Infant Colic Represents Gut Inflammation and Dysbiosis

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Objective To dissect potential confounding effects of breast milk and formula feeding on crying + fussing, fecal calprotectin, and gut microbiota in babies with colic. We hypothesized that infant colic is associated with gut inflammation linked to intestinal dysbiosis.

Study design A nested case-control design of 3 of our studies was used to analyze clinical and laboratory data at presentation, comparing babies with colic with controls. All investigators other than the biostatistician were blinded during data analysis. Subjects were recruited based on their age and crying + fussy time. We screened 65 infants, 37 with colic, as defined by Barr diary (crying + fussing time >3 hours daily), who were compared with 28 noncolicky infants.

Results Fecal calprotectin was elevated in babies with colic. For each mode of infant feeding (breast milk, formula, or breast + formula), infants' fecal calprotectin was higher in babies with colic. Infants with colic had similar levels of fecal alpha diversity (richness) when compared with controls, and alpha diversity was lower in breast-fed babies. Beta diversity at the phylum level revealed significant differences in microbial population. A phylum difference resulted from reduced Actinobacteria (95% of which are Bifidobacilli) in babies with colic. Species significantly associated with colic were Acinetobacter and Lactobacillus iners.

Conclusions Colic is linked with gut inflammation (as determined by fecal calprotectin) and dysbiosis, independent of mode of feeding, with fewer Bifidobacilli. (J Pediatr 2018;169:159-167.)

Trial registration Clinicaltrials.gov: NCT01279265 and NCT01849991.

Infant colic, defined as 3 hours of crying + fussing for more than 3 days/week in an infant <3 months of age, affects 10%-15% of the normal infant population.1 Virtually all reviews conclude that there is no established etiology for colic, although milk allergy, esophageal reflux, migraine headache, sensory processing deficits, and difficulties in effective suckling have been invoked.2-4

Several research groups previously found evidence of dysbiosis, or an abnormal gut microbial community in these babies.5-7 Dysbiosis has been associated with a variety of human gastrointestinal diseases, including irritable bowel syndrome and inflammatory bowel disease.8 We have suggested that microbial alterations in colic are associated with gut inflammation, as measured by fecal calprotectin, an antimicrobial protein released from enteric neutrophils used for the assessment of gut inflammation.6 However, earlier studies have shown similarly high levels of fecal calprotectin in infants with and without colic.9

Sequential calprotectin measurements in babies with colic have consistently showed a reduction as the crying improved,10,11 but some have suggested that this is a normal developmental phenomenon.12,13 One of the confounding factors in the dysbiosis-gut inflammation hypothesis for colic is that babies who are fed breast milk may have higher levels of fecal calprotectin than those fed formula,14 although this has not been found consistently.15 We previously reported that calprotectin levels in breast milk were about 1% of the fecal levels.15 Infants taking human milk may have a different microbial population with reduced alpha-diversity (number of species) compared with those on infant formula, complicating conclusions about colic and microbial diversity.16 In the current report, we addressed 2 questions: Is colic associated with gut inflammation and dysbiosis? Or, alternatively, are elevated fecal calprotectin and abnormal microbiota artifacts produced by the effects of milk type and an incompletely developed neonatal microbiome?

Methods

This nested case-control study of infants (aged 21-90 days) included infants enrolled in our 2 previous double-blind, placebo-controlled, randomized trials6,11 or recruited as age-matched controls who did not cry >3 hours/day. All samples were processed and analyzed identically at baseline before receiving the study product (probiotic or placebo) in both trials (Figure 1; available at www.jpeds.com). All authors had access to the study data and reviewed and approved the final manuscript. We assessed crying by a validated technique, the Barr diary,6,17 and we...
required demonstration of an average of >3 hours of (non-consecutive) crying and fussing time for 2 of 3 days. The studies were approved by the University of Texas Health Science Center at Houston Institutional Review Board (HSC-MS-10-0048 and HSC-MS-11-0203) and registered in ERA Commons (Clinicaltrials.gov: NCT01279265 and NCT01849991).

