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Impact of narrow-spectrum penicillin V on the oral and faecal resistome in a young child treated for otitis media

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ABSTRACT

Objectives: Antibiotic overuse has led to the global emergence of antimicrobial-resistant bacteria, and children are among the most frequent users of antibiotics. Most studies with broad-spectrum antibiotics show a severe impact on resistome development in patients. Although narrow-spectrum antibiotics are believed to have fewer side effects, their impact on the microbiome and resistome is mostly unknown. The aim of this study was to investigate the impact of the narrow-spectrum antibiotic phenoxymethylpenicillin (penicillin V) on the microbiome and resistome of a child treated for acute otitis media.
Methods: Oral and faecal samples were collected from a 1-year-old child before (Day 0) and after (Days 5 and 30) receiving penicillin V for otitis media. Metagenomic sequencing data were analysed to determine taxonomic profiling using Kraken and Bracken software, and resistance profiling using KMA in combination with the ResFinder database.
Results: In the oral samples, antimicrobial resistance genes (ARGs) belonging to four classes were identified at baseline. At Day 5, the abundance of some ARGs was increased, whereas some remained unchanged and others could no longer be detected. At Day 30, most ARGs had returned to baseline levels or lower. In the faecal samples, seven ARGs were observed at baseline and five at Day 5. At Day 30, the number of ARGs had increased to 21.
Conclusions: Following penicillin V, we observed a remarkable enrichment of the aecal resistome, indicating that even narrow-spectrum antibiotics may have important consequences in selecting for a more resistant microbiome.

1. Introduction

The increasing prevalence of antimicrobial-resistant bacteria is now threatening what has been modern medicine's security blanket for over 70 years, namely effective antibiotics [1]. Antibiotics are among the most essential and prescribed drugs in the world, but extensive antibiotic use has led to the global emergence of antimicrobial-resistant bacteria, making bacterial infections once again difficult to treat. Antibiotic use is particularly high in children aged <4 years [2,3]. The leading reason for antibiotic prescription in these children is upper airway infection, including otitis media [3,4]. In Scandinavia, the treatment guidelines for otitis media are much more restrictive than they were some years ago [3,5] and doctors are encouraged to use as few antibiotics as possible in these cases. However, when recommended, the first-line antibiotic is phenoxymethylpenicillin, also known as penicillin V [6,7]. Penicillin V has activity against Gram-positive bacteria but is less active against Gram-negative bacteria. This drug is among the narrowest-spectrum antibiotics in use.

Use of broad-spectrum antibiotics is associated with a reduction in gut microbiome diversity, as demonstrated in several studies [8,9]. The impact on the microbiota in other body sites is less known, but the microbiome in saliva appears to be more resilient to antibiotics than the gut microbiome, at least in healthy volunteers exposed to broad-spectrum antibiotics [10]. In children, loss of diversity is of particular importance given the relevance of the microbiome on immune system maturation [11]. Whilst such antibiotic effects on microbial composition have been the focus of several investigations, less is known about the effects on the overall antimicrobial resistance gene (ARG) load in the human microbiome, known as the resistome [12]. The resistome constitutes an important reservoir of ARGs that can be transferred to pathogens [13]. Emerging data indicate that broad-spectrum

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antibiotics can lead not only to a general increase in resistance genes related to the antibiotic target, but also to other groups of antibiotics [14]. Such resistance genes can in some cases persist for long periods and increase upon cumulative exposure to antibiotics [15]. For narrow-spectrum antibiotics such as penicillin V, the impact on the resistome remains largely unknown.

In the present study, the effect of the narrow-spectrum antibiotic penicillin V on the oral and gut resistome of a young child treated for otitis media was followed. Deep metagenomic sequencing was used to investigate its possible relevance in promoting enrichment of pathogens and ARGs. The results indicate that penicillin V may affect both the taxonomic and resistome profiles of the microbiome, as observed at 25 days after therapy termination.

2. Materials and methods

2.1. Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics—South East ('REK sør-øst'). Written informed consent was obtained from the child's parents. The participants received no compensation.

2.2. Sample collection

Faecal and oral samples from a 1-year-old child presenting with otitis media at the Accident and Emergency Department, Municipal Hospital, South Norway, were collected for shotgun metagenomic sequencing. The child was of Caucasian origin, had no history of travel outside the Nordic Countries, and had previously received antibiotic treatment on one occasion (>1 month ago). Samples were collected at three time points: (i) the same day as the visit to the emergency department (baseline); (ii) immediately after the antibiotic treatment course was completed (Day 5); and (iii) after 25 days post-antibiotic treatment (Day 30). Oral samples were collected using FLOQSwab® (Copan Diagnostics, Murrieta, CA, USA). The swab was stored in 2 mL of TE buffer, was placed directly on ice and DNA was extracted within 2 h of sample collection. Faecal samples were collected in sterile containers and were placed directly in a −20 °C freezer. Faecal DNA was extracted after all samples had been collected.

