Microbiome of the first stool after birth and infantile colic

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Revised submission:

Single file including

Main text 3446 words

Abstract 184 words

Three tables and five figures with figure legends inserted within the main text

Two supplementary tables

Competing interests:

All authors declare no competing interests.

Financial support:

Pediatric Research Foundation, Finland

Academy of Finland

Juho Vainio Foundation, Finland

Alma och K.A. Snellman Stiftelsen, Finland

UniOGS Graduate School, University of Oulu, Finland

Emil Aaltonen Foundation, Finland

Author contributions:

All authors have revised the manuscript for intellectual content, have approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Katja Korpela, MD, performed clinical data collection and data analyses and interpretation of the data, and wrote the first draft of the manuscript.

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Jarmo Salo, MD PhD, planned the study and wrote the research plan, organized statistical data analyses and interpreted the data.

Mysore Tejesvi, PhD, performed 16SrRNA analyses and bioinformatics analyses and was responsible for the quality of the work in the research laboratory.

Pirjo Koivusaari, MSc, performed 16SrRNA analyses and bioinformatics analyses.

Tytti Pokka, MSc, planned and performed all statistical analyses combining microbiome and clinical data and interpreted the data.

Tuula Kaukola, MD PhD, contributed to designing the study and interpreted the clinical analyses.

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Category of the study: Clinical Research Article

Key message and the impact of the study:

- In this population-based prospective cohort study, the microbiome of the first-pass meconium was associated with subsequent infantile colic.
- The diverse microbiome of the first stool, reported earlier in several studies, appears to be related to a clinically significant outcome in infants.
- The pathogenesis of infantile colic may start already at birth. Thus, prevention of infantile colic should start at birth or possibly even in fetal period.

ABSTRACT

Background: Recent studies have shown a diverse microbiome in the first stool after birth. The clinical significance of the microbiome of the first stool is not known. Infantile colic has earlier been associated with the composition of the intestinal microbiome.

Methods: We set out to test whether the microbiome of the first stool is associated with subsequent infantile colic in a prospective, population-based cohort study of 212 consecutive newborn infants. We used next-generation sequencing of the bacterial 16S rRNA gene.

Results: The newborns who later developed infantile colic (n=19) had a lower relative abundance of the genus *Lactobacillus* and the phylum Firmicutes in the first stool than those who remained healthy (n=139). By using all microbiome data, random forest algorithm classified newborn with subsequent colic and those who remained health with area under the curve of 0.66 (SD 0.03) as compared to that of shuffled samples (p-value <0.001).

Conclusions: In this prospective, population-based study, the microbiome of the first-pass meconium was associated with subsequent infantile colic. Our results suggest that the pathogenesis of infantile colic is closely related to the intestinal microbiome at birth.

INTRODUCTION

Infantile colic and its pathogenesis are one of the most thoroughly studied conditions in the field of pediatric microbiome research. The main clinical feature of infantile colic is prolonged crying for an unknown reason >3 h a day for at least 3 days a week (1). Approximately 20% of the children develop infantile colic, and it typically peaks at 5-6 weeks of age (2,3). The etiology of colic is suggested to be multifactorial as gastrointestinal as psychosocial and neurodevelopmental etiologies have been proposed (4). Increasing importance of the role of intestinal microbiome on infantile colic has been assigned, as several cross-sectional and prospective studies among infants have reported an association between aberrant intestinal microbiome and infantile colic, including several typical features such as higher abundance of Proteobacteria and lower abundance of the genera *Bifidobacterium* and *Lactobacillus* and bacterial diversity in the gut microbiome of colicky infants (5). Furthermore, *Lactobacilli*-containing probiotics have been reported to be effective for the treatment of infantile colic in breastfed infants (6). Besides infantile colic, intestinal microbiota has been linked to other gastrointestinal problems, such as irritable bowel syndrome and inflammatory bowel disease (7,8).

