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LAZAREVIC, Vladimir, et al.

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Effects of amoxicillin treatment on the salivary microbiota in children with acute otitis media

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Abstract

Amoxicillin is a first-line antibiotic treatment for acute otitis media in children and one of the most commonly used antibiotics for human bacterial infections. We investigated changes in salivary bacterial communities among children treated with amoxicillin for acute otitis media (n = 18), using a culture-independent approach based on pyrosequencing of the V3 region of the bacterial 16S rRNA gene. The control group consisted of children with acute otitis media who were not given antibiotics (n = 15). One species-level phylotype assigned to the genus Streptococcus was identified across all (n = 99) saliva samples. Two additional species-level phylotypes from the genera Gemella and Granulicatella were shared by all (n = 45) samples of control subjects. Amoxicillin treatment resulted in reduced species richness and diversity, and a significant shift in the relative abundance of 35 taxa at different ranks from phylum to species-level phylotype. At the phylum level, prevalence of TM7 and Actinobacteria decreased at the end of treatment, whereas Proteobacteria had a higher relative abundance post-treatment. Multivariate analysis showed that samples from the same control subject taken over time intervals tended to cluster together. Among antibiotic-treated subjects, samples taken before and at the end of amoxicillin treatment formed two relatively well-separated clusters both of which greatly overlapped with samples taken about 3 weeks post-treatment. Our results point to a substantial but incomplete recovery of the salivary bacterial community from the antibiotic about 3 weeks after the end of treatment.

Keywords: Antibiotics, metagenomics, microbiota, otitis media, saliva

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Introduction

Antibiotic therapy causes changes in the composition and abundance of commensal microbes; however, the long-term effects of these changes remain largely unknown. In children with recurrent acute otitis media (AOM), repeated courses of antibiotics may reduce populations of beneficial bacteria, removing barriers to colonization and growth for potential pathogens [1,2]. Amoxicillin, a first-line antibiotic treatment for AOM, may promote the emergence of Staphylococcus aureus in the nasopharynx when commensal microbes are killed by the antibiotic [1]. Amoxicillin use during the first month of life results in decreased counts of faecal bifidobacteria and Bacteroides fragilis-group species [3]. A single-course of amoxicillin eradicates Helicobacter pylori from the stomach in 20–50% of cases [4].

High-throughput sequencing of metagenome fragments or amplified 16S rRNA has been widely applied to assess the composition and structure of bacterial communities colonizing the human body [5–7]. However, few studies have focused on the effect of antibiotics on the microbiota, and they have included a relatively small number of healthy volunteers [8–10]. Here, we investigated the changes in salivary microbiota in children treated with amoxicillin for AOM. Because of the anatomical proximity of the oral cavity and nasopharynx, the latter being considered as a reservoir of causative agents of
AOM, there is a possibility that the salivary microbiota may be altered by AOM. Therefore, in our study the control group consisted of children with AOM who were not given antibiotics.

Materials and Methods

Patient information and sampling
The study was conducted according to the current version of the Declaration of Helsinki and approved by the Ethics Committee of the Geneva University Hospital (09-006).

This was a prospective cohort study conducted from August 2009 to July 2010 in the emergency department of a tertiary-care paediatric centre. We included children aged from 12 to 72 months with clinically diagnosed AOM whose parents gave informed consent for their participation in the study. Exclusion criteria were pharyngitis and a history of antibiotic treatment in the past 6 months. The amoxicillin group (Group A, n = 18) were patients whose care providers prescribed amoxicillin treatment. The control group (Group C, n = 15) was defined with the same clinical criteria except that their care providers did not prescribe antibiotics. Children in the control group received only non-steroidal anti-inflammatory medications for their AOM.

Saliva was sampled from both groups of patients on three occasions: at the time of initial visit (time point 1), after about 10 days (time point 2, corresponding to the end of the treatment for children prescribed amoxicillin), and about 1 month after the initial visit (time point 3). Saliva was collected by gently rolling a swab under the tongue and between the gum and the cheek. The swabs (Rayon swab tip, Copan 155C; Copan Italia, Brescia, Italy) were placed into the original tubes and stored at −20°C until DNA extraction.

DNA extraction
Fifty microlitres of lysis buffer [Tris 20 mm (pH 8), EDTA 2 mm, 1% Tween-20, 400 mg/L proteinase K (Fermentas, Vilnius, Lithuania)] were applied directly to the swab tip. The swab was inserted back into its container and incubated for 2.5 h at 55°C. Then, the swab head was cut from the stick and placed in the inner tube of the Swab Extraction Tube System (Roche, Basel, Switzerland). Samples were centrifuged for 30 s at 16,000 g. The eluate was incubated at 95°C for 10 min to inactivate proteinase K and stored at −20°C.

