Pediatric RESEARCH

CLINICAL RESEARCH ARTICLE

Antibiotics in early life associate with specific gut microbiota signatures in a prospective longitudinal infant cohort

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BACKGROUND: The effects of antibiotics on infant gut microbiota are unclear. We hypothesized that the use of common antibiotics results in long-term aberration in gut microbiota.

METHODS: Antibiotic-naive infants were prospectively recruited when hospitalized because of a respiratory syncytial virus infection. Composition of fecal microbiota was compared between those receiving antibiotics during follow-up (prescribed at clinicians' discretion because of complications such as otitis media) and those with no antibiotic exposure. Fecal sampling started on day 1, then continued at 2-day intervals during the hospital stay, and at 1, 3 and 6 months at home.

RESULTS: One hundred and sixty-three fecal samples from 40 patients (median age 2.3 months at baseline; 22 exposed to antibiotics) were available for microbiota analyses. A single course of amoxicillin or macrolide resulted in aberration of infant microbiota characterized by variation in the abundance of bifidobacteria, enterobacteria and clostridia, lasting for several months. Recovery from the antibiotics was associated with an increase in clostridia. Occasionally, antibiotic use resulted in microbiota profiles associated with inflammatory conditions.

CONCLUSIONS: Antibiotic use in infants modifies especially bifidobacterial levels. Further studies are warranted whether administration of bifidobacteria will provide health benefits by normalizing the microbiota in infants receiving antibiotics.

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INTRODUCTION

The healthy infant gut microbiota develops in the first years of life according to an apparently biologically determined schedule, yet is depending strongly on external exposures.[1] The microbiota is initially dominated by facultative anaerobic taxa, such as streptococci, enterococci, and enterobacteria. During the first weeks of life, the abundance of bifidobacteria increases and become the dominant taxon in breastfed infants.[1] Antibiotics have been identified as exerting a strong influence on the infant gut microbiota, with a particularly negative impact on bifidobacteria.[2,3] In infancy, the gut microbiota is thought to guide the development of the immune system, via direct contact with host cells at the gut mucosal interface.[4] Owing to their high abundance at this age period, bifidobacteria very likely have an important role in overall infant development, and there is reason to believe that the antibiotic-associated disruption of gut microbiota in infants may have long-term health consequences.

Antibiotic use during the first year of life has been linked with increased risk of, e.g., cow's milk allergy,[5] asthma, eczema, hay fever, wheeze, atopy,[6] and inflammatory bowel disease.[7,8] Experimental mice studies have shown that antibiotic exposure in early life alters the development of the immune system.[9] Furthermore, studies in neonatal mice implicate changes in the gut microbiota as the causal link between antibiotics and altered immune system development.[10] Indeed, an association between the gut microbiota composition during the first months of life and later development of allergic disease has been shown repeatedly in humans.[11]

In addition to the immunological effects, the early-life gut microbiota is emerging as an important factor regulating the overall physiology of the host, especially metabolism.[12] Low abundance of bifidobacteria during the first months of life has been found to be associated with increased body mass index in later childhood.[13] Animal studies showed that metabolites of the gut microbes influence epigenetic programming,[14] and early-life transient disruption of the gut microbiota by antibiotic treatment has revealed long-lasting detrimental metabolic consequences on the host, increasing the susceptibility to diet-induced obesity.[15] Several epidemiologic studies have shown an association between early-life antibiotic exposure and the risk of overweight in later life.[16,17] Intervention studies in low-income countries have indicated that antibiotics have a growth-promoting effect in children.[18,19]

The accumulating evidence of the long-term adverse effects caused by early-life antibiotic treatment increasingly underscores the importance of finding ways to mitigate the effects. The impact of antibiotic use on the development of antibiotic resistance can be reduced by limiting the duration of the antibiotic course,[20] and the same approach could hypothetically alleviate the impact on the gut microbiota. However, the temporal dynamics of the gut microbiota during and after antibiotic treatments in infants are currently not clearly understood. We address this question in a cohort of 40 infants by analyzing the development of the gut microbiota before, during, and after amoxicillin and macrolide treatments and comparing these to control infants with no

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antibiotic treatments. We posed the research question: Does the use of common antibiotics result in long-term aberration in the gut microbiota of infants?. Accordingly, we hypothesized that the use of common antibiotics results in long-term aberration in gut microbiota.