The early steps of intestinal colonization are important for the development of gut barrier function and the modulation of local and systemic immune responses (9). Several reports have shown that the first-pass meconium, the stool formed before birth, contains a diverse microbiome in even healthy pregnancies and that microbiome can be detected in placenta suggesting that the colonization of infant gut may start already in utero (10-12). The concept of fetal microbiome, defined as materno-fetal transmission of microbial DNA or whole microbes during pregnancy, is controversial (13,14) and the implication of the meconium microbiome for later health is not well understood.

As several studies have reported that the intestinal microbiome is associated with infantile colic in a cross-sectional setting, we set out to test whether the microbiome of the first-pass meconium is associated with subsequent infantile colic in a prospective, population-based cohort study of 212 consecutive newborn infants. We collected follow-up stool samples at the age of 1 year to evaluate, whether the potential changes in microbiome with respect to infantile colic had disappeared by the age of 1 year. In addition, we analysed the association of other gastrointestinal symptoms and microbiome at birth and at 1 year of age.

SUBJECTS AND METHODS

Study design

The population is a consecutive sample of infants born in the Central Finland Central Hospital in Jyväskylä, Finland, between February 3 and March 13, 2014. This hospital serves as the sole delivery hospital in the region, with 3000 annual births among a population of 250,000. The study was conducted and all methods were performed in accordance with the relevant guidelines and regulations. The Ethics Committee of the Central Finland Hospital District approved the study plan. All mothers who gave birth during the designated period were invited to participate. The parents received an information letter in the maternity ward, and only those families who provided their written informed consent were enrolled.

We used a questionnaire to record maternal medical history and prenatal factors, combined with details on the pregnancy and delivery recorded by midwives, and collected first-pass meconium samples. For this cohort study, we sent a follow-up questionnaire to the same families concerning the children's health and nutrition and collected a follow-up stool sample when the children were 12 months of age. The families were specifically asked in the questionnaire whether their child had had colic in early infancy, infantile colic being defined as crying for 3 h a day for 3 days a week for ≥3 weeks, in accordance with the current diagnostic criteria (1). Parents also reported whether their child had had symptoms of diarrhoea or constipation or had been diagnosed with reflux disease that had required treatment.

We have previously reported a maternal influence on the composition of the microbiome of the first-pass meconium in the same cohort (15). In brief, the biodiversity of the home environment measured as the presence of furry pets during pregnancy, increased the diversity of microbiome of the first-pass meconium, whereas maternal consumption of antimicrobials during pregnancy increased the proportion of meconium samples that did not amplify sufficiently, i.e. the number of

reads was <1000 per sample. However, delivery mode or exposure to antimicrobials during delivery had no effect on the composition of the first-pass meconium.

Microbiome analyses

All the microbiome analyses were performed blinded for the infants' clinical symptoms. The first-pass meconium samples were collected, stored and analysed as previously reported (15). In brief, the midwives collected the first-pass meconium samples from the diapers of the newborns. The samples were retained in a refrigerator (+4°C to +8 °C) for maximum 24 h, then frozen at < -22°C, before being transferred to the University of Oulu, Finland, where the microbiome analyses were performed. DNA was extracted from the stool samples using the QIAsymphony DSP DNA Mini Kit according to the manufacturer's protocol (Qiagen). Primers F519 and R926 were used to amplify a portion of the 16S small-subunit ribosomal gene. For the collection of a follow-up stool sample, the families were sent two sample tubes, whereupon they mailed the fecal samples to the University of Oulu, Finland, for microbiome analyses performed by 16S rRNA sequencing. Before processing, the samples were stored at -20°C. Fecal DNA extraction, amplification of the bacterial 16S rRNA genes and PCR reactions were performed and the DNA concentrations measured by using the same protocols as in meconium samples (15). The Ion Torrent sequences were processed and analysed with QIIME 1.9.0, using state-of-the-art procedures (16). Principal coordinates analysis (PCoA) was also performed with QIIME, based on the phylogenetic distances between samples, using weighted UniFrac metrics. The raw Ion Torrent data were deposited in NCBI-SRA with the accession number SRP069890. Together with the number of operational taxonomic units (OTUs), the Shannon-Weaver bacterial diversity index was employed to estimate the alpha diversity of the microbiome, equally weighting richness and evenness in each sample. Out of the 158 meconium samples with clinical follow-up data also available, 13 were not amplifiable by PCR, probably owing to a low amount of bacterial DNA. In our previous work, antibiotic use during pregnancy increased the number of samples that did not amplify (15). These samples were

