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Intestinal Microbiota of Infants With Colic: Development and Specific Signatures



WHAT'S KNOWN ON THIS SUBJECT: Colic affects many infants, with incidence rates of up to 25%. The pathogenesis is not well understood. Initial studies based on traditional culturing approaches and in infants >6 weeks of age point at abnormalities in intestinal microbiota.



WHAT THIS STUDY ADDS: Infants with colic showed lower microbiota diversity and stability than did control infants in the first weeks of life. Colic/control differences in the abundance of certain bacteria were also found at age 2 weeks. These microbial signatures possibly explain the excessive crying.

abstract



OBJECTIVES: To provide a comprehensive analysis of the fecal microbiota in infants with colic, as compared with control infants, during their first 100 days of life.

METHODS: Microbial DNA of >200 samples from 12 infants with colic and 12 age-matched control infants was extracted and hybridized to a phylogenetic microarray.

RESULTS: Microbiota diversity gradually increased after birth only in the control group; moreover, in the first weeks, the diversity of the colic group was significantly lower than that of the control group. The stability of the successive samples also appeared to be significantly lower in the infants with colic for the first weeks. Further analyses revealed which bacterial groups were responsible for colic-related differences in microbiota at age 1 or 2 weeks, the earliest ages with significant differences. Proteobacteria were significantly increased in infants with colic compared with control infants, with a relative abundance that was more than twofold. In contrast, bifidobacteria and lactobacilli were significantly reduced in infants with colic. Moreover, the colic phenotype correlated positively with specific groups of proteobacteria, including bacteria related to *Escherichia*, *Klebsiella*, *Serratia*, *Vibrio*, *Yersinia*, and *Pseudomonas*, but negatively with bacteria belonging to the Bacteroidetes and Firmicutes phyla, the latter of which includes some lactobacilli and canonical groups known to produce butyrate and lactate.

CONCLUSIONS: The results indicate the presence of microbial signatures in the first weeks of life in infants who later develop colic. These microbial signatures may be used to understand the excessive crying. The results offer opportunities for early diagnostics as well as for developing specific therapies. *Pediatrics* 2013;131:e550–e558

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KEY WORDS

colic, intestinal microbiota, infants, excessive crying, development

ABBREVIATIONS

HITChip—Human Intestinal Tract Chip

rRNA—ribosomal RNA

Drs de Weerth and Fuentes contributed equally to this work.

Dr de Weerth conceptualized and designed the study, designed the data collection instruments, coordinated and supervised data collection, conducted basic statistical analyses, drafted the initial manuscript, reviewed and revised the manuscript, and approved the final manuscript as submitted; Dr Fuentes conducted the laboratory analyses, conducted and interpreted the advanced statistical analyses, created the figures, reviewed and revised the manuscript, and approved the final manuscript as submitted; Dr Puylaert conducted the laboratory analyses, critically reviewed the manuscript, and approved the final manuscript as submitted; and Dr de Vos coordinated and supervised the laboratory analyses, interpreted the results, drafted, reviewed, and revised the manuscript, and approved the final manuscript as submitted.

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Infant colic, also referred to as excessive crying, affects many infants. By using the widely used, modified Wessel's criteria for defining colic¹ (ie, crying for an average of >3 hours per day), St James-Roberts reported prevalence rates varying from 6.4% to $\leq 29\%$ between 0 and 3 months of age.² Colic causes considerable concern and distress to the parents, and professional help is sought in 1 of 6 cases.³ The pathogenesis of infant colic is not well understood, and the underlying causes of colic remain unexplained. It has even been proposed that, despite the name, colic may not have an intestinal origin but reflects the extreme end of the normal distribution of infantile crying.⁴ A variety of physical, dietary, or drug treatments are in use, but their efficacy is difficult to assess because the excessive crying mostly stops by ~ 4 months of age.⁵ Moreover, a recent review indicated that the clinical evidence for the effectiveness of these treatments is generally very low or moderate.⁴