METHODS

Subjects and fecal sample collection

All participants were prospectively recruited on the first day of their hospitalization because of a respiratory syncytial virus (RSV) infection at Children's Hospital, University of Helsinki, and Turku University Hospital between December 2013 and May 2014. We only included infants who were antibiotic naive at recruitment according to parental report. Fecal sampling started on admission and was thereafter instructed as follows: on day 1 and thereafter at 2-day intervals during hospital stay and at 1, 3, and 6 months at home. Samples were immediately frozen at −20 °C and transported frozen at −70 °C until analyzed. At the time of each sampling, the families filled out a questionnaire on diet, probiotics used, and previous antibiotic therapies as appropriate. Details of birth mode, disease and outcomes, growth, and weight gain were checked on patient files. Of the 58 recruited patients, there were 163 fecal samples from 40 patients available for microbiota analyses (median 4.5, range 1–9 samples per patient). Of these, 14 infants received antibiotics during admission for RSV infection and other 8 during follow-up (Supplementary Fig. S1 and Supplementary Table S1), mostly because of otitis media. The latter eight samples were accordingly instructed to be collected at home. Preterm infants (birth <37 gestational week or birth weight <2500 g) or those with congenital malformations or syndromes were excluded. None of the infants had been traveling abroad. Background data of the study participants are presented in Supplementary Table S1.

Sample processing and sequencing

Bacterial DNA was extracted from ca. 125 mg of fecal samples using repeated bead-beating method[21] with the following modifications for automated DNA purification: After the bead-beating steps, 400 µl of the pooled cell lysates was purified with the RSC Blood DNA Kit AS1400 using Promega Maxwell RCS instrument (Promega, Madison, WI). DNA was quantified using Quanti-iT™ Pico Green dsDNA Assay (Invitrogen, San Diego, CA).

Library preparation and sequencing

Illumina MiSeq paired-end sequencing of the hypervariable V3–V4 regions of the 16S rRNA gene was performed at the sequencing unit of Institute for Molecular Medicine Finland, Helsinki, Finland. The protocol followed that from Illumina except that the libraries were prepared with single-step polymerase chain reaction (PCR), i.e., by combining amplicon and index PCR in the same reaction, and index primers were adapted from Kotzich et al.[22] The PCR reaction comprised of 1 ng/µl template, 1× Phusion® Master Mix (ThermoFisher, F-531L), 0.25 µM V3–V4 16S rRNA gene primers, and 0.375 µM dual-index primers. The PCR was run under the following settings: 98 °C for 30 s, 27 cycles of 98 °C for 10 s, 62 °C for 30 s, 72 °C for 15 s, and finally 10 min at 72 °C, whereafter the samples were stored at 4 °C. The amplicon size (ca. 640 bp) was verified on LabChip GX Touch HT instrument (PerkinElmer, Waltham, MA). The PCR clean-up was performed with AMPure XP beads (Beckman Coulter, Copenhagen, Denmark) and confirmation of the right size of the target was performed on a Bioanalyzer DNA 1000 chip (Agilent Technology, Santa Clara, CA). The pooled libraries were sequenced with an Illumina MiSeq instrument using paired end 2 × 300 bp reads and a MiSeq v3 reagent kit with 5% PhiX as spike-in.

Samples

There were in total 163 fecal samples. Of these, 86 were classified as control samples and included samples from infants never receiving antibiotics and antibiotic-naive samples from infants later receiving antibiotics. Only a single infant in the cohort received a cephalosporin course, so the samples taken after cephalosporin were omitted from the analyses. The samples of the amoxicillin and macrolide groups are shown in Supplementary Fig. S1 and were further divided into groups based on the duration of time since the most recent antibiotic course began (Table 1). There were no samples taken from macrolide-treated infants during the first week, so early comparisons only include amoxicillin-treated infants.

Ethics approval and consent to participate

The study was approved by the ethical committee of The Hospital District of Helsinki and Uusimaa. The guardians of the participants signed an informed consent.

Statistical methods

The comparisons were adjusted for infant age, birth mode, and diet (exclusive breastfeeding, partial breastfeeding, exclusive formula feeding, or solid feeding).

