454 Pyrosequencing Analysis on Faecal Samples from a Randomized DBPC Trial of Colicky Infants Treated with *Lactobacillus reuteri* DSM 17938

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**Abstract**

**Objective:** To analyze the global microbial composition, using large-scale DNA sequencing of 16 S rRNA genes, in faecal samples from colicky infants given *L. reuteri* DSM 17938 or placebo.

**Methods:** Twenty-nine colicky infants (age 10–60 days) were enrolled and randomly assigned to receive either *Lactobacillus reuteri* (10⁸ cfu) or a placebo once daily for 21 days. Responders were defined as subjects with a decrease of 50% in daily crying time at day 21 compared with the starting point. The microbiota of faecal samples from day 1 and 21 were analyzed using 454 pyrosequencing. The primers: Bakt_341F and Bakt_805R, complemented with 454 adapters and sample specific barcodes were used for PCR amplification of the 16 S rRNA genes. The structure of the data was explored by using permutational multivariate analysis of variance and effects of different variables were visualized with ordination analysis.

**Results:** The infants’ faecal microbiota were composed of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes as the four main phyla. The composition of the microbiota in infants with colic had very high inter-individual variability with Firmicutes/Bacteroidetes ratios varying from 4000 to 0.025. On an individual basis, the microbiota was, however, relatively stable over time. Treatment with *L. reuteri* DSM 17938 did not change the global composition of the microbiota, but when comparing responders with non-responders the group responders had an increased relative abundance of the phyla Bacteroidetes and genus Bacteroides at day 21 compared with day 0. Furthermore, the phyla composition of the infants at day 21 could be divided into three enterotype groups, dominated by Firmicutes, Bacteroidetes, and Actinobacteria, respectively.

**Conclusion:** *L. reuteri* DSM 17938 did not affect the global composition of the microbiota. However, the increase of Bacteroidetes in the responder infants indicated that a decrease in colicky symptoms was linked to changes of the microbiota.

**Trial Registration:** ClinicalTrials.gov NCT00893711.

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**Introduction**

Infantile colic is one of the most common problems in paediatric medicine. It is a clinical condition characterized by incontestable crying, fussing and irritability in otherwise healthy newborn infants during the first 3 months of life. The diagnosis is clinical according to an old definition based on crying time, known as the “Wessel’s rule of three”: unexplained episodes of paroxysmal crying for more than 3 h/day for 3 days/week for at least 3 weeks [1]. A recent study identified some perinatal predisposing factors including maternal education, smoking habits, cheese consumption, hostility scores and domestic violence [2]. Despite, however, many studies conducted to determine the cause of this condition, its pathogenesis remains unclear and is most likely multifactorial [3,4].

Increasing importance has been recently assigned to the role of an aberrant gut microbiota in infants suffering from infantile colic, suggesting its influence on gut motor function and gas production.
had a diagnosis of infantile colic according to Wessel’s criteria: episodes of crying lasting more than 3 h a day, for 3 or more days in the 1 week prior to enrolment. They were all born at term, adequate for gestational age, with a birth weight between 2500 and 4000 g, and were aged 10–60 days at recruitment. Only exclusively breastfed infants were enrolled in order to prevent variability in the intestinal microbiota caused by diet. Exclusion criteria were clinical evidence of chronic illness or gastrointestinal disorders, any intake of probiotics and/or antibiotics in the week preceding recruitment and mixed breast- and formula-feeding.

The sample size was calculated post-hoc to find a clinically relevant difference in the reduction in daily average crying time of 50% between day 1 and day 21. With $\alpha = 0.05$, $\beta = 0.40$, 13 patients were needed per group.

For the specific analysis of the faecal microbiota, we evaluated 29 infants. The protocol was approved by the Ethics Committee of the Azienda Ospedaliera, OIRM S. Anna – Ospedale Mauriziano (Turin, Italy). Characteristics of enrolled subjects are reported in Table 1, and no differences were found between the two groups of subjects, besides the response to the treatment. Thus, the previous reported effect of L. reuteri DSM 17938 [10] was evident also in this subgroup of subjects. A written informed consent was obtained from both parents before the inclusion of infants. At enrolment, each infant underwent a medical examination and parents completed a questionnaire to obtain data about subjects, including also type of delivery (vaginally or caesarean). Infants were recruited and randomly assigned to receive the probiotic L. reuteri DSM 17938 or placebo daily for 21 days. Patients in each of the two groups were defined as “responders” or “non responders”, a responder displaying a reduction of 50% of crying time on day 21. Faecal samples (10–15 g) were collected from each subject, directly from the diaper or anus, at enrolment and on day 21. Samples (blinded) were immediately placed at $-20^\circ$C and then later stored at $-70^\circ$C until analysis of the gut microbiota by 454 pyrosequencing.