Recruitment took place through local offices of pediatricians and the University of Texas-Houston clinics. Advertising was via mailings, media coverage, and a website. The parents of noncolicky infants also provided a Barr diary. We excluded infants with <32 weeks of gestation, failure to thrive, lung disease, diarrhea, fever, or previous consumption of a probiotic or antibiotic. Breastfed infants were supplemented with vitamin D, with no other supplements. Screenings included 65 individuals, 37 with colic and 28 without colic. Evaluators of samples and data were blinded upon processing and analysis for each individual. All stool samples were processed for fecal microbiota analysis; 13 were excluded from fecal calprotectin analysis because of an inadequate quantity for testing (Figure 1).

**Stool Microbial Community Analysis**

Parents collected a stool sample within 48 hours of the visit. Stools were subdivided and stored at ~80°C. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, California). DNA sequencing was performed by the Louisiana State University School of Medicine Microbial Genomics Resource Group. The 16S rDNA hypervariable region V4 was amplified by polymerase chain reaction using gene-specific primers with Illumina adaptors: 515F GTGCCAGCMGCCGCGGTAA and 805R GGACTACHVGGGTWTCTAA. Taxonomic identification and analysis of the V4 16S rDNA gene sequences was accomplished using QIIME (http://qiime.org) and R (R version 3.4.3) with the Phyloseq (v1.22.3; https://github.com/joey711/phyloseq), and Tidyverse (v1.2.1; https://www.tidyverse.org/contribute) libraries. It should be noted that, although stools were collected at baseline from different studies, they were sequenced in the same manner.

**Fecal Calprotectin**

Stools were processed and analyzed according to manufacturer’s protocol by fecal calprotectin enzyme-linked immunosorbent assay kit. The level of calprotectin was expressed as µg/g of stool weight. The assays were done with the same kit (Eagle Biosciences, Nashua, New Hampshire), an assay with high reproducibility for frozen stools.11,15

**Statistical Analyses**

Patient characteristics were compared using the 2 sample t test when normally distributed (variables were reported as mean ± SD) or using Wilcoxon rank sum test if variables were not normally distributed (reported as medians with IQR). The correlation of fecal calprotectin and crying + fussing time was performed by fitting a linear regression model to the normalized data and reported using a Pearson correlation coefficient. Spearman correlations were used to describe relationships between nonnormally distributed continuous variables. Differences in the microbial composition between groups of samples were determined using multiple metrics, including alpha diversity by the number of observed species and Shannon and Simpson diversity indices. Beta diversity was evaluated using the nonparametric analysis of molecular variance as implemented in the Vegan R package (v2.4.5). Taxa abundances were compared using Wilcoxon rank sum tests for pairwise comparison and Kruskal-Wallace rank sum tests for comparisons involving >2 groups. The Benjamin-Hochberg correction was used to control for multiple testing. Random Forrest classification models included relative operational taxonomic unit (OTU) abundance, age, acid blocker usage, fecal calprotectin, and feeding type using the random Forest R package and 20 000 trees and 9 variables tried at each split. A subanalysis was also performed for cases and controls matched by age (±5 days). Fecal calprotectin levels were compared between the matched pairs using a Wilcoxon matched-pairs signed-rank test. Statistical significance was assumed at a type 1 error rate of 5%.

**Results**

Baseline characteristics of the eligible infants for fecal microbiota analysis demonstrated no significant difference between the colic and normal groups in terms of gestational age, age, sex, race, and ethnicity, although there was a trend toward more Caucasian babies in the colic group (Table). All infants were term or late preterm and approximately 2 months old at enrollment. Crying + fussing time was approximately 5 hours in the group with colic and <1 hour in the control group.

**Fecal Calprotectin in Infants with Colic**

To test for intestinal inflammation, we analyzed fecal calprotectin levels as a function of colic (Figure 2, A). In univariable analyses, calprotectin levels were 7-fold higher among breast-fed infants (median, 183; IQR, 234) compared with formula-fed babies (median, 23.7, IQR, 97.7) or breast + formula-fed (median, 107; IQR, 109) infants (P = .031; Figure 2, B). However, multivariable models adjusted for both colic and feeding demonstrated an independent association of calprotectin with colic (P < .001), but not with milk source.

| Table. Demographic distribution between infants with and without colic |
|--------------------------|--------------------------|
|                          | Controls (n = 28)         | Colic (n = 37) |
| Female                   | 8 (38)                   | 10 (36)       |
| Age, d                   | 72 (37-81.5)             | 46.5 (34-60) |
| Race                     |                          |               |
| Non-Hispanic white       | 3 (14)                   | 12 (41)       |
| Hispanic                 | 5 (24)                   | 9 (31)        |
| Black                    | 9 (43)                   | 3 (10)        |
| Asian                    | 3 (14)                   | 4 (14)        |
| Other/mix                | 1 (5)                    | 1 (3)         |