PCR and sequencing
We amplified the VI–3 region of the bacterial 16S rRNA gene corresponding to Escherichia coli 16S rDNA positions 28–514 (excluding primer sequences). The PCRs included 3 µL of DNA extract, 25 pmol of each forward primer 5'-gcgttccgagcgcctcag-ac-GAGTTTGATCMTGCGCTCAG and a barcoded reverse primer 5'-gcctccccgctgcatcag-NNNNNNNNN-NNN-NNN-CCGGCRTGCTGGCAC in 50 µL of Primestar HS Premix (TaKaRa, Shiga, Japan). The structure of composite PCR primers has been explained previously [11]. All PCRs were carried out in duplicate for 30 cycles using the following parameters: 98°C for 10 s, 56°C for 15 s and 72°C for 1 min. Two replicate PCRs were then pooled, column-purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and eluted in 25 µL water. One hundred and fifty nanograms of each of the purified samples was pooled and the amplicon library was sequenced on a Genome Sequencer FLX system (Roche) from the Fusion Primer A.

Sequence analysis
Sequence filtering and analysis of sequence data were performed using the MOTHUR software package [12]. We removed sequences using any of the following criteria: (i) they were <290 or >600 nucleotides in length; (ii) contained ambiguous bases; (iii) contained more than one mismatch in the reverse primer sequence (excluding the adaptor A sequence); (iv) contained runs of ≥10 identical nucleotides; and (v) had a minimum quality score <35 over a 50-nucleotide window. Sequences were aligned (align.seq) against the SILVA reference alignment set [13] and truncated to include bases corresponding to E. coli 16S rDNA positions 257–514. Removal of noise and removal of potentially chimeric sequences were performed using MOTHUR’s commands pre.cluster [14] and chimera.slayer [15], respectively. Sequences were assigned to representative 97% identity phylotypes (operational taxonomic units, OTUs) using CD-HIT [16]. Taxonomic assignments of phylotypes were made using the RDP CLASSIFIER [17] with an 80% cut-off. A trimmed dataset (212,852 reads) was deposited in MG-RAST [18] under accession number 4496634.3.

Clustering of bacterial communities, ecological indices and statistical analysis
Multiple SILVA-based alignment of representative OTUs was imported into FastTree [19] to construct a tree that was then used as input file for the Fast Unifrac web interface [20]. A Bray–Curtis similarity matrix was generated using square-root-transformed abundance of OTUs. Principal coordinate analysis was applied on the UniFrac distance matrix of all pairwise combinations of communities. Chi-square test, Student’s t test, Mann–Whitney U test, Wilcoxon signed-rank test and permutation multivariate analysis of variance (PERMANOVA) were used...
to assess statistical significance. The PRIMER-E package (Primer-E Ltd., Plymouth, UK) was used to calculate species richness and diversity.

**Results**

**Subjects and pyrosequencing results**

Saliva samples were taken on three occasions (1–3) from children with AOM, treated (A) or not treated (C) with amoxicillin. Patient characteristics are presented in Table 1 and in Supplementary material, Table S1.

A total of 212 852 filtered pyrosequencing reads were analysed. Trimmed sequences corresponded to the 16S rDNA hypervariable region V3 and a part of the conserved region between V2 and V3 (positions 257–514 in the *E. coli* 16S rRNA gene) and had an average length of 257 nucleotides. We extracted this region from the SILVA Bacterial reference set (14 956 16S rRNA gene sequences) [13] to assess the accuracy of taxonomic assignments. The proportion of such simulated 454 reads correctly and erroneously assigned at the genus level, using RDP CLASSIFIER at a bootstrap cut-off of 80%, reached 76% and 2%, respectively (see Supplementary material, Fig. S1).

**Taxon abundance**

The six phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, TM7 and Fusobacteria), commonly found in salivary bacterial communities [11,21–24], dominated the samples in our study (Fig. 1 and Supplementary material, Table S2). Averaging of the data from our study and published studies [11,21–24] showed a trend of a higher proportion of Firmicutes and a decrease in the frequency of six other major phyla in saliva from children/adolescents in comparison to adults (Fig. 1).

Two out of 81 genera identified, *Amaricoccus* and *Moritella*, have not been previously found in the salivary microbiota [11,21–24], but each of them had only one occurrence. This supports the claim that the most frequent and widespread members of the human salivary microbiome in the western world have already been described [25]. However, the analysis of samples from other geographic locations still identifies new relatively abundant genera [26].