2.3. DNA extraction and sequencing

DNA from oral samples was extracted using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA). Sample material in TE buffer was pelleted by centrifugation (10 000 rpm for 10 min) and was extracted following the manufacturer's protocol. Faecal DNA extraction was performed using a PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Minor adjustments to the protocol were implemented according to the Human Microbiome Project (Core Microbiome Sampling Protocol A, HMP Protocol #07-001) [16]. The amount of extracted DNA was measured using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was evaluated by agarose gel electrophoresis. Metagenomic shotgun sequencing was conducted at the Norwegian Sequencing Centre (Oslo, Norway) on an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) using a paired-end sequencing approach with a targeted read length of 125 bp in high-output mode.

2.4. Quality control and pre-processing of metagenomic data

The quality of raw sequencing reads was assessed using the FastQC tool [17], with all samples passing commonly applied quality criteria including base quality, GC content, nucleotide distribution and duplication rate (mean Phred score >20; read length >100 bp; nucleotide distribution %A = %T and %G = %C with a constant proportion over the length of the read; duplicated sequences should make up <50% of the total reads). Low-quality reads and adapter sequences were trimmed out using Trimmomatic [18], followed by removal of human DNA sequences by extracting all reads mapping to an assembly of the human genome (version GRch38, downloaded from NCBI GenBank) using BowTie2 [19]. The remaining reads were subsequently used to characterise the microbiome and resistome in oral and faecal samples.

2.5. Taxonomic profiling

Paired non-host reads were classified and assigned taxonomic labels using Kraken v.1 software [20]. Reads (k-mers) were aligned against the prebuilt MiniKraken DB_8GB database, which encompasses complete archaeal, bacterial and viral genomes in NCBI's RefSeq (18 October 2017). Kraken assigns the most specific taxonomic label to each read in a given set of metagenomic reads for classification. However, certain times the label assignment is not at the species level. Especially in the case of nearly identical reads that are found in more than one species, Kraken will assign it to the lowest common ancestor, which could be genus level or higher. Therefore, the number of species-specific reads would be underestimated in samples containing one or few highly similar species. To overcome this issue, the species abundance after classification with KrakEN was re-estimated using Bracken (Bayesian Reestimation of Abundance after Classification with KrakEN) [21]. Furthermore, Shannon and Chao1 diversity indexes were calculated at species level for oral and faecal samples. The samples were rarefied to even sequencing depth (per million reads) for comparative analysis. Only reads that were taxonomically classified were used in downstream analyses.

2.6. Identification and quantification of antimicrobial resistance genes

The KMA program [22] was used in combination with the ResFinder 2.1 database [23] to identify ARGs in the metagenomic data set. The ResFinder database consists of ∼2200 resistance genes and is curated from existing databases and published literature. Resistant phenotypes attributed to mutations within an antibiotic target gene [target resistance alleles (TRAs)] were not included in the analysis since read-based approaches using short reads are still limited in providing reliable identifications of TRAs [24]. KMA was selected over other alignment tools (SRST2, MGmapper etc.) because of its accuracy in aligning short reads against redundant databases. In addition, this method is able to resolve the issue of multiple read matches by evaluating and statistically testing the global alignment scores. Quality-controlled reads were mapped against the ResFinder database using KMA, with 80% identity threshold for resistance gene identification (see Supplementary Table S1 for detailed information). To reduce the number of false positives, only genes present at significant levels ($P < 0.05$) with a coverage >50% were used for further analysis. For co-occurrence analyses, the source (organism) of genes (identified template) was manually searched through their accession ID in NCBI and the Comprehensive Antibiotic Resistance Database (CARD). On the background of this search, genes described in multiple bacterial species or defined by CARD as present in mobile elements were not included in the co-occurrence analysis.

Furthermore, the given depth/abundance of each resistance gene and the classes they belong to were normalised using RPKM (reads per kilobase million mapped reads) for comparison across samples. The abundance profiles are represented in the form of a

heatmap both for oral and faecal samples. Heatmaps were generated using 'pheatmap' package in R. Likewise, all of the co-occurrence or correlation line plots between the ARGs and potential microbial taxa were created in R.

3. Results

3.1. Sequencing results

A total of six samples were sequenced and analysed. The samples were collected from two body sites (O = oral and F = faecal) and from three different time points (Day 0 = d0, Day 5 = d5 and Day 30 = d30). For all six samples, a total of 260 million paired-reads were generated from shotgun metagenomic sequencing. The mean Phred quality score of raw reads across all samples was 37.3. Host DNA contamination was higher in oral compared with faecal samples. On average, 66% (28M) and 8% (3M) reads were identified as human DNA in oral and faecal samples, respectively. Human DNA was filtered from the sequencing data during analysis as described in Section 2.4.