Aberrancies in the infant intestinal microbiota have been proposed to affect gut motor function and gas production, leading in turn to abdominal pain and colicky behavior.⁶ Some initial studies indicated that the intestinal microbiota in infants with colic differed from that of healthy control infants. For example, infants with colic displayed a less diverse fecal microbiota, which is associated with increased calprotectin levels that are indicative of intestinal inflammation.⁷ Moreover, infants with colic were found to have lower counts of lactobacilli and higher numbers of Gram-negative bacteria in their stools.⁷⁻⁹ However, these reports described differences in infants already diagnosed with colic and who were usually >6 weeks of age. Also, with 1 exception,⁷ they were based on traditional culturing approaches that do not represent the full complexity of the

intestinal microbiota.¹⁰ The recent development of high-throughput and molecular approaches allows a view on the intestinal microbiota and its function in which bias is greatly reduced.¹¹ The application of these molecular methods has shown that the intestinal tract is colonized rapidly in early life by a developing microbial community that displays specific succession patterns.¹²⁻¹⁴ Whereas at birth the intestinal tract is virtually sterile, already within a few hours, bacteria start to appear in fecal samples. These first settlers are often facultative anaerobic bacteria that are rapidly replaced by anaerobes with decreasing levels of oxygen tolerance.¹⁵ After several years of life, a complex microbial ecosystem is established, resembling that of adults.¹⁶ Although we do not yet know the exact order and impact of this microbial succession, it can be envisaged that deviations in the early intestinal colonization process could have an impact in later life. These deviations could also apply to the etiology of infant colic. If so, such deviations should appear early in life, preceding the excessive crying that is known to peak at ~ 6 weeks.¹⁷

The current study was initiated to prospectively follow the temporal development of the intestinal microbiota in a group of infants with and without colic. We collected samples in the first month after birth, which precedes the peak of colic at ~ 6 weeks of age, and again at ~ 3 to 4 months of age. By focusing on the first 100 days of life we looked at the buildup of the colic phenotype as well as at a follow-up period, when colic had most probably resolved. In contrast to earlier studies that focused on specific and often cultured bacteria, we addressed here the total intestinal microbiota composition by extracting fecal DNA and analyzing it with a phylogenetic microarray that allows a global, and comprehensive analysis of >1000 intestinal phylotypes.¹⁸ We hypothesized

that infants with colic would display a less diverse intestinal microbiota with a specific microbial signature and that these differences compared with control infants would be observable early in life.

METHODS

Participants and Study Design

This study is part of a prospective longitudinal project (termed the Bibo Study) that investigates the influences of early-caregiving factors on the development of children. The design of this study was described in detail elsewhere.¹⁹ All parents provided written informed consent, and the study was approved by the Ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen (ECG/AvdK/07.563). For the current study, the parents of a group of 160 healthy term infants collected 9 stool samples from birth until ~ 100 days of life. Inclusion criteria were as follows: an uncomplicated and singleton pregnancy, no drug use during pregnancy, no pre- and post-natal maternal physical health problems (eg, diabetes and heart disease) or mental health problems (eg, major depression), a term delivery (≥ 37 weeks) without major complications, and a normal 5-minute infant Apgar score (≥ 7). Four samples were collected in the first month of life at 2 (the meconium sample), 7, 14, and 28 days and 5 samples were collected at 3 to 5 months of age. The mean (SD) collection days were as follows: 2.1 (1.0), 6.5 (0.5), 13.8 (0.9), 27.6 (0.7), 83.9 (18.3), 87.9 (18.2), 92.8 (18.3), 100.1 (18.6), and 114.1 (18.2).

Determination of Colic

The parents reported crying by means of a well-validated 4-day diary at 6 weeks of age (41 ± 5 days).²⁰ The mean daily minutes of crying (ie, sum of crying, fussing, and unsoothable crying) were calculated. Criteria for colic were fulfilled in 25.4% of the group (ie,

modified Wessel's criteria for colic¹: crying for an average of >180 minutes per day over the 4 diary days). From the group of infants for whom a complete series of fecal samples was available ($N = 106$, 24.7% fulfilling the criteria for colic), the 12 infants with the highest levels of daily crying and the 12 with the lowest levels of daily crying were selected. The 2 groups showed large differences in the daily time spent crying (Table 1). Other than the excessive crying in 1 of the groups, the infants were healthy and the cohort representative of normally delivered infants in a Western country with low antibiotic use. The most salient health issues during the study period were as follows: antibiotic use (1 control infant), chickenpox (1 infant with colic), and 1-week hospitalization for fracture (1 control infant).

Fecal DNA Extraction and Phylogenetic Microarray Analysis

Fecal samples were collected by the parents at home and stored at -20°C . The samples were transported in cool-

TABLE 1 Characteristics of the Infant Cohort Described in This Study

	Colic Group ($n = 12$)	Control Group ($n = 12$)
Gender of infant		
Male	7	8
Female	5	4
Place of delivery		
Home	5	4
Hospital	7	8
Type of delivery		
Vaginal, unassisted	10	11
Vacuum pump	1	1
Cesarean delivery	1	0
Birth wt, g	3686.3 (417.7)	3603.0 (383.9)
Breastfeeding, mo ^a	4.3 (3.8)	6.3 (4.5)
Center-based child care		
Yes	8	5
No	4	7
Crying, min/d ^b	221.3 (30.5)	70.9 (19.0)*

Data are presented as means (SD) or n .