Sequence read processing and statistical analysis was conducted using the R package mare,[23] which uses usearch[24] for sequence processing, and the R packages vegan,[25] MASS,[26] nlme,[27] and gstat[28] for data analysis. The statistical test was individually adapted to each bacterial taxon to make sure the data fulfill the assumptions of the test. The specific statistical test used for each taxon is presented in Supplementary Tables S2 and S3. Microbiota diversity was calculated as the inverse Simpson index and richness was calculated as the number of operational taxonomical units (OTUs). Microbiota maturity index was calculated based on the bacterial taxa that were significantly associated with age in the control infants. A generalized linear model with the negative binomial distribution was used to model age (months) as a function of the microbiota composition at the family level in the control samples. First, the associations of all family-level taxa were assessed and the taxa that were significantly associated with age ($p < 0.01$) were selected and combined into a single model. The full model was then reduced using Akaike Information Criterion to obtain the final model:

$$\text{Maturity} = 2.721 - 3.560 \times \textit{Verrucomicrobiaceae} + 2.653 \times \textit{Ruminococcaceae} + 27.760 \times \textit{Peptostreptococcaceae} + 1.754 \times \textit{Lachnospiraceae} - 22.642 \times \textit{Carnobacteriaceae} - 2.088 \times \textit{Porphyromonadaceae}.$$

Table 1. Number of samples per time group and amoxicillin and macrolide groups[a].

Days since the beginning of course	0	1	2–3	4	5–8	12–40	46–115	>169
Amoxicillin (no. of infants = 20; total number of samples = 66)	5	5	7	7	8	12	12	10
Macrolide (no. of infants = 4; total number of samples = 10)	0	0	0	0	0	6	3	1

[a]For comparisons in each time group, all control samples were used ($N = 86$ samples prior to any antibiotic from 22 infants). A single sample after cephalosporin therapy not included.

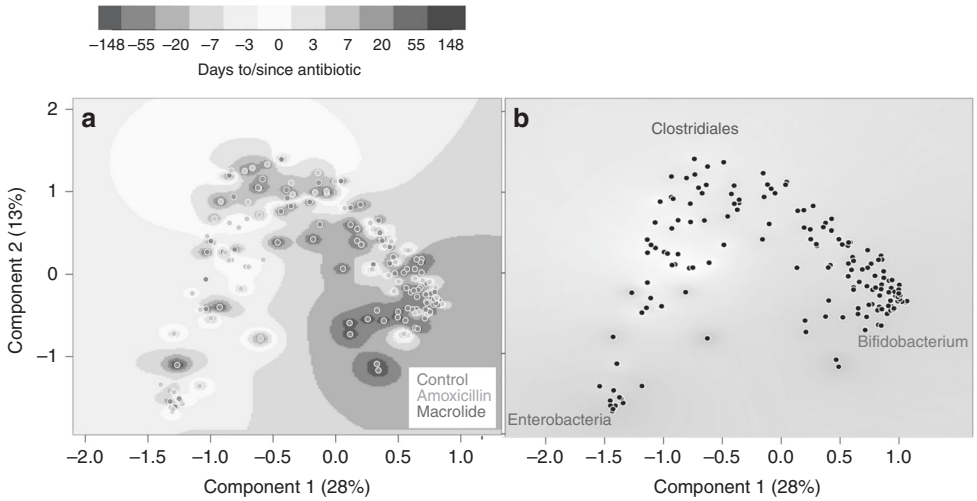

Fig. 1 Principal coordinates analysis (PCoA; Bray–Curtis dissimilarities) of the infant gut microbiota. Both panels show the same first two PCoA components. The background colors show the interpolated value of time to/since antibiotic treatment (**a**) and the abundances of three major bacterial taxa, each in different color (**b**). In **a**, the sample color represents the most recent antibiotic. For the purpose of the picture, the time to antibiotic was set to 148 days for the control infants who had not received any antibiotic.