3.2. Oral microbiome composition

On average, 48% reads in oral samples remained unclassified. The abundance of the remaining taxonomically classified reads are illustrated as a stacked bar chart in Fig. 1. The results suggest that the taxonomic composition of the oral metagenomes following antibiotic treatment varied slightly with respect to genus and species, but less with respect to phylum. All of the oral samples were dominated by Gram-positive bacteria, most of them belonging to the phylum Firmicutes. The baseline (O-d0) sample contained around 67% Firmicutes, with strong dominance of the genus *Streptococcus* (64%). The majority of reads were assigned to *Streptococcus mitis* (24%) at species level. In addition, 15%, 12% and 5% of the reads were also assigned to bacteria from the phyla Proteobacteria, Actinobacteria and Bacteroidetes, respectively. The second most abundant species in the baseline sample was *Rothia mucilaginosa* (11%), belonging to the phylum Actinobacteria. The sample taken at Day 5 (O-d5), corresponding to the day of therapy termination, shows a pattern that was slightly different from O-d0. Firmicutes was still the dominating phylum. However, the relative proportion of Proteobacteria was doubled compared with O-d0. Actinobacteria and Bacteroidetes were both reduced in their relative abundance compared with baseline (O-d0). The most abundant species was still *S. mitis*, but the proportion of some of the Gram-negative species such as *Haemophilus parainfluenzae* increased from 5% in O-d0 to 12% in the O-d5-sample. The sample collected 25 days after the end of antibiotic exposure (O-d30) revealed a taxonomic composition that was more similar to O-d0 than O-d5. Whilst the fraction of reads belonging to Proteobacteria was still high as in O-d5, the relative proportion of Bacteroidetes was restored to the level shown in O-d0. Within the Proteobacteria, the highest proportion of reads were assigned to the *Neisseria* and *Haemophilus* genera. Also, the relative abundance of *Neisseria* was found to be highest at O-d30 (12%). Furthermore, the Chao1 (richness) and Shannon (evenness) species diversity measurements decreased drastically at O-d5 compared with O-d0 and increased again at O-d30 (Fig. 2). The statistical significance of such changes could not be measured due to only one child (case) in the study.

3.3. Faecal microbiome composition

Large differences in microbiota composition were observed in the faecal samples before antibiotic treatment (F-d0), at the termination of antibiotic treatment (F-d5) and 25 days after antibiotic termination (F-d30) (Fig. 1). The faecal baseline sample (F-d0) was strongly dominated by the phyla Actinobacteria (62%) and Firmicutes (36%). However, after 5 days of antibiotic treatment the taxonomic profile showed a shift towards the phylum Proteobacteria completely dominating the sample with a contribution of >76% in total reads compared with <1% at baseline. A massive increase in abundance of *Escherichia coli* (49%) contributed particularly to the high proportion of Proteobacteria. In the F-d0 sample, species belonging to the Firmicutes were mainly from the genus *Streptococcus* or *Anaerostipes*. *Enterococcus* (especially *Enterococcus faecalis*) was the leading genus in the Firmicutes in the F-d5 sample. In the follow-up sample from Day 30 (F-d30), the relative abundance of Proteobacteria decreased to levels that approached those at baseline, with Actinobacteria again dominating the sample. The Firmicutes showed also a tendency of returning to baseline values, except for *Streptococcus thermophilus*, whilst the phylum Bacteroidetes, with its most abundant species *Bacteroides fragilis* (17%), became a substantial part of the gut microbiome. Interestingly, *B. fragilis* was not detected in F-d0 or F-d5 samples. Furthermore, a considerably larger portion of unmapped reads was observed for F-d30 than for F-d0 and F-d05. For F-d05, only 10% of the reads were not mapped to a known reference compared with 58% for F-d30 and 40% for F-d0. Similar to the oral samples, the species richness and evenness in the faecal samples were reduced at F-d5 compared with F-d0 (Fig. 2). Both oral and faecal samples had the highest species diversity at d30.

3.4. Oral resistome

For oral metagenomes, seven ARGs were found that were present at all time points, i.e. O-d0, O-d05 and O-d30 (Fig. 3B). At baseline, a total of 11 annotated ARGs were identified. These genes encoded resistance to antibiotics belonging to four different classes, representing three different mechanisms of resistance. The largest group of ARGs at baseline were those conferring resistance to β-lactams. In total, five different genes belonging to the β-lactamase group ($bla_{\text{TEM-1B}}$,*cfxA3*, $bla_{\text{OXA-85}}$, $bla_{\text{SPU-1}}$ and *penA*) were detected before antibiotic treatment started. The TEM-1 enzyme was described already in the early 1960s [25] and is one of the most prevalent β-lactamase enzymes in Gram-negative bacteria. It is found on transferable genetic elements and is able to inactivate penicillins and narrow-spectrum cephalosporins [26]. In addition to the β-lactamases, the efflux pump *mef(A)* conferring resistance to macrolides was detected at O-d0, together with *msr(D)*, which is a macrolide efflux protein expressed on the same operon as *mef(A)* [27]. The *lsa(C)* gene confers resistance to lincosamides, streptogramins and pleuromutilins, whilst *erm(F)* confers resistance to the macrolide–lincosamide–streptogramin B (MLS_B) group. Two genes conferring tetracycline resistance [*tet(M)* and *tet(Q)*] were also detected at O-d0. Most ARGs discovered at O-d0 were also identified at O-d5. Levels of $bla_{\text{OXA-85}}$, *lsa(C)* and *tet(Q)* were similar to O-d0, whereas the abundance of $bla_{\text{TEM-1B}}$, *cfxA3*, *mef(A)* and *msr(D)* were increased at O-d5. Of the four genes with an increased abundance after 5 days, different responses were observed at O-d30: *mef(A)* and *msr(D)* both decreased in abundance below their baseline level, whilst *cfxA3* increased even more at O-d30. $bla_{\text{TEM-1B}}$ decreased from the high abundance at O-d5, but not to the low levels observed at Day 0. Thus, in oral samples most of the observed changes at O-d5 showed a tendency to return to baseline levels at O-d30 (Fig. 3B).