^a Four infants from the colic group and 2 from the control group received no breastfeeding at all.

^b Sum of crying, fussing, and unsoothable crying.

* Significant difference between the groups ($T = -14.5$, $P < .001$).

ers with freezing cartridges or dry ice for further processing. Total DNA was extracted from fecal material by the repeated bead beating procedure by using a modified protocol of the QiaAmp DNA Mini Stool Kit (Qiagen, Hilden, Germany) essentially as described previously.²¹ The subsequent analysis of 16S ribosomal RNA (rRNA) bacterial amplicons by using the Human Intestinal Tract Chip (HITChip) was performed in duplicate as described earlier.¹⁸ The HITChip is a comprehensive and highly reproducible phylogenetic microarray that enables the parallel profiling and semiquantitative analysis of 1140 phylotypes representing all major intestinal phyla grouped in 131 genus-like taxa described for the human intestinal microbiota.¹⁸ This high-throughput HITChip has been benchmarked with ultra-deep pyrosequencing of 16S rRNA²² and next-generation parallel sequencing of intestinal metagenomes.²³ Its taxonomic read-outs are linked to a recent overview of the human intestinal microbiota²⁴ and have been described in detail.¹⁸

The HITChip microarray hybridization was considered satisfactory if 2 independent hybridizations for each sample correlated >95% (Pearson's correlations). The HITChip microarrays showed a dynamic range of >10 000-fold, and >200 independent microarray readouts were used in this study. Ward's minimum variance method was used for the generation of hierarchical clustering of the total microbiota probe profiles, whereas the distance matrix between the samples was based on complete observation correlations.

Quantification of total bacteria by real-time quantitative polymerase chain reaction was performed as described previously.²¹

Data Analysis

The stability of the total microbiota composition was assessed by calculating Pearson's moving window correlation

between the log-transformed hybridization signals for 3699 unique HITChip probes¹⁸ obtained for pairs of 2 consecutive samples in time.

The diversity of the total microbiota or its subgroups was assessed by calculating the Simpson's reciprocal index of diversity (1/D).²⁵ The diversity was calculated at the HITChip probe level as detailed previously.¹⁸ To evaluate the significance of the differences between the colic and control microbiota, 2-tailed Student's t tests were calculated.

Wilcoxon signed-rank test corrected for false-discovery rate by the Benjamini and Hochberg method was applied to determine significant differences of individual genus-level groups between the study groups.

For comprehensive multivariate statistical analyses, Canoco software for Windows 4.5 (Wageningen, The Netherlands) was used.²⁶ Redundancy analysis was used to assess correlations between the microbial groups detected by the HITChip analysis and the sample characteristics. The log-transformed hybridization signals of 131 genus-level phylogenetic groups targeted by the HITChip were used as biological variables. As environmental confounder variables we included breastfeeding (weeks), gender, birth weight, home versus hospital delivery, sampling age in days, and crying (infants with colic or control infants). The Monte Carlo Permutation Procedure was used to assess significance of the variation in large data sets.

RESULTS

Colic and Control Infant Characteristics

The 12 infants with colic and 12 control infants showed highly similar gender distribution, birth weight, place and mode of delivery, and breastfeeding duration (Table 1). From all infants, 9 fecal samples were obtained.

Phylogenetic Profiling, Temporal Clustering, and Composition Analysis of the Global Fecal Microbiota

Six of the 216 total fecal samples did not contain enough fecal material for analysis. Total DNA was readily recovered from the remaining 210 samples, but from the meconium the total bacterial count as based on the quantitative polymerase chain reaction analysis was generally 100 to 1000-fold lower than from the other samples.

Subsequently, total bacterial 16S rRNA genes were amplified from the extracted DNA and labeled and hybridized to the phylogenetic microarray containing 3699 distinct HITChip oligonucleotide probes.¹⁸ All samples were analyzed twice in a dye swap experiment, and in all cases a high reproducibility (>95% Pearson's coefficient) was obtained, which substantiated the accuracy of the used high-throughput approach. Moreover, hierarchical clustering revealed high similarities between the fecal samples from the same infant except for the meconium samples, which often clustered together (data not shown). Typically, such unsupervised clustering exposed a proper temporal ordering of the samples with the meconium sample being the most different (Fig 1A). The immediate and permanent presence of actinobacteria (mainly bifidobacteria; see below) was evident. Whereas bacilli were found in early samples, their abundance was reduced after 75 days when the proteobacteria and Bacteroides increased in numbers. This shift was further quantified in the overall composition based on the cumulative signals for the level 1 groups, representing genus- or group-level taxa (Fig 1B). A total of 210 phylogenetic microarray analyses were performed, and the overall bacterial composition of the samples revealed a highly variable temporal composition (Supplemental Figs 5 and 6). Careful