The dataset was represented by 11 028 distinct sequences (100%-ID phylotypes) and 1 486 OTUs. In a recent study of the salivary microbiome, which included 264 samples from 107 individuals aged from 8 to 24 years, two OTUs belonging to the genus *Streptococcus* were found in all but one sample and corresponded to 10.5% of all sequence reads [27]. Here, on a cohort including 33 subjects aged 1–6 years, 18 of which were exposed to the antibiotic, an OTU was identified across all time-point samples of all individuals (99 samples). It was also assigned to the genus *Streptococcus* and represented on average 44.6% of the total number of sequence reads per sample. Two additional OTUs from the genera *Gemella* and *Granulicatella* were shared by all (n = 45) samples of control subjects. After combining the sequence reads of the three time-point samples from the same individual into a single

![FIG. 1. Relative abundance of seven major phyla across different studies of the salivary microbiome. Data obtained for different individuals (and different time-points if available) were averaged for each study except that of Keijser et al. [24] in which individual non-bar-coded amplicon libraries were pooled in equimolar amounts before sequencing. 'A' and 'C' correspond to the treated and untreated group of children analysed in this study, respectively. 'Children/adolescents AVG' and 'Adults AVG' were obtained by averaging data from the relevant studies.](image)

<table>
<thead>
<tr>
<th>TABLE 1. Patients’ characteristics</th>
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<tr>
<td><strong>Antibiotic group</strong></td>
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<td>---------------------</td>
</tr>
<tr>
<td>Mean age (months ± SD)</td>
</tr>
<tr>
<td>Male sex</td>
</tr>
<tr>
<td>Antibiotic treatment in last 12 months</td>
</tr>
</tbody>
</table>

*Chi-square test was used to compare proportions between groups. A Student t test was used to compare continuous variables (mean age). A level of p < 0.05 was considered significant.
dataset, the ‘universal core’ of the entire cohort reached an average size of 69% and included 11 OTUs belonging to the genera Streptococcus (4), Granulicatella (2), Gemella (2), Veillonella (1), Coprococcus (1) and Fusobacterium (1).

The assessment of differences in relative taxon abundance at different visits (1, 2 and 3) revealed 39 significant changes within the antibiotic-treated (A) group and none within the control (C) group (Table 2). In all cases (31) where a statistically significant shift in the average taxon abundance was found between pre-treatment (A1) and end-of-treatment (A2) samples, we also observed the opposite direction of change between end-of-treatment and post-treatment (A3) samples, which points to a recovery from antibiotic treatment.

The end of treatment correlated among others with a reduced proportion of the phyla TM7 and Actinobacteria, genera Corynebacterium, Rothia, Abiotrophia, Prevotella, Solobacterium and Fusobacterium, as well as OTUs assigned to most of these genera but also to Streptococcus and Gemella. The proportion of two OTUs, assigned respectively to Abiotrophia and order Burkholderiales, increased at the end of treatment. Given the antibiotic spectrum of amoxicillin [28], the reduction in Streptococcus, Corynebacterium and Prevotella was expected. In contrast, genera Neisseria and Haemophilus (and OTUs assigned to them) were not consistently reduced at the end of treatment.

Among 31 taxa with statistically significant changes in the relative abundance between A1 and A2 samples, 13 had the same direction of change (increase or decrease) in all relevant individuals. For each of the remaining 18 taxa, the change in one direction prevailed over the other (Table 2). This was also the case with all the taxa showing significant changes in relative abundance in A1–A3 and A2–A3 sample comparisons.

At the post-treatment sampling point, Proteobacteria were significantly more abundant, whereas phylum TM7, class Clostridia, order Coriobacteriales and genus Prevotella had lower relative abundances compared with the baseline (A1) levels. It has been previously reported using the culture method that amoxicillin treatment reduced the number of Prevotella spp. isolates considered to interfere with the growth of potential pathogens in the nasopharynx [1].

Microbiota clustering
We compared the saliva bacterial communities in terms of their phylogeny within the antibiotic-treated and control groups using UniFrac. Principal coordinate analysis of unweighted UniFrac distances in group C showed that samples taken from the same individual over time tend to cluster together (Fig. 2). In group A, the samples taken before and at the end of the amoxicillin treatment formed two relatively well-separated clusters both of which partly overlapped with the cluster formed by the post-treatment samples (Fig. 2). The effect of antibiotic is clearly seen in the first two principal coordinates where 16 (of 18) end-of-treatment samples (A2) were displaced towards the left relative to the corresponding pre-treatment samples (A1) and all their post-treatment (A3) counterparts returned right. Similarly, 16 A2 samples were displaced downwards relative to A1 and 11 of them moved upwards at post-treatment.