**DNA Extraction, PCRs and 454 Pyrosequencing**

DNA was extracted from 250 mg of faeces using the MoBio Power Soil DNA Kit (Sunnyvale, CA, USA) according to the manufacturer’s instruction with the modification that the bead-beating step was performed 2 x 43 s, at level 5 on a FastPrep® -24 device (MP Biomedicals, Solon, OH, USA).

The 16S rRNA genes were PCR amplified using broad range bacterial primers. The primers: Bakt_341F (CTTAGGAGTTGATATCTAATCC) were complemented with 454 bacterial primers. The primers: Bakt_341F (CTTAGGAGTTGATATCTAATCC) were complemented with 454 adapters and sample specific barcodes [13]. DNA was amplified with PCR (DreamTaq™ Mastermix, Thermo Fisher Scientific, Fermentas GmbH, St. Leon-Rot, Germany) under the following conditions.

**Table 1. Characteristics of subjects.**

| Variable                      | Placebo (n = 14) | L. reuteri (n = 15) | P value |
|-------------------------------|-----------------|---------------------|---------|
| Type of delivery (caesarean), n (%) | 3 (21.4) | 6 (40) | 0.427$^a$ |
| Male, n (%)                  | 7 (50)          | 9 (60)              | 0.715$^a$ |
| Age at entry (days), median (SD) | 29 ± 12.9   | 29 ± 11.7           | 0.960$^a$ |
| Responders, n (%)            | 7 (50)          | 14 (93)             | 0.013$^a$ |

$^a$Fisher’s Exact Test; $^b$ Wilcoxon Rank Sum Test; $^c$Significant difference.

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running conditions: initial denaturation at 95°C for 5 min, 30
cycles of 40 s at 95°C, 40 s at 50°C, 1 min at 72°C, and a final
elongation step for 7 min at 72°C. PCR products were confirmed
using agarose gel electrophoresis and these were subsequently
isolated from the gel and purified using GenElute™ gel extraction
kit (Thermo Fisher Scientific). Purified products were quantified
using a NanoVue spectrophotometer (GE HealthCare, Uppsala,
Sweden) and mixed into equimolar amounts. The mixed pool of
PCR products was sequenced from the reverse primer direction
at the Swedish Institute for Infectious Disease Control in Solna, using
the Roche/454 GS Titanium technology platform (Branford, CT,
USA).

Taxonomic Analysis
The sequence processing was mainly performed as described in
Herlemann et al. [13]. In brief, sequences were checked for
quality and those that were less than 300 bp in length excluding
the primer sequence, contained incorrect primer sequences, or
contained any ambiguous base were discarded. Remaining
sequences were then subjected to complete linkage clustering
using the pyrosequencing pipeline at RDP [17] with a conservative
5% dissimilarity to define OTUs. The most abundant sequence
from each OTU was selected as a representative sequence and was
taxonomically classified by BLAST searching against a local
BLAST database comprised of 600, 316 bacterial 16 S rRNA gene
sequences longer than 1,200 bp with good Pintail scores from RDP
v. 10.7. The OTU inherited the taxonomy (down to genus
level) of the best scoring RDP hit fulfilling the criteria of ≥95%
identity over an alignment of length ≥200 bp. Assignment of
sequences to samples was based on the 5-bp barcode.

Statistical Analyses
Comparisons of characteristics of subjects were made using
Fisher’s exact test and Wilcoxon rank sum test (SAS V9.2).
Changes in microbial community composition during the
intervention, for treatment type, response and type of birth, and
interactions of these factors were tested using the “adonis”
function in the vegan package for R [18]. Adonis is a
permutational multivariate analysis of variance using a distance
matrix as response variable. We used Euclidean distance and 999
permutations. The change of composition was also tested using
principal component analysis (PCA) on a matrix on the difference
in abundance of the different phyla or genus after and before
untreatment, using the vegan package [18]. Confidence ellipses
around the centroids of the result categories were used to assess the
differentiation of responders and non-responders [18]. In all
multivariate analyses, data was transformed by taking the arcsine
of the square root of the proportional abundance. Comparison of
specific changes in relative abundance during the intervention
period was done with a Mann Whitney test using Past [19]
(http://folk.uio.no/ohammer/past/). We also explored the struc-
ture of the data from the two time points with PCA using Canoco
4.5 and the validity of clustering patterns was confirmed with a
distance based MANOVA (Bray Curtis distance; Past software).