inspection indicated that, in many cases, most of the different members of Firmicutes, including many strict anaerobes, were already present at a low level even in the first week of life (see also Fig 1). As early as in the first weeks of life, significant differences could be found in the microbiota of control infants and infants with colic (see Supplemental Table 3). Bacteroidetes appeared to be lower in the infants with colic for the entire study period, with various levels of significance in the first 2 months. In contrast, proteobacteria (including bacterial groups related to *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas*, or *Yersinia*) were significantly increased in infants with colic compared with control infants in the first 2 months, with a more than doubled relative abundance after 2 weeks. Remarkably, the intestinal bifidobacteria and lactobacilli (including bacteria related to *Lactobacillus gasseri* and *Lactobacillus plantarum*) were found to be similarly increased in control infants compared with infants with colic after 1 and 2 weeks, respectively (see Supplemental Table 4).

Diversity and Stability Analyses Revealed Early Differences Between Colic and Control Samples

To further address the differences between infants with colic and control infants, the microbiota diversity was determined on the basis of all hybridization signals. In the control infants, microbiota diversity increased slightly with time. However, the diversity of the microbiota in the infants with colic appeared to have a different temporal development and stayed rather low during the first 100 days of life (Fig 2). On postnatal days 14 and 28, the diversity of the microbiota was significantly lower in infants with colic than in control infants ($P < .02$ and $P < .01$, respectively). This difference could be mainly attributed to the decreased bacterial evenness in the colic sam-

ples (ie, how similar the amounts of the different bacterial groups in the samples are), whereas the richness (ie, number of different species found in the samples) was similar to that in the control infants (data not shown).

A further analysis on the stability of the microbiota was performed by comparing the similarity between successive samples. The lowest similarity in all investigated samples is that between the meconium sample and the first fecal sample. The results indicated that the control infants showed a higher stability than did the infants with colic (see Fig 3). Moreover, the similarity between the samples taken at 1 and 2 weeks of age was significantly lower in the infants with colic compared with the control infants ($P < .04$, Fig 3).

Multivariate Statistical Analysis of Fecal Microbiota in Infants With Colic and Control Infants

To detail the differences in the microbiota between infants with colic and control infants, we performed a multivariate cluster analysis on the microbiota composition and other data sets. Comparisons were performed with data from all time points. These analyses revealed significant differences occurring at 2 weeks of age (Fig 4); at that age, the microbiota of both groups could be separated with the environmental variable "crying," which significantly influenced the sample distribution ($P = .03$, Monte Carlo Permutation Procedure). The variable crying was also found to be associated (although not significantly) with the infants with colic and specific bacterial groups (notably the proteobacteria) in the earlier life samples. The separation of the colic and control groups and the bacterial associations were not as marked in later life samples as at age 2 weeks (data not shown).