not included in the analyses for Shannon diversity index. For other analyses, such as the number of operational taxonomic units and the relative abundances of bacterial phyla and genera, these samples were coded as zero.

In addition to conventional statistical analysis, we performed analysis using machine-learning approach. Weighted random forest classifiers were trained on relative abundance tables to distinguish between colic and non-colic samples for meconium and 12-month samples (17). Model building was done with a stratified nested cross-validation approach to ensure close to even splitting of folds. Models were built using 14x15 and 13x14 fold set up with meconium and 12-month samples, respectively. Random forests hyperparameters were tuned by grid-searching values related to the depth, number of features and class weight parameters. Best performing models on each iteration were selected for testing on the outer-fold test set. Receiver operating characteristic area-under-the-curve (ROC AUC) was chosen to evaluate the model. Dummy classifiers were used to generate baseline random chance predictions. Nested cross-validation was repeated 20 times and averaged ROC curves were generated from the test results. Permutation tests were employed to test the nested cross-validation approach against random chance using the permutation_test_score function in Scikit-learn on each permutation of the process (18). Independent p-values generated from permutation tests were combined using the Fisher's method. The machine-learning analysis was done with custom python scripts, employing the Scikit-learn package (18). In addition, we compared the mean relative abundances of the most important bacterial genera for the meconium and 1-year sample classifier between infants with and without colic.

Statistical analysis

The Mann-Whitney *U* test was used to compare the relative abundance of the selected bacterial groups and diversity indices of the first-pass meconium samples between the infants who did and did not develop subsequent symptoms of infantile colic, gastro-oesophageal reflux disease,

constipation and diarrhoea. Samples that were not amplifiable by PCR, because of a low amount of bacterial DNA, were not included in the analyses for the Shannon diversity index and for other analyses they were recorded as zero. The traditional P-level threshold of 0.05 (alpha error) was employed for testing four pre-existing hypotheses involving factors that have previously been reported to be linked to infantile colic, namely a high abundance of Proteobacteria, low abundance of Actinobacteria, Bacteroidetes and Firmicutes phyla and genera *Bifidobacterium* and Lactobacillus (5,19,20). For other comparisons, Bonferroni correction of the P-value was used to compensate for the multiple testing problem, i.e. the cut-off threshold for statistical significance. In other words, the corrected alpha error was calculated by dividing the alpha error of 0.05 by the number of comparisons used for each outcome measure. Thus P-values of < 0.0031 ($\alpha /16$) were considered to be statistically significant in the analyses. The statistical analyses were performed with SPSS version 24 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Study population

The study population was a consecutive sample of 312 children born at the hospital during the period concerned. We received informed consent from 218 families and first-pass meconium samples from 212 newborn infants (Figure 1). All infants were term or near term (Table 1). Caesarean section was performed in 40 deliveries (19%). At the age of one year, we received clinical follow-up questionnaires from 160 children (Figure 1, Table 1) and a follow-up fecal sample was received from 96 infants.