Results are shown as means (IQR) or n (%). No difference was found for sex (P = .55), age (P = .08), gestational age (P = .90), or race (P = .051).
Because crying + fussing time was the determinant for classification of colic, it was also associated with fecal calprotectin in univariable correlation or regression models. However, when stratified by colic status, there was no significant correlation between crying + fussing time and calprotectin levels (Figure 3).

Although age was also not associated with calprotectin level in our models (Figure 4; available at www.jpeds.com), a subanalysis was performed to confirm that the small statistically nonsignificant age difference between the cases and controls was not confounding the results. Matching of cases and controls by age (±5 days) yielded 13 matched pairs, including 8 pairs (62%) that had an age difference within a ±1 day range. Calprotectin levels was higher in cases compared with their age-matched controls (median difference, 204.9 μg/g stool; range, -349.1 to 1002.1; \( P = .016 \)).

Multivariable unpaired models of square root–transformed calprotectin levels, adjusting for independent effects of colic status, feeding type, crying + fussing time and age, and interactive effects of colic and feeding type, yielded colic as the only independent predictor of fecal calprotectin levels (\( \beta = 9.74; 95\% \) confidence interval, 2.96-16.53). That translates to calprotectin levels that were 95 μg/g higher in colicky infants compared with noncolicky infants.

**Figure 2.** Relationship between fecal calprotectin, colic, and feeding modality in infants. A, Infants with colic (n = 29) have significantly higher levels of fecal calprotectin than normal controls (n = 25; \( P < .0001 \), Wilcoxon rank-sum test). B, Infants with colic had higher levels of calprotectin than controls when separated by feeding group (\( P = .019 \), Kruskal-Wallis with post hoc Dunn test and correction for multiple comparisons).

**Microbiota.** We noted that the stool samples had very high sample-to-sample variability, which we have previously reported,\(^1\) likely owing to the nature of the neonatal microbiome. We examined the 99.99% most abundant OTUs, which reduced our data to 5 phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*. The stools of colicky babies were characterized by a significant decrease in the relative abundance of *Actinobacteria* (\( P = .032 \)) and a marginal but not statistically significant increase in *Proteobacteria* (\( P = .07 \)) when compared with normal infants (Figure 5, C). There was no effect of feeding mode on the composition among the 5 major phyla at this young age (data not shown).

More than 95% of phylum *Actinobacteria* was attributable to *Bifidobacterium* in these infants. The relative abundance of genus *Bifidobacterium* among the total bacteria was reduced from a median of 10.1% (IQR, 19.4) in normal infants to 0.3% (IQR, 5.8) in infants with colic (\( P = .049 \)). At the OTU level, OTUs classifying as a *Lactobacillus iners* and an *Acinetobacter* with unknown species level name were found in significantly
higher proportions of colic samples and at a significantly higher abundance than control samples (using 2-sample test for equality of proportions and Wilcoxon rank sum tests). L iners was detected in 26 of 37 babies (70%) with colic, but in only 8 of 28 control samples (29%; \( P = .002 \)). Acinetobacter was detected in 30 of 37 colicky babies’ samples (81%) and 11 of 28 of controls (39%; \( P = .0014 \); Figure 6; available at www.jpeds.com).

**Beta Diversity.** Unweighted unifrac distances were used to assess the beta diversity between samples. Differences between groups were assessed using a nonparametric analysis of molecular variance. Results showed that the fecal microbiota of infants with colic (purple dotted area) overlapped with but was distinctly separated from the composition of normal infants (gold dotted area; Figure 5, D; \( P < .0001 \); \( r^2 = 0.051 \)). Thus, we found a transition of microbiota from normal infants to infants with colic. Beta diversity was affected to a mild extent by feeding type as well (\( P = .016 \); \( r^2 = 0.050 \)).