RESULTS

We recruited antibiotic-naive infants ($n = 40$) and the effects of antibiotics on their gut microbiota. The infants were admitted because of RSV infections, and during follow-up, 22 received antibiotics (mostly because of otitis media), the introduction, dose, and duration of the therapy prescribed at clinicians' discretion. Most infants received amoxicillin. Macrolide was the second most frequently used antibiotic drug. In total, there were 163 fecal samples for analyses from patients (median age 2.3 months at baseline; median 4.5 samples per patient; details in Supplementary Table S1 and Supplementary Fig. S1). The microbiota composition in the samples was characterized by variation in the abundance of bifidobacteria, enterobacteria, and clostridia (Fig. 1 and Supplementary Fig. S2 showing individual variation). Samples taken before antibiotic courses clustered in the high-bifidobacteria end of the principal coordinates plot (Fig. 1). Samples taken at the beginning of the antibiotic course (available in the amoxicillin group only) were characterized by high abundance of enterobacteria. Recovery from the antibiotics was not associated with a return of the microbiota to the original *Bifidobacterium*-dominated composition but rather with an increase in clostridia. This pattern was evident in both the amoxicillin- and the macrolide-treated infants.

Infants treated with amoxicillin had initially, before the beginning of the course, increased relative abundances of *Bacteroidaceae* (mainly *Bacteroides*, $p = 0.0001$) *Bifidobacterium* ($p < 0.0001$), *Enterococcus* ($p < 0.0001$), *Bacillus* ($p = 0.014$), and *Haemophilus* ($p = 0.003$) compared to the group not introduced to antibiotics (Supplementary Table S2). After the onset of the amoxicillin treatment, the disruption of the microbiota composition was rapid, evident already on the first day (Fig. 2), when the relative abundance of bifidobacteria was reduced by 50%. By day 4, bifidobacteria were nearly completely depleted, to 1.4% of the abundance in control samples ($p < 0.0001$) and replaced by enterobacteria as the dominant group (6-fold increase in enterobacteria, $p = 0.0008$, Supplementary Table S3). The single child with samples after introduction of amoxicillin–clavulanic acid showed a somewhat different pattern but rapid recovery already on day 6 (Supplementary Fig. S2). In addition to the change in the dominant bacteria, the relative abundances of several other taxa changed (Fig. 3): amoxicillin treatment resulted in a rapid, long-term decline in enterococci ($p < 0.0001$), as well as temporary declines in *Bacteroidaceae* (42-fold decrease, $p < 0.0001$) *Clostridiaceae* (37-fold decrease, $p = 0.0003$), *Coriobacteriaceae* (77-fold

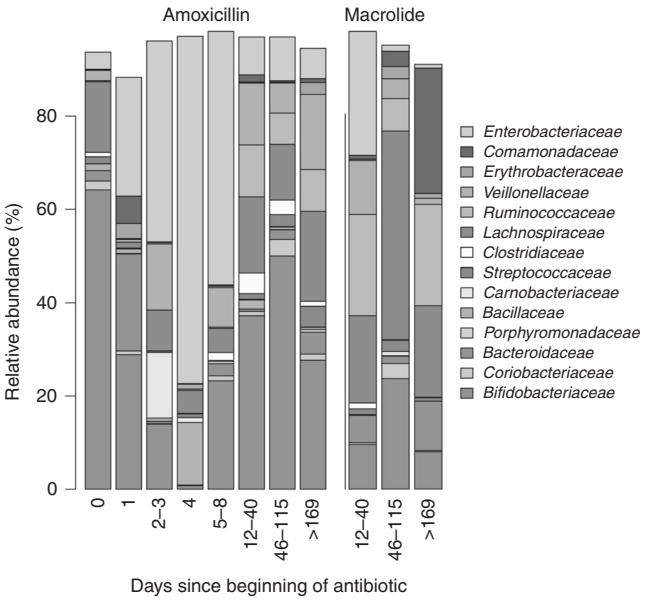

Fig. 2 Average microbiota composition at family level by time since the recent amoxicillin or macrolide course. Day 0 denotes the beginning of the course, with the fecal sample collected before the administration of the antibiotic.

decrease, $p < 0.0001$), *Streptococcaceae* (3.8-fold decrease, $p = 0.02$), and *Veillonellaceae* (37-fold decrease, $p = 0.02$). The findings were not related to probiotic use (data not shown).