3.5. Faecal resistome

In the faecal samples, in total 25 different ARGs were detected. At baseline, seven different ARGs were identified, conferring resistance to four different antibiotic classes (Fig. 3A). *ant(6)-Ia*

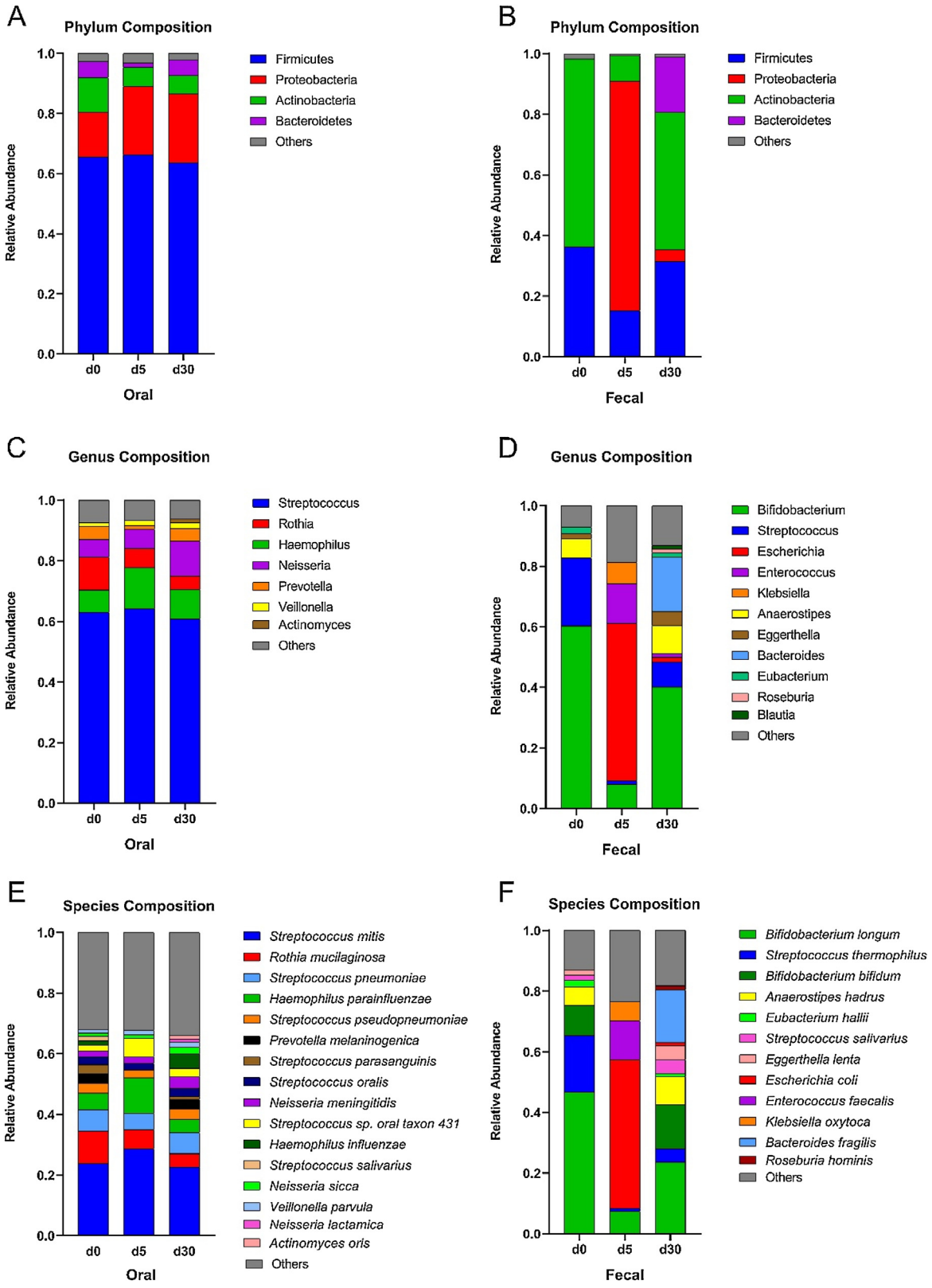

Fig. 1. Taxonomic profile of the oral (left) and faecal (right) metagenomes. Relative abundance of taxa from three time points, namely baseline (d0), immediately after treatment completion at Day 5 (d5) and 25 days post-antibiotic treatment (d30), presented at phylum (top), genus (middle) and species (bottom) levels. Those found at <1% are grouped as 'Others'.