**Potential Influence of Acid Blockers on Microbial Composition or Fecal Calprotectin**

Because babies with colic frequently vomit, acid blockers are often prescribed. Seven infants with colic were taking lansoprazole, 1 was taking ranitidine, and 1 took both. We examined whether these medications could have influenced results and reanalyzed the data with these 9 infants excluded. Results for crying + fussing time, fecal calprotectin, and microbial beta diversity remained unchanged, with identical conclusions.

We used Random Forest Analysis to determine the most important factors other than crying + fussing time for classifying babies into controls versus those with colic. Taking into consideration fecal microbiota, feeding type, age, use of acid blockers, and fecal calprotectin, we found that by far the most important variable was fecal calprotectin, with age and presence of Acinetobacter being distant second and third most important variables, respectively (Figure 7; available at www.jpeds.com).

**Discussion**

“The sound of a crying baby . . . is just about the most disturbing, demanding, shattering noise we can hear.”18 Colic has been attributed to a number of causes other than gut inflammation, such as stress during pregnancy and negative experiences during childbirth,19 allergy to milk proteins,20 and an infant’s need for additional months of womb-like nurturing.21 However, in the landmark article describing colic in 1954, Wessel et al clearly pointed toward the gastrointestinal tract, citing pain caused by “excessive proteoids like those of the bean which rapidly undergo gaseous decomposition. . .[which] causes violent peristalsis.”1 He also pointed out that “enemas may decrease putrefactive fermentation” in this condition.

Olafsdottir et al compared fecal calprotectin in stools from normal babies with that of babies without colic.9 They reported no differences in fecal calprotectin in the 2 groups, noting very high levels in all babies.9 We and others have reported longitudinal decreases in calprotectin as the colic resolves,11,22,23 but an association between calprotectin and colic has not been established. One problem is that the source of milk may influence calprotectin. Whether or not formula feeding is associated with a decreased level of fecal calprotectin compared with breast milk has been a point of debate.14,24-26 Our results clearly show that the calprotectin level is
associated with the presence or absence of colic, regardless of source of milk. Savino et al confirmed a higher level of calprotectin in babies with colic.\textsuperscript{27} Further support for inflammation in babies with colic comes from Partty et al, who showed low-grade, systemic inflammation in these babies, based on circulating levels of interleukin-8, macrophage inhibitory factor-1-β, and monocyte chemotactic peptide-1.\textsuperscript{28}

Dysbiosis generally refers to a difference in microbial community composition when comparing a diseased group with healthy controls. Microbiota composition of the stool can be programmed by postnatal maturation, medications, dietary choices, antibiotics, lifestyle characteristics, and psychological stress.\textsuperscript{29} Differential colonization of a relatively sterile gut with anti-inflammatory bacteria (\textit{Bifidobacteria}) or proinflammatory taxa (\textit{Proteobacteria}) may underlie the gut inflammation seen in the newborn. Differences in beta diversity have been found in 2 prospective studies of necrotizing enterocolitis in premature infants.\textsuperscript{30,31}

Savino et al investigated if colic could be related to gut dysbiosis and found reduced lactobacilli and increased...
Escherichia coli in colicky babies using polymerase chain reaction.\textsuperscript{3,22} Subsequently, our observations (using denaturing gradient gel electrophoresis) showed an increase in fecal Klebsiella in 50% of babies with colic, compared with 6% of control infants.\textsuperscript{6} In a study that used 16S rDNA microbial sequencing, De Weerth found reduced Bifidobacteria, Lactobacilli, and Bacteroides concomitant with increased Proteobacteria in babies with colic.\textsuperscript{2} Another group took the stools from 14 infants, 7 with colic, and infused a “fecal transplant” into mice, to measure visceral hypersensitivity to colorectal distension using electromyography of the abdominal muscles.\textsuperscript{23} The authors were able to show that the stools from infants with the highest crying time produced the greatest electromyography changes, indicating visceral hypersensitivity, clearly linked to Bacteroides vulgatus and Bilophila wadsworthia.