After the amoxicillin course ended (by days 5–8, mean duration 5 days), bifidobacteria began to recover, but even after 6 months their relative abundance was 50% lower than that in the control samples ($p = 0.03$, Figs 2 and 3, Supplementary Table S2). During the recovery, enterobacteria were replaced by Firmicutes (2-fold increase, $p = 0.015$, mostly the families *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridiaceae*, Fig. 2). After approximately 1 month from the amoxicillin treatment, the abundance of *Coprococcus (Lachnospiraceae)* was 10-fold increased ($p = 0.02$), *Dialister* (*Veillonellaceae*) was 20-fold increased ($p = 0.01$), and *Megasphaera* was 100-fold increased ($p = 0.0006$), compared to controls. After ca. 6 months, the total

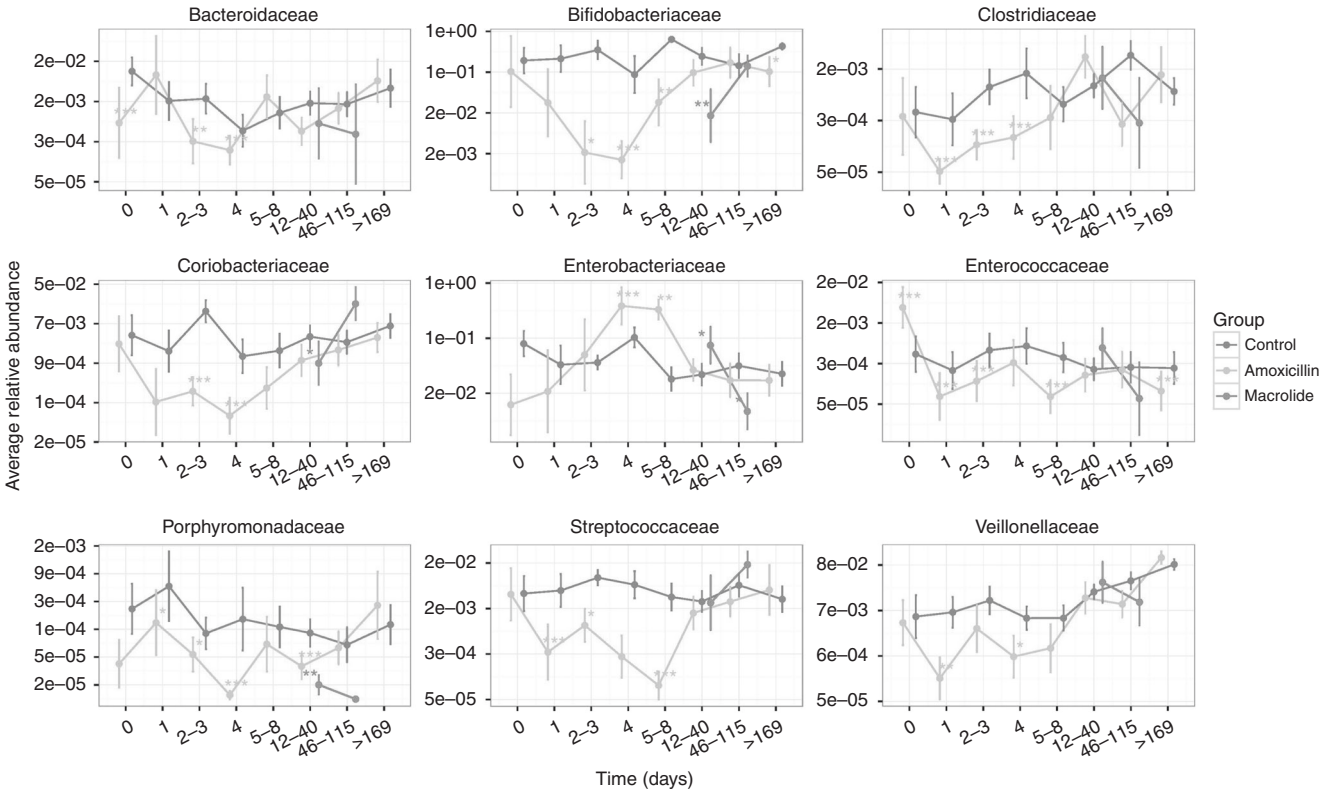

Fig. 3 Statistically significant changes in gut microbiota composition before, during, and after antibiotic courses. The antibiotic groups at each time point are compared to the control group. The group means and standard errors of relative abundance are shown.