None of the other variables (gender, birth weight, breastfeeding, home/hospital

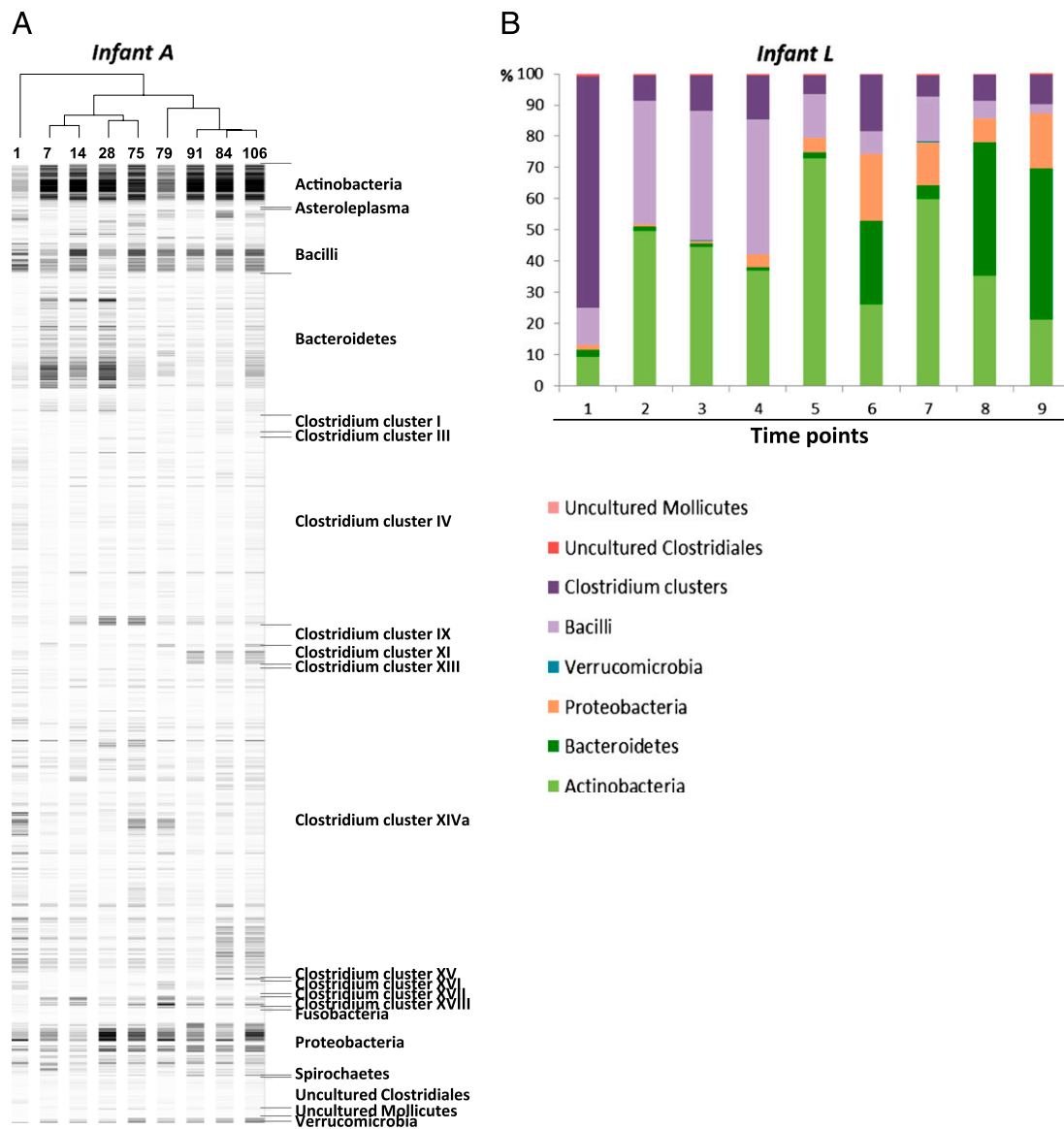


FIGURE 1

A, Hierarchical clustering with a heat map of the HITChip profiles of a representative control infant (infant A) taken at days 1, 7, 14, 28, 75, 79, 84, 91, and 106. The darkness of the lines represents the bacterial abundance in the sample. The highest phylogenetic levels represented are shown on the right side of the figure. B, Temporal dynamics of the relative abundance (%) of the most abundant phyla/classes in fecal samples from a representative control infant taken at the 9 different time points.

delivery, and sampling age) significantly influenced the sample separation at this time point (data not shown). The observed separation (Fig 4) was found to explain $\geq 18\%$ of the variation in the abundance of 34 bacterial groups. These bacterial groups are included in the plot (gray arrows) and listed separately (Table 2). The 8 bacterial groups that were positively associated with crying included potentially pathogenic Gram-negative bacteria such as

bacteria related to *Escherichia*, *Klebsiella*, *Serratia*, *Vibrio*, *Yersinia*, and *Pseudomonas*. In contrast, the groups that were negatively associated with crying contained relatively abundant bacteria. These included bacteria belonging to the butyrate-producing species *Butyrivibrio crossotus*, *Eubacterium rectale*, and *Eubacterium hallii*, which were found to be consistently more (~ 1.5 -fold) abundant in healthy infants than in the infants with colic (data not shown).

DISCUSSION

In this study we followed the temporal development of the intestinal microbiota from birth onward in infants with and without colic by using the HITChip, a phylogenetic microarray that allows a highly reproducible and comprehensive analysis of the known intestinal microbiota.¹⁸ Colic was determined at 6 weeks of age with a cry diary. The infants with colic were characterized by a microbiota that developed slower