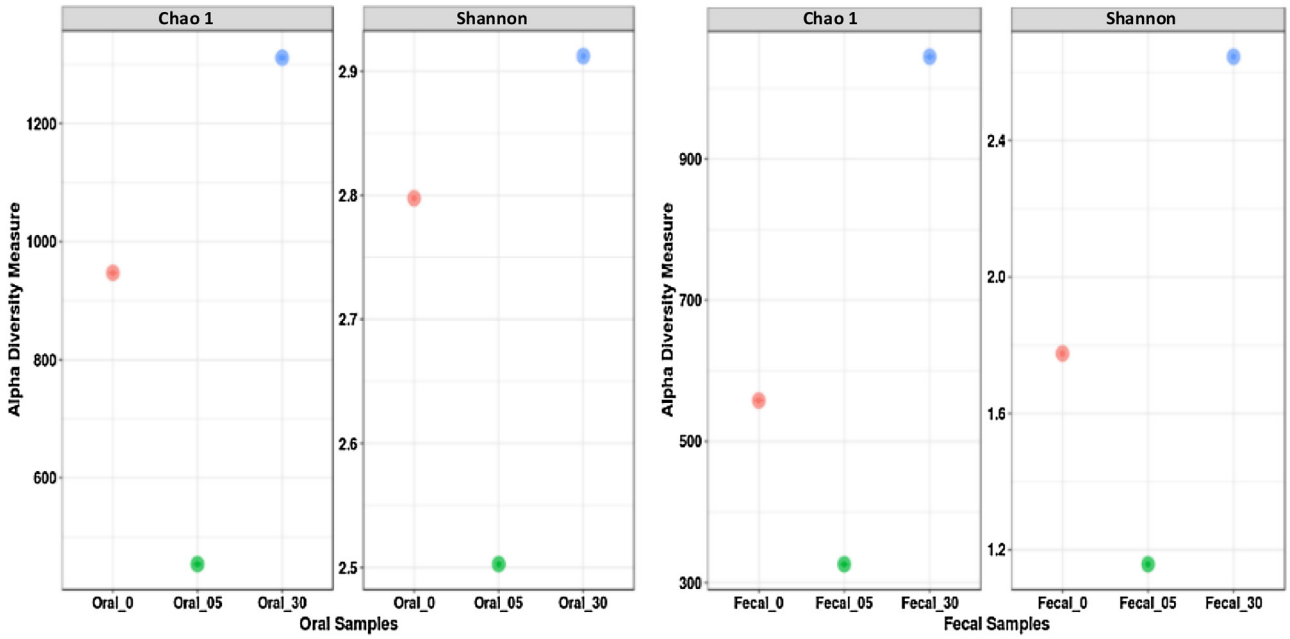

Fig. 2. Alpha diversity measured by Chao1 (richness) and Shannon (evenness) diversity indexes plotted for oral and faecal samples at three time points, namely baseline (0), immediately after treatment completion at Day 5 (05) and 25 days post-antibiotic treatment (30). Both oral and faecal samples have been rarefied to even sequencing depth for diversity estimation.

confers aminoglycoside resistance, *mef(A)* encodes a macrolide efflux pump, *msr(D)* is associated with resistance to the MLS_B group, and *tet(M)*, *tet(W)*, *tet(O)* and *tet(32)* encode ribosomal protection proteins preventing damage from tetracyclines. Only one of these genes [*tet(M)*] was identified again after 5 days (F-d5), thus showing a shift in the faecal resistome at Day 5. The abundance of *tet(M)* was increased at F-d5 compared with the baseline sample. In addition to *tet(M)*, four ARGs not present at baseline were identified at F-d5. One β-lactamase (bla_{OXY-2}), a fosfomycin resistance gene (*fosA*), and two efflux pumps [*lsa(A)* and *oqxB*] conferring resistance to macrolides and fluoroquinolones, respectively. In the last sample, 21 ARGs were identified in total, showing a considerably increase from the first two faecal samples. At F-d30, ARGs from seven different antibiotic classes were identified, including genes encoding resistance to vancomycin that were not detected at F-d0 or F-d5. *tet(M)* was the only gene detected at all timepoints, with its highest abundance at F-d5. Interestingly, 14 ARGs were solely identified at F-d30, 25 days after the last antibiotic exposure. Hence, the faecal resistome showed an extensive shift in composition and increase in diversity following antibiotic treatment (Fig. 3A).