Intestinal microbiome and infantile colic

The infants who later developed infantile colic symptoms (n = 19, 12%) had a different intestinal microbiome in the first-pass meconium from those who did not develop such symptoms (n = 139, 88%) (Figure 2, Table 2). Infants with subsequent infantile colic had a lower relative abundance of the phylum Firmicutes (27% [SD 30, 95% confidence interval (CI) of the mean 13-41%] *vs* 46% [SD 32, 95% CI of the mean 40-52%], $P = 0.008$) and the genus *Lactobacillus* (0.54% [SD 1.1, 95% CI of the mean 0.02-1.1%] *vs* 4.6% [SD 15, 95% CI of the mean 2.1-7.1%], $P = 0.04$) (Figure 2, Table 2) in the first-pass meconium. The differences in the relative abundance of main phyla and genera between the infants with and without colic were no longer observed in the 1-year stool samples.

Random forest classifiers trained on the meconium and 12-month microbiome profiles showed a difference in test performance of colic *vs* non-colic samples, when compared to random chance in permutation tests (Figure 3). Using a machine learning approach, a random forest classifier analysis, the microbiome of the first stool predicted subsequent infantile colic with an area under the curve (AUC) of 0.66 (SD 0.04) as compared to that to random chance (P value $1.59*10^{-6}$). The

most important bacteria for the classifier at birth were both previously reported colonizers of the gut and bacteria typically found in terrestrial environment. The previously reported early colonizers of the gut such as *Bacteroides, Streptococcus, Lactobacillus, Staphylococcus, Faecalibacterium* and *Enterococcus,* were more abundant in the infants who remained healthy, whereas infants with colic had more genera typical for terrestrial environments, such as *Bradyrhizobium, Stenotrophomonas,* and *Ralstonia* (Figure 4A, Supplementary Table 1). Infants with colic had lower relative abundance of *Lactobacillus* in their meconium microbiome, but after Bonferroni correction, differences in other genera were not statistically significant. The microbiome at the age of one year classified earlier infantile colic with an AUC of 0.66 (SD 0.04) as compared to random chance (p-value $5.65*10^{-5}$). The most important bacterial genera for the classifier at one year of age were *Bacteroides, Prevotella, Ruminococcus*, *Clostridium*, *Lactobacillus, Oscillospira* and *Blautia* (Figure 4 B).

Microbial diversity and infantile colic

No statistically significant differences were found in the number of OTUs or the Shannon bacterial diversity index with respect to subsequent infantile colic, nor was any clustering of colicky or healthy children observed in the principal coordinates analysis (PCoA) of the first-pass meconium samples (Figure 5). Out of the 158 meconium samples with follow-up data available, 13 were not amplifiable by polymerase chain reaction (PCR), and four of these 13 children (31%) developed infantile colic, while 15 (10%) of the children with samples that could be amplified developed infantile colic, but the difference was not statistically significant ($P = 0.053$). The number of OTU's and the Shannon-Weaver bacterial diversity index at the microbiome at 1 year were not associated with infantile colic.

Epidemiological background factors of infants with subsequent colic

There were no significant differences in the gender of the children, mode of delivery, use of antimicrobials or probiotics during pregnancy or the duration of breastfeeding between the children with and without subsequent infantile colic. There was a difference in their distribution according to maternal education level (Table 3).

Other gastrointestinal symptoms and the microbiome

No major differences were seen in the main phyla and genera, in the number of operational taxonomic units or in the bacterial diversity indices of the microbiome of the first-pass meconium between the children who were treated for gastro-oesophageal reflux disease (n = 10, 6%) and those who remained healthy (n = 147, 94%) (Supplementary Table 2). Nor was the composition of the meconium microbiome associated with later constipation (n = 26) or parent-reported diarrhoea (n = 11). Similarly, we did not observe any changes in the gut microbiome at 12 months in relation to later gastro-oesophageal reflux disease or constipation. The relative abundance of the genus *Lactobacillus* in the 12-month stool sample was greater in the children with reported diarrhoea than in those without (1.1% [SD 2.4] *vs* 0.28% [SD 1.3], $P = 0.001$) (Supplementary Table 2). There was no difference in the use of probiotics between the infants with and without diarrhoea.