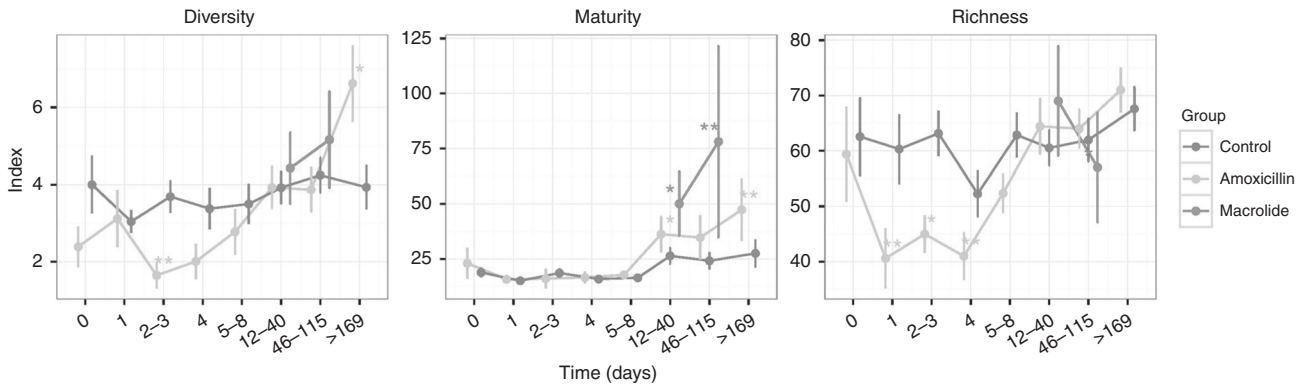

Fig. 4 Changes in microbiota related to diversity, maturity, and richness before, during, and after antibiotic courses. The antibiotic groups at each time point are compared to the control group. The group means and standard errors in statistical significant changes are shown.

abundance of Firmicutes was still 2.5-fold increased ($p = 0.03$), *Coprococcus* was 21-fold increased ($p = 0.007$), and *Dialister* was 43-fold increased ($p = 0.01$).

In the macrolide-treated infants, after 1 month (the first available samples), the relative abundance of bifidobacteria was still 59-fold lower than that in the control samples ($p = 0.004$, Supplementary Table S3), *Subdoligranulum* (*Ruminococcaceae*) was 26-fold increased ($p = 0.007$), and *Salmonella* was 45-fold increased ($p < 0.0001$). After ca. 3 months, *Bacillales* were 20-fold increased ($p = 0.004$), and Alphaproteobacteria were 25-fold increased ($p = 0.03$).

As indicators of overall microbiota development, we calculated microbial diversity (inverse Simpson index), maturity (based on a model of age-associated bacterial taxa), and richness (number of OTUs). Diversity initially declined during the amoxicillin course (Fig. 4, $p = 0.009$) but recovered rapidly and exceeded the expected values by 6 months post-antibiotic ($p = 0.018$). Microbiota maturity was not affected immediately by the amoxicillin course but increased during the recovery period, reaching a significantly higher level than in the controls at 1 month after the course ($p = 0.004$). The increase in microbiota maturity was particularly dramatic in the macrolide-treated infants ($p = 0.001$). Microbiota richness declined rapidly during the first day of amoxicillin treatment ($p = 0.01$), which was associated with a loss of 20 OTUs on average (30% of the original 60 OTUs). After the cessation of the antibiotic, the microbial richness recovered quickly.

The temporal development of the microbiota composition in infants given amoxicillin and in infants not given any antibiotic is shown in Supplementary Figs S3 and S4, respectively.

DISCUSSION

Analyzing the temporal dynamics of the infant gut microbiota before, during, and after antibiotic treatment, we have shown that a single course of amoxicillin, the most commonly used antibiotic in the pediatric population, results in the long-term disruption of the gut microbiota composition in infants. Rather than returning to the original composition after the course, the microbiota begins an accelerated maturation toward low abundance of bifidobacteria and increased abundance of clostridia. Hence, amoxicillin does not just temporarily affect the relative abundance of dominant bacteria sensitive to this drug[29] but appear to profoundly alter the ecology of the early microbiota succession in the developing infant.

The observed changes are unlikely to be caused by the infection for which the antibiotic was prescribed, since the changes occurred after the beginning of the antibiotic course and showed a clear temporal pattern matching with the onset and end of the antibiotic treatments. Furthermore, all children in our cohort, including the controls, had an RSV infection.