In the present study, we found lower microbial diversity in breastfed than in formula-fed infants, similar to previous reports,\textsuperscript{34,35} but no major differences in alpha diversity were attributable to colic. We did find significant difference in community composition in babies with colic. Reduced anti-inflammatory Bifidobacilli would be predicted to be associated with increased inflammation; and reduced levels have been recently linked to prenatal maternal stress.\textsuperscript{36} Previously, neither of the species identified as a potential pathobiont in the current study has been linked to colic. L iners is a member of the vaginal flora that has been associated with bacterial vaginosis; it produces a toxin linked to adverse pregnancy outcome.\textsuperscript{37,38} Acinetobacter is also a vaginal organism and an opportunistic pathogen, which has been associated with sepsis.\textsuperscript{39}

A weakness of the study is the relatively small number of infants recruited. We enrolled all infants seen in our general pediatrics and continuity clinics or referred from collaborating private clinics whose parents were willing to participate. We found that some parents were reluctant to fill out the Barr diaries, particularly in the control group, and others were too stressed or busy to participate. The age range of 37–82 days in controls and 34–60 days in those with colic overlapped. The difference in the median value of 3 weeks (statistically non-significant) would not be predicted to have an impact on either of the major 2 outcome variables, fecal calprotectin or microbiota. Figure 4 shows that the fecal calprotectin level shows no trend at all in the age range of 34–82 days. Li et al showed that there is a decrease in fecal calprotectin, comparing children aged 3–6 months with those aged 1–3 months, but that is a much greater age range, at a time during which weaning is taking place and solid foods are being introduced.\textsuperscript{12}

The difference in fecal microbiota during our 3-month timeframe would also not be likely to be significant. We previously studied sequential stools at 4 intervals during this timeframe (in 2 randomized, controlled trials). There is a chaotic composition of the microbiota during this time period; and we have been unable to detect an evolution of a “core” microbiota during the first 90 days. This is particularly evident in Figure 3 (grouped data) and Figure 5 (individual data) in our study of formula-fed babies with colic.\textsuperscript{11}

Other weaknesses of this study include that we did not analyze fecal metabolite levels to identify whether particular bacterial products could be associated with colic. Baldassarre et al found that a number of fecal metabolites increased more in babies with colic who took a placebo than in responders to an 8-organism probiotic.\textsuperscript{40} Metabolites possibly related to colic in their analysis included 2-hydroxy isovalerate, alanine, and 2-oxo-isocaproate. Finally, upper endoscopy or colonoscopy could have been helpful in identifying a site of inflammation, but this would have required general anesthesia and would likely have been rejected by the internal review board.

In summary, our results strongly support the inflammation-dysbiosis theory for infant colic and indicate that the source of milk does not bias these findings. These results may provide a conceptual framework to account for the beneficial effects of the probiotic L reuteri in infants with colic.\textsuperscript{41–43} We thank our participants and their parents, for their visits to the Texas Medical Center and dedication to the study. We also thank Memorial Hermann Hospital/UT Health Clinical Research Unit, Houston, research staff and UT clinical staff and director (Robert Vetman, MD) for their referrals and support during these studies. We thank Alisa Sanders (RN, IBCLC, RLC) of the University of Texas Health Science Center Lactation Clinic and the Lactation Foundation, as well as Helen Sheena, MD, of the Kelsey Seybold Clinic for help in recruitment. We also thank Meredith Rayne and Deborah Lake of McGovern Medical School Media Relations, who publicized the studies.

References

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Normal cohort identified through study 2 (n = 28)

Colic cohort identified through study 111 (n = 18)

Fecal calprotectin analysis
Excluded subjects
Samples not Adequate (n = 13)

Subjects data screened (n = 65)

Fecal Microbiome
Excluded subjects cutoff point (<1000 16S rDNA reads) (n = 0)

Criteria met

Fecal calprotectin analysis
Eligible subjects (n = 52)
(colic = 27; normal = 25)

Fecal micro biome analysis
Eligible subjects (n = 65)
(colic = 38; normal = 27)

Qualitative analysis of included subjects fecal microbiota and fecal calprotectin, complete set analysis (n = 52)

Figure 1. CONSORT flow diagram showing recruitment and data analysis.
Figure 4. There was no relationship found between age and calprotectin level ($P = .403; R^2 = -0.005$).

Figure 6. Differences at the species level between controls and babies with colic. Pairwise 2-sided Wilcoxon rank sum tests with subsequent correction for multiple comparisons showed 2 operational taxonomic units to be significantly different between the colic and control groups: *Lactobacillus iners* ($P = .002$) and *Acinetobacter spp* ($P = .014$).
Figure 7. Random Forest plot of the most important variables, comparing infants with colic and their controls. Mean decrease in the Gini coefficient (>0.5) of attributes as assigned by the random forest model.