 ± 1.8	1.7 ± 0.7	.35*
Lymphocytes (%)	68.4 ± 9.7	66.5 ± 11.6	.69*	66.6 ± 14.3	76.6 ± 9.4	.18*
Eosinophils (%)	5.5 ± 3.4	4.1 ± 1.6	.30*	3.3 ± 1.5	3.2 ± 2.2	.96*
Platelets	421.2 ± 154.5	392.1 ± 117.1	.66*	439.2 ± 120.3	481.8 ± 129.9	.53*
CRP	2.4 ± 1.1	2.1 ± 1.3	.63*	2.3 ± 1.8	2.9 ± 0.0	.36*
SGOT	41.1 ± 15.1	43.0 ± 22.6	.82*	44.2 ± 13.9	40.8 ± 11.7	.64*
SGPT	43.6 ± 14.5	39.4 ± 13.5	.53*	44.1 ± 15.1	44.2 ± 12.9	.99*
Bilirubin	2.4 ± 3.2	3.6 ± 3.9	.45*	0.7 ± 1.0	0.5 ± 0.3	.64*
Blood urea nitrogen	5.2 ± 1.4	6.7 ± 2.7	.10*	5.8 ± 2.9	5.8 ± 1.9	1.00*
Creatinine	0.3 ± 0.1	0.2 ± 0.1	.53*	0.2 ± 0.1	0.3 ± 0.1	.56*
Bicarbonate	22.5 ± 2.2	22.1 ± 3.4	.80*	24.8 ± 5.8	22.2 ± 0.8	.19*

CRP, C-reactive protein; *HCT*, hematocrit; *Hgb*, hemoglobin; *SGOT*, serum glutamic oxaloacetic transaminase; *SGPT*, serum glutamic pyruvic transaminase; *WBC*, white blood cell.
*Denotes *P* values obtained by 2-sample *t* test.

Table III. Comparison of immune markers data by treatment group at baseline visit and day 42

	Baseline: pretreatment, median (Q1, Q3)			Day 42: post-treatment, median (Q1, Q3)		
	L reuteri strain DSM 17938 (n = 13)	Placebo (n = 6)	*P* value	*L reuteri* strain DSM 17938 (n = 11)	Placebo (n = 5)	*P* value
IL-1β	0.1 (0.0, 0.1)	0.0 (0.0, 0.0)	.34*	0.0 (0.0, 0.1)	0.0 (0.0, 0.0)	.14*
TNF-α	3.5 (3.1, 3.9)	3.5 (3.4, 4.3)	.79*	4.1 (3.0, 4.8)	3.8 (3.3, 4.9)	.95*
IL-10	0.7 (0.6, 0.9)	0.6 (0.6, 0.7)	.66*	0.6 (0.4, 1.2)	0.8 (0.5, 0.9)	.78*
IL-2	0.2 (0.2, 0.4)	0.3 (0.2, 0.3)	.44*	0.1 (0.1, 0.2)	0.2 (0.2, 0.2)	.05*
OPG	355.6 (312.3, 388.1)	367.2 (200.8, 441.0)	.76*	359.5 (308.0, 456.0)	394.2 (336.6, 476.0)	.69*
TIMP-1	135 699.8 (124 171.9, 146 316.9)	152 836.2 (116 720.4, 168 662.1)	.79*	126 898.2 (98 393.5, 219 956.6)	148 937.0 (129 529.2, 153 964.6)	.69*
TWEAK	363.4 (341.3, 441.0)	425.3 (324.8, 447.8)	.63*	384.5 (309.5, 524.2)	476.0 (394.2, 477.4)	.86*
CD4+Foxp3+ within CD4+	7.2 (6.5, 7.8)	6.2 (4.7, 7.2)	.39*	6.6 (4.4, 7.8)	7.1 (6.1, 10.6)	.25*
$CD4^+Foxp3^+CD25^+$ within $CD4^+Foxp3+$	70.0 (68.0, 73.5)	68.0 (58.3, 84.2)	.72*	56.8 (43.9, 74.0)	77.5 (76.5, 78.0)	.01*
$CD4^+Foxp3^+HELIOS^+$ within $CD4^+Foxp3^+$	87.7 (86.4, 89.2)	82.9 (76.7, 88.4)	.19*	82.4 (66.1, 87.2)	90.2 (88.8, 91.0)	.04*

OPG, osteoprotegerin; *TIMP-1*, tissue inhibitor of metalloproteinase-1; *TNF-α*, tumor necrosis factor-α; *TWEAK*, tumor necrosis factor–like weak inducer of apoptosis.
*Denotes *P* values obtained by Wilcoxon rank sum test.

Table V. Major OTUs at enrollment and changes following 42 days of treatment with *L reuteri* strain DSM 17938 or placebo

Subjects	Major OTU (%)	Second major OTU (%)	Third major OTU (%)
L1	*E coli* (39%→34%)	*Staphylococcus* (21%→0%)	*Proteus* (17%→4%)
L2	*Bacteroides* (65%→30%)	*E coli* (25%→22%)	*Oscillospira* (4%→11%)
L3	*Klebsiella* (62%→2%)	*Hemophilus* (27%→1%)	*E coli* (6%→57%)
L4	Lachnospiraceae (33%→21%)	*E coli* (23%→12%)	*Roseburia* (9%→2%)
L5	*Bacteroides* (63%→2%)	*Bifidobacterium bifidum* (21%→33%)	*E coli* (12%→47%)
L6	*Veillonella* (58%→45%)	*E coli* (31%→45%)	*Clostridium* (9%→2%)
L7	*E coli* (81%→0%)	*Clostridium* (19%→0%)	*B bifidum* (15%→29%)
L8	*Clostridium* (35%→3%)	*E coli* (21%→42%)	*Veillonella* (19%→0)
P1	*Prevotella* (78%→40%)	*Ruminococcus* (8%→1%)	*Bacteroides* (7%→20%)
P2	*Bacteroides* (62%→95%)	*Parabacteroides* (17%→1%)	*Ruminococcus* (10%→2%)
P3	*Bacteroides* (84%→72%)	*E coli* (16%→12%)	None other (all <1%)
P4	*Bacteroides* (87%→56%)	*Ruminococcus* (5%→1%)	None other (all <1%)

OTU, operational taxonomic unit.
Arrow reflects the changes in the relative abundance from the beginning to the end of study. Only results for infants who had fecal samples available at baseline and day 42 are shown.