3.6. Relationship between the resistome and microbiome

Some of the observed ARGs, such as bla_{BRO-1}, bla_{OXA-85} and *penA* in oral samples, and $bla_{OXY-2-7}$ and the *van* genes in faecal samples, are found intrinsically in specific species. Others, such as bla_{TEM-1}, *tet* genes and most *erm* genes, are found either intrinsically in several bacteria or on mobile DNA elements. A correlation analysis was performed between intrinsic resistance genes and the bacterial species in which the resistance genes have been identified. For this, their abundances (co-occurrence) were plotted across the three different time points in oral and faecal samples (Fig. 4). In the oral samples, the rise in abundance of bla_{BRO-1}, a resistance gene found in the chromosome of *Moraxella catarrhalis*, correlated with the increase in *M. catarrhalis* abundance on Day 5. The same is true for $bla_{OXY-2-7}$ and the *van* genes that are intrinsic to *Klebsiella oxytoca* and *Enterococcus casseliflavus*, respectively. *Enterococcus casseliflavus* is known to harbour intrinsic, non-transferable resistance to vancomycin owing to a chromosomally encoded *vanC* gene cluster [28,29]. All five vancomycin resistance genes found in this study are gene variants found in this cluster. *Enterococcus casseliflavus* is not as well described as *E. faecalis* and *Enterococcus faecium*, however it can cause serious infections and, due to its high level of intrinsic resistance to several antibiotics, it is a difficult bacteria to treat [30].

Not all genes followed the expected source of origin. The resistance genes *penA* and bla_{OXA-85} (also named bla_{FUS-1}), which usually are found in *Neisseria meningitidis* and *Fusobacterium nucleatum*, respectively, did not follow the abundance profile of their expected host, suggesting that these genes may also be intrinsic in other bacteria or that the genes can be found on mobile elements. For genes that are found intrinsically in several bacteria or on mobile DNA elements, it is not possible to correlate them with bacterial species with the methods applied in this study.

4. Discussion

Young children are frequent users of antibiotics. Antibiotic use in Scandinavian countries is still lower than in many other European countries and the low usage most likely contributes to the low prevalence of antibiotic resistance that is observed [31]. Penicillin V is recommended in Norwegian treatment guidelines as a first-line drug for empirical treatment of otitis media [6].

In this study, a sequence-based metagenomic approach was used to elucidate the impact of penicillin V on the oral and faecal microbiome and resistome of a child living in Norway. Supporting previous findings [10], the results of the current study showed that the oral microbiota was more resilient to the effects of antibiotics than the faecal microbiota. However, previous observations have generally been done with broader-spectrum antibiotics, in healthy adults, and not in young children with an infection such as in the present study.

Microbial diversity both in oral and faecal samples decreased after 5 days of treatment. After 30 days there was a tendency for higher diversity than at baseline. This is an observation that is in line with recent results showing that in children the faecal