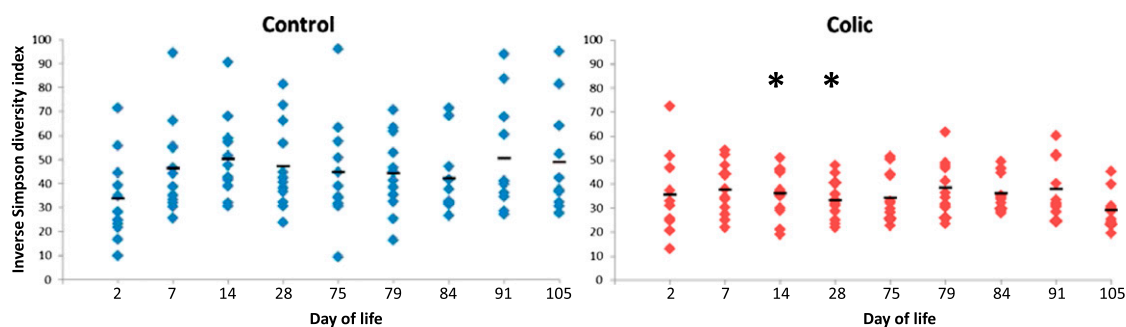


FIGURE 2

Temporal development of the inverse Simpson's diversity index (highest observed value set to 100 for each individual) in the first 100 days of life of control infants and infants with colic, calculated with all of the HITChip probe signals. Diversity was reduced in the colic group and was significantly different (*) between groups at days 14 and 28 ($P = .03$ and $P = .02$, respectively).

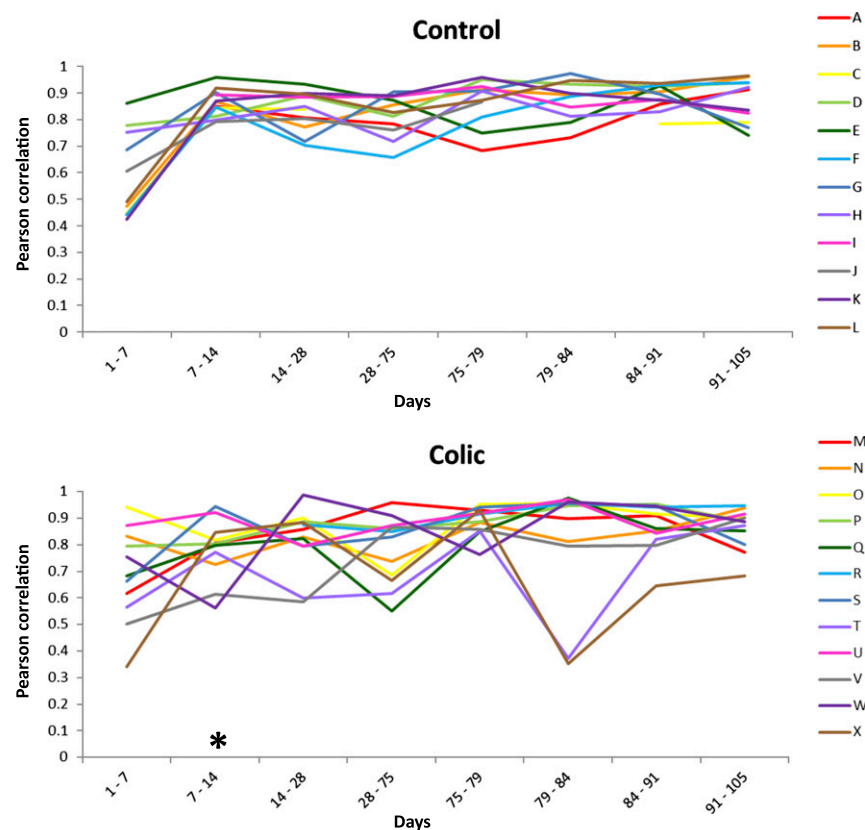


FIGURE 3

Moving window analysis of similarity indexes of the microbial composition of control infants and infants with colic (indexes calculated for pairs of successive sampling days). The colic group revealed a less stable pattern in the first weeks, and the similarity indexes were significantly different (*) in the control group between 7 and 14 days of age ($P = .04$).

than that of the control infants and that also showed a reduced temporal stability. Infants with colic also showed a significantly reduced microbiota diversity at 14 and 28 days of life. In addition, already in the first 2 weeks of life, specific significant differences between both groups were found;

proteobacteria were increased in infants with colic, with a more than doubled relative abundance, whereas bifidobacteria and lactobacilli were increased in control infants. Moreover, samples from infants with colic were found to contain fewer bacteria related to butyrate-producing species.

The observed differences confirm and extend earlier studies at older ages (ie, at ~6 weeks) with traditional culturing approaches and a focus on specific bacteria and revealed an increased level of coliform bacteria (notably gas-producing *Escherichia* and *Klebsiella* spp.) in infants with colic.⁷⁻⁹ The results are also in line with those of the first study showing that colic is linked to reduced lactobacilli,²⁷ and those of a recent study that found that colic is linked to inflammation and reduced microbial diversity.⁷ In addition, a recent study that assessed continuous levels of crying in healthy infants reported that the abundance of *Lactobacillus* spp. at 3 weeks of age was inversely associated with total infant distress at 7 weeks.²⁸ However, the type of lactobacilli was not specified in that study.