DISCUSSION

In this prospective, population-based study, the microbiome of the first-pass meconium was associated with subsequent infantile colic. The relative abundance of *Lactobacillus* and of the phylum Firmicutes was lower in newborn infants with later infantile colic than in that of healthy infants. Thus, the earlier reported associations of gut microbiome and infantile colic appeared to be present already at birth.

If the microbiome of the first-pass meconium is regarded as a proxy for fetal microbiome, this prospective, population-based cohort study may link the suggested fetal microbiome to the subsequent infantile colic. Several recent reports have detected diverse bacterial DNA in the meconium, placenta and umbilical cord, suggesting that the colonization process starts during fetal period (10-12,21), but the validity of the findings have been questioned (13,14). However, the early steps of intestinal colonization are important for establishment and development of the gut microbiome and furthermore for local and systemic immune responses (9), and several clinical studies have shown an association between intestinal microbiome in infancy and asthma, inflammatory bowel disease and other immune-mediated disorders (22), The primary colonizers of infant gastrointestinal tract are typically facultative anaerobes belonging to phylum Firmicutes such as *Enterococcus, Lactobacillus Staphylococcus* and *Streptococcus* following by *Bifidobacterium, Clostridium* and *Bacteroides spp.* and colonization process can be affected by delivery mode, maternal microbiome and feeding pattern (23,24). Also intrapartum antibiotic prophylaxis has been showed to disturb the establishment of gut microbiota of the infant (25). Furthermore, intrapartum antibiotic administration and neonatal antibiotic treatment have been associated with infantile colic also suggesting the role of early colonization in the pathogenesis of infantile colic (26,27). Perinatal period has been shown to be a critical time for the antibiotic treatment to disturb murine microbiota and development of immune responses (28).

Our results expand the findings obtained in earlier cross-sectional and prospective studies that have investigated the gut microbiome in infants. In a longitudinal prospective study by de Weerth et al., children with infantile colic had an increased abundance of the phylum Proteobacteria and decreased abundance of the genera *Lactobacillus* and *Bifidobacterium* compared with control children at the age of two weeks. Infantile colic was also associated with slower bacterial colonization and reduced bacterial diversity (19). In cross-sectional studies where fecal samples were collected at an older age, infantile colic has been associated with decreased bacterial diversity, a lower total bacteria count and a higher relative abundance of coliform bacteria (29-31).

As the decreased lactobacilli and Firmicutes in the meconium microbiome preceded the symptoms of infantile colic in our cohort study, our results support the idea that the lack of lactobacilli is crucial for the pathogenesis of infantile colic. A decreased proportion of *Bifidobacterium* and a lower prevalence of *Lactobacillus* have been found to be associated with increased infant crying and fussing (32). Supplementation with *L. reuteri* DSM 17938 reportedly reduced crying time in breastfed children who suffered from infantile colic (6). Furthermore, in a randomized controlled trial of infants with colic, relative abundance of phylum Bacteroidetes and genus *Bacteroides* increased in children who responded to treatment with *L. reuteri* DSM 1793 (33). In newborn, breastfed mice, *L. reuteri* DSM 17938 has been shown to increase the proportion of regulatory T-cells in the intestinal mucosa and to increase the bacterial diversity and the relative abundance of phylum Firmicutes and decreasing Bacteroidetes (34). Lactobacilli have been implicated in the development of local and systemic immune responses, and a few *Lactobacillus* strains have been reported to possess antimicrobial effects against some gas-producing coliforms (35,36). Infantile colic has been associated with low-grade systemic inflammation and increased fecal calprotectin levels (29,32), while intestinal gas production has been considered to be a cause of abdominal pain that leads to colicky behaviour, since colicky children are reported to have more gas-producing coliforms (30).

Proteobacteria, especially coliform bacteria of the genera *Escherichia* and *Klebsiella*, have been found to be more abundant in colicky children (19,29). Although there was no difference in the relative abundance of Proteobacteria with respect to later colic symptoms in our results, a decreased abundance of Firmicutes was noted, implying that the relative abundance of the other main bacterial phyla must have simultaneously been higher.