For 12 individuals the A1–A2 and A2–A3 vectors on principal coordinate analysis had opposite directions. Of these, in ten cases (#A4, 5, 6, 15, 20, 21, 23, 24, 27, 32) the A2 points moved down and left relative to A1 (see Supplementary material, Fig. S2), whereas in two cases (#A7, A10) other patterns were observed. For the remaining six individuals (#A11, 12, 13, 16, 18, 28), the A1–A2 and A2–A3 vectors were not in opposite directions; the only five individuals who had the A1–A3 UniFrac distance greater than the A1–A2 one were found among these six subjects. Separation of pre-treatment and end-of-treatment samples was less clear using weighted UniFrac analysis (see Supplementary material, Fig. S3), although two-thirds of both A2 and A3 samples were located outside the area formed by the A1 samples.

Variations of salivary microbiota among time-points for the same control subject were smaller than those between different control subjects at the same time point (Fig. 3 and see Supplementary material, Fig. S4). In the antibiotic-treated group, however, the average within-individual variations were not significantly different from between-individual variations (Fig. 3). Clearly, this was a result of the perturbation of the microbiota by the antibiotic.

The results of PERMANOVA of unweighted UniFrac distances confirmed the marked effect of the antibiotic on the salivary microbiota (see Supplementary material, Table S3). Samples taken at the end of amoxicillin treatment (A2) were significantly different from the five other groups of samples defined by the treatment and medical visit (A1, A3, C1 C2, C3). Similar results were obtained with PERMANOVA of Bray–Curtis similarities. This test was based on square-root transformed abundance of OTUs and did not take into account the phylogenetic distance as was the case using UniFrac.

It has been shown that the oral microbiota is more stable compared with other human microbiota [6], but most important changes occur in childhood [23,27]. Our study did not include children younger than 1 year, a period when dramatic microbiota changes occur [22]. The result of PERMANOVA (see Supplementary material, Table S4) suggested that, at the time of the first visit, the salivary microbiota were not significantly different between: (i) children belonging to
### TABLE 2. Changes in taxa abundance at different visits.

<table>
<thead>
<tr>
<th>Rank and taxon</th>
<th>Taxonomic information (phylum; class; order; family; genus)</th>
<th>Relative abundance</th>
<th>p</th>
<th>Number of subjects with increased/decreased abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td></td>
<td></td>
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<tr>
<td>Actinobacteria</td>
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<tr>
<td>Class</td>
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</table>

*Number of samples with increased abundance/Number of samples with decreased abundance.*

A, treated subjects; 1, 2 and 3 correspond to the first, second and third medical visit, respectively; Avg, average; Med, median.

Only taxa found in ≥0.25% in at least one of the six groups of samples (A1, A2, A3, C1, C2, C3) were analysed and those with significant changes (p < 0.05) are presented. The statistical analysis was a Wilcoxon signed-rank test with Benjamini-Hochberg correction for multiple comparisons (for each taxonomic level individually).

Only significant p values are presented.
different age groups defined by 1-year increments (see Supplementary material, Fig. S5), except when 1–2-year-old children were compared with those who were 5–6 years old; (ii) genders; and (iii) patients prescribed (A1) and not prescribed (C1) amoxicillin. Previous AOM episodes, recorded in 11 patients, nine of which were treated by antibiotics, showed a weak effect on the salivary microbiota when a PERMANOVA test based on unweighted UNIFRAC distances was used (Pseudo-$F = 1.5193$, p $< 0.043$).

**FIG. 2.** Principal coordinate analysis of unweighted UNIFRAC distance matrices. Blue squares, orange circles and grey-contour triangles correspond to samples taken at the time of the first, second and third visit, respectively. The area comprising samples taken at the time of the first and second visit for treated (a) and control (b) subjects are blue and orange shaded, respectively.

**FIG. 3.** Average within-subject and between-subject unweighted UNIFRAC distances. 'A' and 'C' correspond to the treated and untreated groups of children. Standard error bars are given and the Mann–Whitney U-test significance is indicated as follows: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

**FIG. 4.** Richness and diversity indices of the salivary bacterial communities. Margalef richness (d) and Shannon diversity (H') indices were calculated for the six groups of samples. Each sample was normalized to 283 sequences before analysis. Average values and standard error bars are presented. The statistical analysis was Wilcoxon signed-rank test.