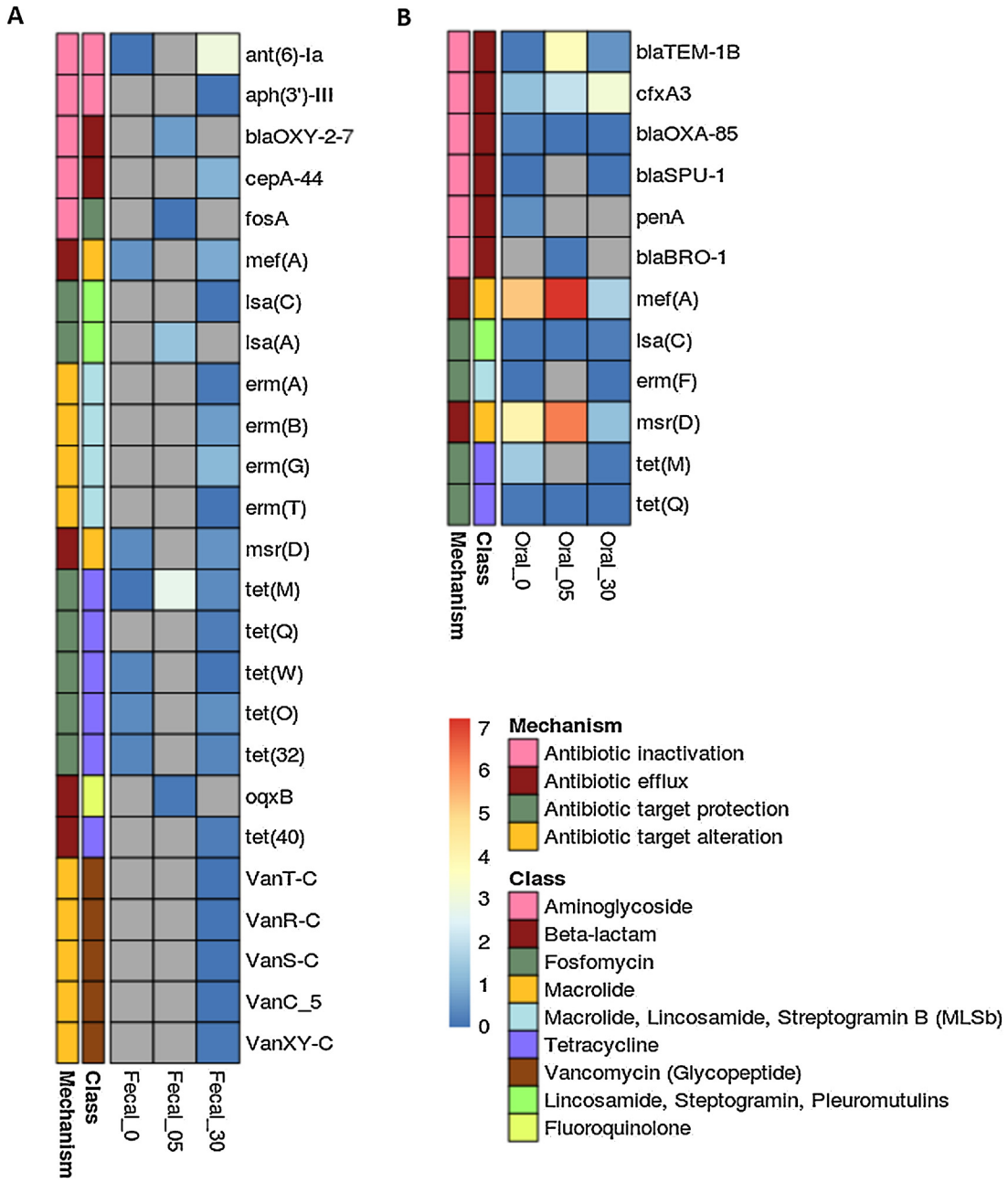

Fig. 3. Antimicrobial resistance genes (ARGs) identified in (A) faecal and (B) oral samples. Heatmap showing distribution of ARGs detected within the metagenomic samples at three time points, namely baseline (0), immediately after treatment completion at Day 5 (05) and 25 days post-antibiotic treatment (30). Genes are grouped by antibiotic class and mechanism of action. Colours shown in the legend (right) indicated the abundance of ARGs scaled from 'blue' (minimum) to 'red' (maximum). The scale 0–7 are normalised levels for the abundance of genes based on sequencing depth and gene length as determined by RPKM (reads per kilobase million mapped reads). Genes that have not been detected or have been excluded based on cut-off criteria are coloured grey.

microbial diversity continues to increase until it stabilises at around 3 years of age independent of antibiotic use [32]. Hence, the increase in diversity at Day 30 compared with baseline is not necessarily a result of antibiotic exposure.

The taxonomic profile of the oral microbiota at the three different time points was relatively stable despite the fact that penicillin V has antimicrobial activity against Gram-positive bacteria, which predominate in the oral cavity. In contrast, the taxonomic alterations observed in the faecal samples were prominent, especially at Day 5. In the faecal baseline sample, *Bifidobacterium* spp. predominated. After 5 days of treatment the potentially pathogenic Enterobacteriaceae were the dominating family and the abundance of *Bifidobacterium* spp. was reduced. This is likely an undesirable outcome, since high levels of *Bifidobacterium* are associated with several health benefits [33], including reduced levels of antimicrobial resistance in early life [34]. Conversely, the Enterobacteriaceae are a group of bacteria known to harbour high levels of ARGs and they have been assigned the highest priority by the World Health Organization (WHO) in the fight against antibiotic-resistant bacteria [35]. The observed reduction of Bifidobacteriaceae and the increase in Enterobacteriaceae during penicillin V treatment supports previous studies investigating the effect of other β-lactams of broader spectrum on the faecal microbiome [36,37].