In our study, meconium microbiome was not associated with later constipation, diarrhoea or gastroesophageal reflux disease. There could be overlapping between these conditions and infantile colic, and thus they were solicited separately. It has been estimated that other gastrointestinal disorders, such as gastroesophageal reflux disease and allergies, explain less than 10% of the infantile colic cases (37).

The strength of this work lies in its population-based, prospective design, extending from the first-pass meconium to the clinical follow-up. The prospective setting avoided the reverse causation problems of a case-control study design, where symptoms could lead to different nutrition and thus to an altered microbiome. Our sample size is one of the largest in populations assessing the microbiome of the first stool after birth. The incidence of infantile colic observed during the follow-up was within the range of earlier point estimates of the incidence for infantile colic (2). The limitation of the study was that, the families only reported the occurrence of infantile colic in a questionnaire, but the infant cry behaviour was not recorded in a journal. The follow-up sample and questionnaire were both collected at 1 year of age to assess whether possible changes in the intestinal microbiome had persisted, and at this age, the intestinal microbiome starts to resemble that of an adult, being more complex and diverse, finally reaching a constant, adult-like state at 2-3 years of age (23,24). Recall bias, which is present in studies assessing reported epidemiological risk factors instead of objective measurements as in this study, was avoided as both the parents and the physician collecting the clinical data were unaware of the microbiome findings. The scope of the study was not an epidemiological analysis of risk factors of infantile colic, which often require a

case-control study design to achieve statistical power for risk factor comparisons.

Our results show that bacterial DNA present in the first-pass meconium is associated with infantile colic. 16S rRNA sequencing allows comprehensive microbiota analysis, but it does not allow a quantitative measurement of the bacteria. It shows whether there is bacterial DNA present but doesn't indicate that active bacteria reside in the gut. Also, in low-biomass samples, such as the meconium, contamination from laboratory reagents can become a major problem (13). We performed a quality control analysis as earlier reported (15). In our work, the differences were seen in bacterial genera that are known to be the first colonizers of infant gut (24).

We used machine-learning approach, in addition to conventional analysis, in the present study. Machine learning is a novel way to analyse microbiome data (38,39). Our models found a pattern to differentiate colic state of the samples with increased performance compared to random chance. Although performance was increased compared to random chance, the models indicate the infantile colic question to be of complex nature. Ideally, combining data from heterogenous sources, such as patients gene abundance data or environmental variables, could be of interest for future studies (40).

In this prospective, cohort study, the microbiome of the first-pass meconium was associated with subsequent infantile colic. Our results suggest that the pathogenesis of infantile colic is closely related to the intestinal microbiome at birth.

Acknowledgments: We thank the nurse who assisted in our study, Leena Okkonen, and the staff at the Central Finland Central Hospital

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Figure legends

Figure 1. Study design.

Figure 2. Relative abundance of the phylum Firmicutes and the genus *Lactobacillus* in the first-pass meconium at birth in newborn infants with subsequent infantile colic and those who remained healthy. The mean value of each group is indicated with a line, and each dot.

Figure 3. Prediction performance of the random forest classifiers for subsequent infantile colic compared with healthy infants. Solid lines indicate ROC curve of real classifier and dotted lines indicate random chance. Green lines indicate meconium samples and blue lines the samples at one year of age.

Figure 4. Comparison of the mean relative abundances of the most important bacterial genera for the random forest classifier between infants with colic and those who remained healthy. Bacterial genera with relative abundance >1% are shown. A) Microbiome of the first-pass meconium. B) Microbiome at 1 year of age.

Figure 5. Microbiome composition of the first-pass meconium according to weighted and unweighted principal coordinates analysis (PCoA). Red squares = colic, blue dots = no colic. A) First model, weighted PCoA. B) Second model, unweighted PCoA.