The initial response of the infant gut microbiota to amoxicillin was rapid and dramatic. Many significant changes occurred already on the first day, and by day 4, the microbiota composition was completely changed. Since each successive day of treatment was associated with further exacerbation of the alterations, it is clear that minimizing the duration of the course would help to reduce the damage on the microbiota. The most dramatic immediate effect of the amoxicillin treatment was the replacement of bifidobacteria with enterobacteria. Although the abundance of enterobacteria quickly returned to normal levels after the antibiotic course, the overall microbiota composition did not return to the original state.

In healthy, breastfed infants, bifidobacteria form the dominant taxon, beginning to slowly decline after weaning, being replaced by adult-type clostridia.[1] The time window for *Bifidobacterium* dominance is thus normally between the first and the sixth month of age. At the time of the antibiotic treatments, the infants in our cohort were on average almost 4 months old. Our results show that antibiotic treatments during this age period cause a severe depletion of bifidobacteria that continues at least for 6 months, i.e., past the time window for *Bifidobacterium* dominance. In other words, antibiotics during this age period end the *Bifidobacterium* phase abruptly and prematurely.

We have previously shown that macrolide, but not amoxicillin, treatments have a long-term detrimental impact on the relative abundance of bifidobacteria in 2–7-year-old children, recovery taking at least 2 years.[30] In this older age group, amoxicillin had modest effects on the microbiota compared to macrolides and appeared as a safer alternative. The current results indicate that the infant microbiota may be more fragile than that of older children to the effects of antibiotics, since both amoxicillin and macrolide antibiotics had similar, very strong effects. Therefore, amoxicillin cannot be considered a microbiota-friendly antibiotic in young infants. The difference could also be due to different sampling time frame: in the previous study, the samples were taken several months and even years after the antibiotic course.

The pattern of low bifidobacterial and high clostridial abundance in infants has previously been linked with increased risk of allergic diseases in later life.[31] Bifidobacteria can modulate several immunological pathways on both innate and adaptive immunity, promoting mucosal and systemic immune homeostasis.[32] They also make pili that interact with the gut epithelium and stimulate proliferation.[33,34]

These results suggest that antibiotic treatment, which causes a replacement of bifidobacterial first with lipopolysaccharide-producing enterobacteria and then with clostridia, whose immunological effects are very different from those of bifidobacteria, likely results in increased inflammatory responsiveness of the host. Interestingly, pediatric patients with inflammatory bowel disease often have an increased abundance of enterobacteria[35,36] or decreased abundance of bifidobacterial,[37] and we recently found that the abundance of bifidobacteria predicts positive treatment response in these patients.[36]

Intestinal microbiota is a key source of microbial signals that are required to the balanced postnatal development of innate and adaptive immune systems.[38,39] The extent and duration of early microbiota perturbations that may lead to deviated physiological state in humans is unclear. However, the accumulating evidence of negative long-term health consequences of early antibiotic treatments indicate that the early disruption of normal gut microbiota development may be detrimental to host health. Intriguingly, we have recently shown that supplementation with bifidobacteria is able to reduce the impact of antibiotics on the infant gut microbiota.[40] Thus further studies are needed to study whether specific bifidobacterial strains given during and after antibiotic treatments in infants are able to restore the microbiota to age-dependent maturational stage.

As a limitation, the number of available samples, however, was low, like in most pediatric studies, and especially in the control group, there were few long-term samples. Also, the data available did not allow any conclusions related to the dosing of antibiotics.

CONCLUSIONS

A single course of common antibiotics resulted in aberration of infant microbiota lasting for several months. Antibiotic use may result in increased inflammatory responsiveness of the host as suggested by the replacement of bifidobacteria with clostridia and enterobacteria. As antibiotic use in infants modifies especially bifidobacterial levels, further studies are warranted whether administration of bifidobacteria will provide health benefits by normalizing the microbiota during antibiotic courses.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are included within the article and its additional files. With publication, deidentified data on microbiota analyses will be made available on request at the email address: katri.korpela@helsinki.fi.

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AUTHOR CONTRIBUTIONS

K.K. designed the study together with K.-L.K., H.S., A.S., and W.d.V., analyzed the data on microbiota, performed the statistical analyses, and wrote the first draft of the article. A.N. recruited the patients and collected the samples and demographics. V.P. participated in the study design and was responsible for the recruitment of patients in Turku University Hospital. T.J. participated in the patient recruitment. All authors critically revised the manuscript and approved the final submission.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-020-0761-5) contains supplementary material, which is available to authorized users.

Competing interests The authors declare no competing interests.

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