Diversity of the salivary microbiota

Diversity and richness estimates are considered to be sensitive to the number of sequences analysed [29], so we normalized the data of each sample to 283 sequences, as it was the lowest number of sequences in any sample. Margalef richness and Shannon diversity indices were lower in 17 and 16 (of 18) A2 samples, respectively, in comparison to their A1 counterparts.
(see Supplementary material, Table S5). The average values for both indices in A3 samples were between values found in A1 and A2 samples (Fig. 4). In the control group, the average diversity and richness indices were somewhat reduced in samples taken at the time of the second visit in comparison to the baseline and third-visit samples, but these differences were not statistically significant. Decrease in Shannon diversity and Margalef richness indices, observed in 13 and 11 (of 15) control subjects, respectively, may be the result of some changes in diet, hygiene or immunological status linked to the illness.

Discussion

The present paper represents the only controlled study showing the impact of antibiotic treatment on the human salivary microbiome in the context of AOM. In spite of the inter-individual differences in the salivary microbiota profiles at baseline, common themes in microbiota perturbations caused by the amoxicillin treatment were revealed.

Salivary bacterial communities were in great part recovered 3 weeks after the course of amoxicillin, but some effects of the antibiotic treatment were still seen, e.g. lower species richness and diversity.

At the phylum level amoxicillin effect was the strongest on TM7 and Actinobacteria. The proportion of TM7, identified in 17 (of 18) individuals at pre-treatment, dropped below the detection limit in 88% and 41% of these subjects at the end of treatment and post-treatment, respectively. The biology of TM7 bacteria, which so far have no culturable representatives, their interaction with other members of the oral microbial consortium, and their possible impact on oral (and general) health are unknown. After averaging the data from several salivary microbiomes [11,21–24], TM7 stands as the sixth most abundant phylum in both adults and children/adolescents, with a frequency higher than that of Spirochaetes (Fig. 1). Data from our study show that, in some samples, the sequences assigned to TM7 may represent up to 20% of the total number of reads in the salivary 16S amplicon libraries.

Responses of the salivary microbiota to amoxicillin were individualized as it has been previously shown for the gut microbiota of ciprofloxacin-treated individuals [9]. These variations may be a result of: (i) differences among individuals in pharmacokinetics and pharmacodynamics of amoxicillin; (ii) various duration of antibiotic therapy and time intervals between visits at which the participants were sampled; (iii) lifestyle differences between patients; (iv) some seasonal phenomena during the 10-month study duration; and (v) different patterns of subject colonization by antibiotic-resistant strains. Indeed, a wide variety of amoxicillin-resistant bacteria were commonly identified in the dental plaque of children [30].

Not only do antibiotics alter the microbiota membership and composition, they also increase the prevalence of antibiotic resistance. Resistance to antibiotics prescribed in primary care may persist for up to 4 years [31]. Number of courses of an antibiotic, its doses and course duration may influence the prevalence of antibiotic resistance and induce changes that may influence the response to later antibiotic-driven perturbations [9,32]. It would be of interest to include these aspects of the microbiota changes in future studies of the salivary as well as the nasopharyngeal bacterial communities, the latter being considered as a reservoir of causative agents of AOM.

All the patients enrolled in our study had recovered at the time of the second visit, regardless of the use of amoxicillin. According to the current recommendations [33], amoxicillin treatment was mostly limited to children with severe AOM. Long-term effects of antibiotics on the oral (and other) human microbiota may be regarded as an additional argument to further reduce their prescription in AOM.

Acknowledgements

These results were presented, in part, at the Annual Congress 2012 of the Swiss Society for Microbiology, St Gallen, Switzerland.

Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Accuracy of taxonomic assignments for the V3 region of the SILVA Bacterial reference 16S rRNA gene sequences.

Figure S2. Samples from ten treated individuals showing similar trajectories in the first two ordination axes in the principal coordinate analysis plot of unweighted UniFrac distances.

Figure S3. Principal coordinate analysis of weighted UniFrac distance matrices.

Figure S4. Intra-individual and inter-individual unweighted UniFrac distances in the treated (a) and control (b) patient group.
Figure S5. Principal coordinate analysis of unweighted UNIFRAC distances between salivary microbial communities at the first medical visit.

Table S1. Details of the patients’ characteristics and treatment.

Table S2. Relative abundance of taxa in saliva samples.

Table S3. Pairwise comparisons between the group of samples defined by the treatment status and medical visit.

Table S4. Differences between groups of samples taken at baseline (first medical visit), identified by different factors.

Table S5. Number of sequences assigned to different levels in the taxonomy, diversity estimates and barcodes for pyrosequencing.

References