Results and Discussion
The 454 pyrosequencing generated in total 412 000 sequences
and the number of sequences per sample were between 1593–
21322 (average 6538, median 5997). Phylogenetic classification
revealed that the prevalence of the four major phyla varied
considerably between the samples (Table 2). All four were found at
abundances varying from less than 2% to more than 50%,
indicating that none of the phyla was discriminated by the method.

| Phyla         | Mean | Median | Max | Min |
|---------------|------|--------|-----|-----|
| Proteobacteria| 15.4%| 12.1%  | 52.0%| 0.04%|
| Actinobacteria| 30.2%| 30.3%  | 91.4%| 0.1% |
| Firmicutes    | 37.7%| 31.7%  | 97.4%| 1.9% |
| Bacteroidetes | 16.3%| 0.3%   | 78.6%| Not detected |

The infants’ faecal microbiota were composed of Proteobacteria,
Firmicutes, Actinobacteria and Bacteroidetes as the four main phyla (Figure S1). The microbiota compositions were shown to have very
high inter-individual variability with Firmicutes/Bacteroidetes ratios
varying from 0.025 to 4000 in line with previously published
studies on gut microbiota establishment in neonates [20,21]. On
an individual basis, the microbiota was, however, for most
individuals relatively stable over time (Figure S1). Although the
distribution between the phyla differs slightly from the present
investigation, other studies have shown that composition of the
microbial communities varies widely from baby to baby [20,22],
but also that distinct structures of each individuals microbial
community were noticeable [20].

Treatment with L. reuteri DSM 17938 did not affect the global
composition of the microbiota (Table 3). However, when comparing
responders (>50% reduction in crying time; n = 21; 14 in the L. reuteri group and 7 in placebo) with non-responders
(n = 8; 1 in the L. reuteri group and 7 in placebo) the change of
composition at the phyla and genus levels differed between the
groups (Table 3). Ordination analyses revealed that this difference
depended on an increase of the phylum Bacteroidetes (Figure S2) and
genus Bacteroides (data not shown) during the intervention in the
group of responders. Although some individuals only had a small
change and the variation was large the difference was significant
(p<0.01) and the abundance of Bacteroides increased on average
with 8% in responders but decreased with 18% in non-responders
(Figure S3). This is the first time that a high abundance of
Bacteroides has been linked to a decrease in colicky symptoms,
although increased diversity and relative abundance of Bacteroidetes
and Bacteroides have been shown to correlate with absence of IgE-
associated eczema [22] and this is in line with a reported
correlation between eczema and colic [23]. Those groups of
bacteria are also indicators of a gastrointestinal microbial
ecosystem that has maturated and participates in the breakdown
of complex plant polysaccharides [24], which could improve a
poor digestion. Furthermore, in a study on patients with severe
systemic inflammatory response syndrome, Shimizu et al. [25]
reported that gastrointestinal dysmotility was associated with
altered gut microbiota, and faecal analysis showed that patients
with feeding intolerance had significantly lower numbers of total
obligate anaerobes including Bacteroides than those in patients
without feeding intolerance.

A principal component analysis on the microbial composition
on day 21 revealed a pattern where three types of microbial
compositions (enterotypes) could be seen (Figure S4). This
clustering pattern was also evident when samples from day 1
were included in the ordination analysis (data not shown).
The validity of the clustering pattern was confirmed with a distance
based MANOVA (Bray Curtis distance; p<0.00001). In one type
(A; n = 12), the relative abundance of Actinobacteria was over 40%
and Bacteroidetes below 1% (11 of 12); in the second type (B; n = 9),...
the relative abundance of Bacteroidetes was above 25%; and in the third type (F; n = 8), the relative abundance of Firmicutes was above 45%, Actinobacteria below 2% and Bacteroidetes below 1% (7 of 8). Notably, 8 of the 9 type B samples, with a high abundance of Bacteroidetes, came from the group responders, and 7 of those came from infants with spontaneous delivery (Figure S4). The existence of different host-microbial symbiotic states in adults was recently described [14] and it is intriguing to see “enterotype-like” clustering in colicky infants and that correlates to the improvement in colic symptoms to some extent.