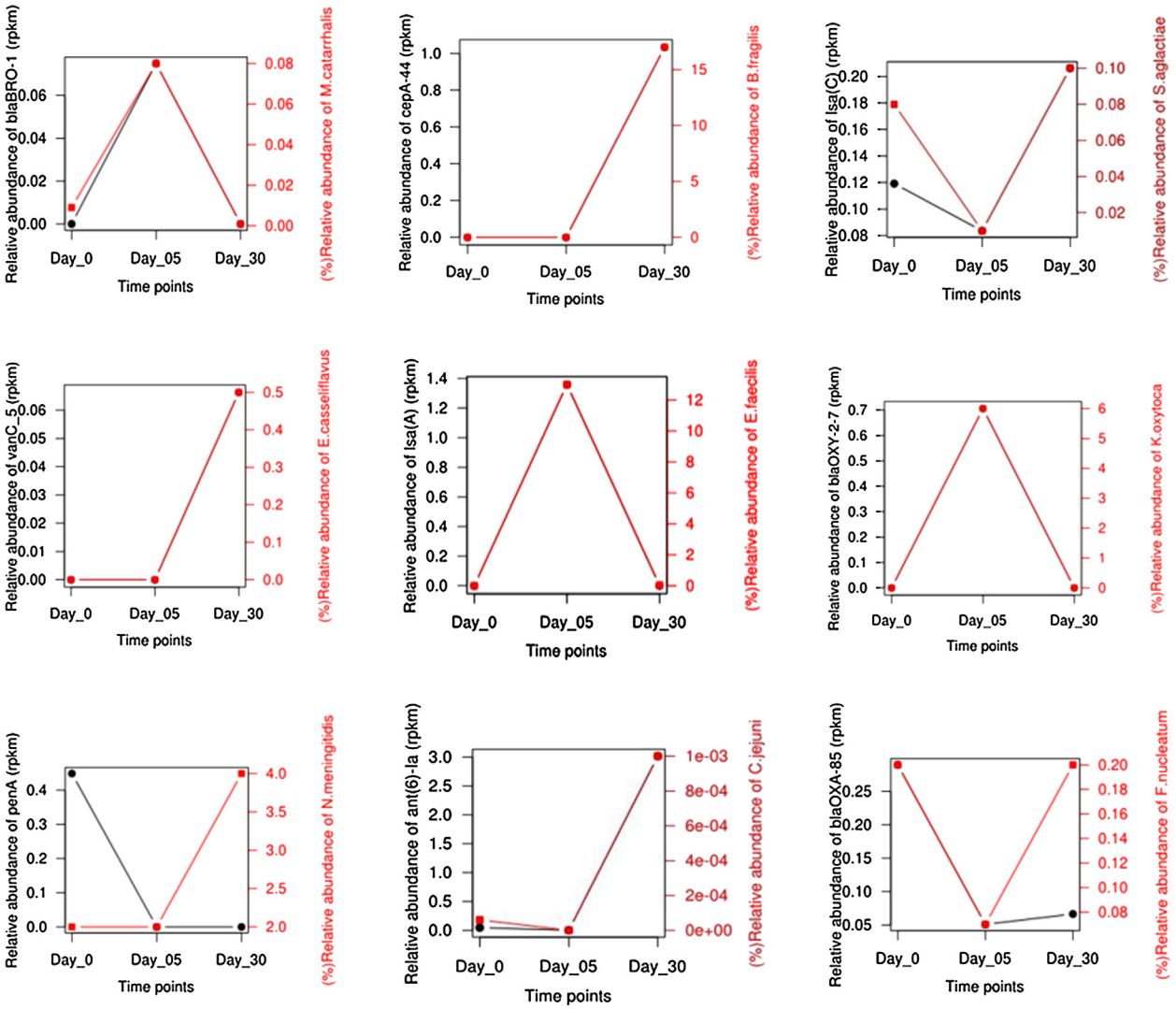

Fig. 4. Line plots showing the abundance of antimicrobial resistance genes (ARGs) (reads per kilobase million mapped reads) in the oral and faecal metagenome samples (black), together with the relative abundance of selected species potentially carrying the ARGs (based on annotation by KMA) (red).

Five ARGs [*mef(A)*, *lsa(C)*, *msr(D)*, *tetM* and *tet(Q)*] were observed both in the oral and faecal microbiome. Four of these [*mef(A)*, *msr(D)*, *tetM* and *tet(Q)*] can be found in several bacteria on mobile elements and are therefore of particular significance for spreading via horizontal gene transfer [38]. The impact of penicillin V on the resistome was greater in faecal samples than in oral samples. Whilst in the oral samples the 10 ARGs detected at O-d30 were also observed at baseline (Fig. 3B), in the faecalsamples 14 genes were observed that were exclusive to d30 samples (Fig. 3A). Interestingly, only one of the faecal ARGs identified at Day 30 conferred resistance to β-lactam antibiotics, which was used to treat the patient. ARGs conferring resistance to β-lactam antibiotics were also observed in oral samples, but these were not enriched following antibiotic therapy. The extensive impact on the faecal resistome at Day 30 compared with the modest effects on the oral sample might be explained by a larger taxonomic shift in the faecal samples. Newly appeared taxa or taxa with higher abundance at the last time point, such as *Clostridium* spp., *B. fragilis* and *E. casseliflavus* (Supplementary Table S2), may be contributing to new genes such as *ant(6)-Ia* and *aph(3′)-III*, *cepA-44* and the *van* genes, respectively, in the resistome. Also, some of the enriched ARGs are known to cluster together, such as *mef(A)* and *msr(D)*, and will consequently be co-selected. However, the large difference in the resistome of the faecal samples may also be a result of higher antibiotic selection pressure and horizontal gene transfer within the already existing microbiota [39].

Although definitive conclusions require a larger and ideally randomised study, this case report indicates that even narrow-spectrum antibiotics may have a significant effect on the faecal microbiome and resistome, thus warranting further investigations. Overall, acute and recurrent otitis media is among the most predominant conditions associated with antibiotic prescription and thus studies on these patients should be encouraged.

Data availability

The metagenome sequences generated in this work were deposited in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena) under accession no. PRJEB31957.

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Competing interests

None declared.

Ethical approval

This study was approved by the Regional Committee for Medical and Health Research Ethics [2014/1946/REK sør-øst C]. Written informed consent was obtained from the participating child's parents.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.08.004.

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