Our results indicate the importance of the first weeks of life for microbiota development. The reduced diversity and specific microbiota signature observed in infants with colic already in the first weeks of age could suggest a role of microbiota development in the etiology of colic, as both well precede the usual colic peak (ie, ~6 weeks after birth). Moreover, the findings may aid the future development of tests designed to predict the development of colic as well as specific therapies for prevention of colic. The results could also help explain why the administration of probiotics can result in a decrease in colic symptoms^{29,30};

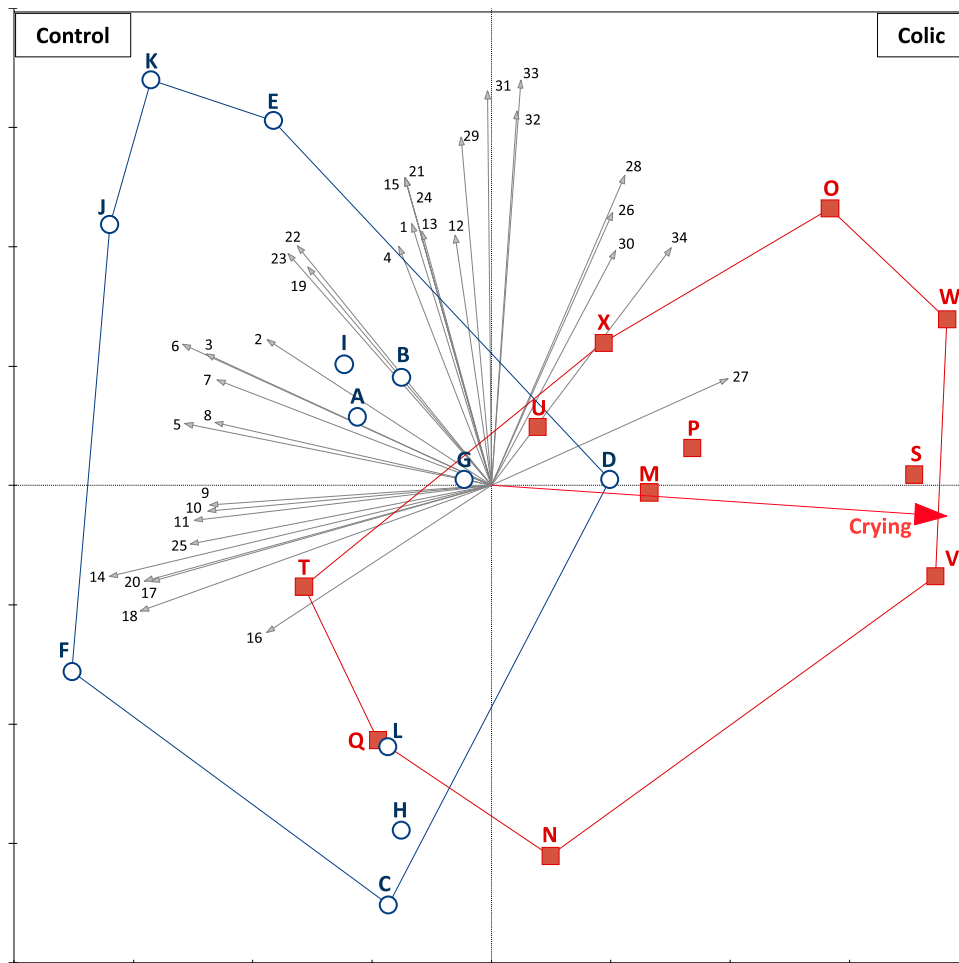


FIGURE 4

Redundancy analysis of samples taken at age 14 days from control infants (blue circles) and infants with colic (red squares). Capital letters indicate the different infants. Gray arrows indicate the bacterial groups associated with the different samples (see Table 2). The first and second ordination axes are plotted and explained 17.5% of the variability in the data set. Crying was the only environmental variable significantly related to the sample distribution ($P = .03$, Monte Carlo Permutation Procedure with forward selection).

the probiotics might change the microbiota thereby displacing the colic-associated bacteria.