Vaginally delivery of the infants was overrepresented in enterotype group B and several other studies have reported that method of delivery influenced the microbiota in the first period of life. Caesarean section has been shown to delay colonization by Escherichia coli, Bacteroides and Bifidobacterium [26] and a negative correlation between Bacteroides and cesarean section has been described [27]. Recently Jost et al. [28] performed a study, using pyrosequencing, to investigate the establishment of pioneer gut microbiota during the first month of life of vaginally and exclusively breastfed infants. An early onset of Bacteroides was observed in 4 of 7 subjects, demonstrating that those strict anaerobes can reach high densities already during the first weeks of life. In this regards conflicting findings have been published previously [29].

Our current knowledge of the intestinal microbiota and its role in infant colic is weak. The question is if infantile colic is a cause or consequence of an alteration in gut microbiota. Illustrating the complexity of the interaction, the gut microbiota has been described to be involved in the regulation of various host metabolic pathways, giving rise to interactive host-microbiota metabolic, signaling, and immune-inflammatory axes that physiologically link the gut to the brain [30]. The change of the composition of the microbiota detected in the responders could not be attributed to treatment with L. reuteri. Consequently, the mechanisms by which this bacterium reduces colic symptoms may be either by modulating of the microbiota in a way that is not detected by 454 pyrosequencing, or by a direct effect on the infant. Preidis et al. [31] reported that L. reuteri DSM 17938, but not an other L. reuteri strain, considerably increased enterocyte migration, proliferation and crypt height in an experimental model on neonatal mice. The possible role of these mechanisms in decreasing colic symptoms is not known, but they may represent novel mechanisms for L. reuteri strain-specific effects. Although no effect on the microbiota could be attributed to supplementation with L. reuteri DSM 17938, the results of the study may provide the basis for further investigation of the role of the intestinal microbiota in infants with colic.

### Supporting Information

**Figure S1** Bacterial composition of 29 infants at the phylum level. The first column in each pair shows the initial composition and the second the composition at day 21. The different phyla are indicated by different colors and the infants responding to the treatment as well as the individuals given L. reuteri are marked in the figure.

**Figure S2** Principal component analysis on the difference in abundance of different phyla before and after treatment. Ellipses show 95% confidence around the centroids of the two outcomes of the treatment. Filled circles are responders and empty non-responders.

**Figure S3** Change in relative abundance of Bacteroidetes during the intervention period. The group of responders had an increase of this bacterial phylum and differed from the group of non-responders (P = 0.004; Mann Whitney test).

**Figure S4** PCA plot from faecal samples on day 21. Bacterial phyla indicated in circles: black, Actinobacteria; red, Bacteroidetes; blue, Proteobacteria, and green, Firmicutes. The small inlaid figure describes the features of the samples: purple, responders; green, non-responders; triangles, spontaneous delivery; and squares, caesarean delivery.

### Checklist S1 The CONSORT 2010 checklist shows all the information about the randomised trial.

**Protocol S1** Trial Protocol.

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### Author Contributions

Conceived and designed the experiments: FS SR. Performed the experiments: SR JD. Analyzed the data: VT EL FR. Contributed reagents/materials/analysis tools: SR JD UG. Wrote the paper: FS SR VT EL FR.

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Table 3. Pseudo F-ratios from tests using “adonis” of effects in microbial community composition between treatment group, response of treatment, and type of delivery, on a matrix on the difference in abundance before and after treatment.

| Phyla       | Taxa | OTU |
|-------------|------|-----|
| Treatment   | 1.02 | 0.81| 0.99|
| Response    | 4.09*| 2.49*| 1.30|
| Delivery    | 0.89 | 0.82| 0.99|
| Treatment × Responders | 2.51 | 0.92 | 1.18|
| Treatment × Delivery | 0.56 | 0.44 | 0.51|
| Responders × Delivery | 3.17 | 3.66 | 2.22|

The analyses were performed on three different taxonomic resolutions: Phyla, Taxa and OTU.

p<0.1, *p<0.05.

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