With respect to the mechanisms underpinning this possible relation, the twofold relative abundance of proteobacteria may be of relevance. These included bacteria known as potential pathogens that might cause inflammation. Remarkably, we have previously described that the abundance of similar pathobionts at the other extreme end of life, namely in centenarians, is associated with inflammation and a lower abundance of butyrate-producing bacteria.³¹ The butyrate-producing bacteria identified here as correlating with the absence of crying (*Butyrivibrio crossotus*

et rel., *Coprococcus eutactus* et rel.) appear to be different species than in those described in the study in centenarians. However, the mechanism by which they act may be similar because it has been shown that butyrate reduces the pain sensation in adults.³² The substantially reduced level of lactobacilli in infants with colic compared with healthy infants is of specific interest, notably because this reduction was found to be limited to bacteria related to *L. gasseri* and *L. plantarum*. Both of these are considered to be mucosal lactobacilli, suggesting that they may signal to the host. We recently established that exponentially growing *L. plantarum* cells induce expression of

antiinflammatory genes in the upper intestinal tract of adults.³³ Moreover, strains of *L. gasseri* are known to induce an antiinflammatory response. Hence, we propose that the excessive crying may be caused by increased inflammation by an increased level of pathogens and by a reduction in antiinflammatory lactobacilli.

But why do certain infants show these delays and aberrancies in microbiota colonization patterns? Possible early-life candidate factors that could be distinguishing future infants with colic from those without colic are genetics, epigenetics (eg, from the prenatal environment), postnatal environment (eg, household and caregiving

TABLE 2 Bacterial Groups (and Related Species) That Are Negatively (Left) or Positively (Right) Associated With the Environmental Variable Crying

Negatively Associated		Positively Associated	
Phylum and Group No.	Phylum (Class)/Genus-like	Phylum and Group No.	Phylum (Class)/Genus-like
Actinobacteria	Actinobacteria	Proteobacteria	Proteobacteria
1	<i>Eggerthella lenta</i> et rel.	26	<i>Anaerobiospirillum</i> spp.
Bacteroidetes	Bacteroidetes	27	<i>Enterobacter aerogenes</i> et rel.
2	<i>Bacteroides intestinalis</i> et rel.	28	<i>Escherichia coli</i> et rel.
3	<i>Bacteroides plebeius</i> et rel.	29	<i>Haemophilus</i>
4	<i>Bacteroides splachnicus</i> et rel.	30	<i>Klebsiella pneumoniae</i> et rel.
5	<i>Bacteroides vulgatus</i> et rel.	31	<i>Pseudomonas</i> spp.
6	<i>Prevotella oralis</i> et rel.	32	<i>Serratia</i> spp.
7	<i>Prevotella tanneriae</i> et rel.	33	<i>Vibrio</i> spp.
Firmicutes	Bacilli	34	<i>Yersinia</i> et rel.
8	<i>Gemella</i> spp.		
9	<i>Streptococcus bovis</i> et rel.		
10	<i>Streptococcus intermedius</i> et rel.		
11	<i>Streptococcus mitis</i> et rel.		
12	<i>Weissella</i> spp.		
	<i>Clostridium</i> cluster I		
13	<i>Clostridia</i>		
	<i>Clostridium</i> cluster IV		
14	<i>Clostridium orbiscindens</i> et rel.		
	<i>Clostridium</i> cluster IX		
15	<i>Peptococcus niger</i> et rel.		
16	<i>Veillonella</i>		
	<i>Clostridium</i> cluster XIVa		
17	<i>Bryantella formatexigens</i> et rel.		
18	<i>Butyrivibrio crossotus</i> et rel.		
19	<i>Clostridium symbiosum</i> et rel.		
20	<i>Coprococcus eutactus</i> et rel.		
21	<i>Eubacterium ventriosum</i> et rel.		
22	<i>Lachnospira pectinoschiza</i> et rel.		
23	<i>Ruminococcus gnavus</i> et rel.		
	<i>Clostridium</i> cluster XVI		
24	<i>Eubacterium cylindroides</i> et rel.		
Proteobacteria	Proteobacteria		
25	<i>Oxalobacter formigenes</i> et rel.		

Numbers correspond to species arrows in Fig 4.

factors), and even fortuitous encounters with specific bacteria in the neonatal period. Future prospective research concentrating on the early-life microbiota and host functions should shed light on this question.

Remarkably, the differences between colic and control microbiota were all seen in the first month of life, before the colic peak takes place. At ~3 to 4 months

of age, when the colic phenotype has usually disappeared, there were no longer detectable differences between our 2 study groups. This could indicate that the colic phenotype is associated with a delayed and somewhat aberrant microbiota development, but that it is only temporary and not indicative of a permanently altered intestinal microbiota. However, longitudinal studies

with samples taken at later ages are required to clarify this issue.

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