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ORIGINAL ARTICLE

The initial state of the human gut microbiome determines its reshaping by antibiotics

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Microbiome studies have demonstrated the high inter-individual diversity of the gut microbiota.
However, how the initial composition of the microbiome affects the impact of antibiotics on microbial
communities is relatively unexplored. To specifically address this question, we administered a
second-generation cephalosporin, cefprozil, to healthy volunteers. Stool samples gathered before
antibiotic exposure, at the end of the treatment and 3 months later were analysed using shotgun
metagenomic sequencing. On average, 15 billion nucleotides were sequenced for each sample. We
show that standard antibiotic treatment can alter the gut microbiome in a specific, reproducible and
predictable manner. The most consistent effect of the antibiotic was the increase of *Lachnoclos-
tridium bolteae* in 16 out of the 18 cefprozil-exposed participants. Strikingly, we identified a subgroup
of participants who were enriched in the opportunistic pathogen *Enterobacter cloacae* after exposure
to the antibiotic, an effect linked to lower initial microbiome diversity and to a *Bacteroides*
enterotype. Although the resistance gene content of participants' microbiomes was altered by the
antibiotic, the impact of cefprozil remained specific to individual participants. Resistance genes that
were not detectable prior to treatment were observed after a 7-day course of antibiotic administration.
Specifically, point mutations in beta-lactamase $bla_{\mathrm{CfxA-6}}$ were enriched after antibiotic treatment in
several participants. This suggests that monitoring the initial composition of the microbiome before
treatment could assist in the prevention of some of the adverse effects associated with antibiotics or
other treatments.
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Introduction

The human digestive tract is a complex ecosystem in which communities of microorganisms interact with each other and with their host. Unbalancing this environment modifies these endemic microbial communities and can promote the emergence of opportunistic infections such as *Clostridium difficile* (Chang *et al.*, 2008; Manges *et al.*, 2010), affect weight and obesity (Turnbaugh *et al.*, 2009) and even influence mental health (Hsiao *et al.*, 2013). The administration of antibiotics can perturb this micro-flora temporarily and in certain cases permanently (Dethlefsen *et al.*, 2008; Dethlefsen and Relman, 2011; Hernández *et al.*, 2013; Pérez-Cobas *et al.*, 2013). Bacterial resistance to antibiotics is a current

public health concern as it can transform diseases that were easily treatable 30 years ago into deadly threats (WHO, 2014). Indeed, microorganisms themselves can produce antibiotics (Donia *et al.*, 2014) with resistance to these natural molecules being a selective factor aiding the survival of these strains in this environment (Forsberg *et al.*, 2012, 2014). As most clinically relevant antibiotics are derived from natural molecules, bacterial gene evolution potentially allows the development of resistance to current drugs. Mobility of resistance genes from commensal species to pathogens through conjugation, transduction and transformation has, as a consequence, rendered part of our therapeutic arsenal inefficient and in some cases obsolete. Our responsibility for enhancing resistance is demonstrated by the clinical and agricultural use of antibiotics throughout the world, which increases the incidence of resistance genes in the gut microbiota (Ghosh *et al.*, 2013; Forslund *et al.*, 2013, 2014).

Several studies have examined the impact of antibiotics on the microbiota of individuals

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(Pallav *et al.*, 2014; Dethlefsen and Relman, 2011; Pérez-Cobas *et al.*, 2013) using 16S rRNA gene sequencing alone or combined with metatranscriptomics (Maurice *et al.*, 2013). As expected, the composition of microbial communities found in the gut and the regulation of bacterial genes were shown to be affected by antibiotics. However, the high inter-individual heterogeneity of the human gut microbiome and its quick response to changes in lifestyle and diet affect the impact of these compounds and of diseases on gut microbiota (David *et al.*, 2014). In this study, we exposed 18 healthy volunteers (E) selected using stringent inclusion and exclusion criteria, to a therapeutic dosage of the antibiotic cefprozil, a commonly used second-generation cephalosporin clinically recognized for having minimal side effects (Lode *et al.*, 1992; Wise, 1994; Edlund and Nord, 2000). Using the same selective criteria, we enrolled six controls (C) who received no antibiotic. Stool samples were collected before treatment (E0 and C0: initial state), at the end of the treatment (E7 and C7: peak effects) and 3 months after the end of the treatment (E90 and C90: long-term effects). Metagenomic DNA was extracted from the stools and sequenced at an average of 15 Gb per sample (Supplementary Table S1). Shotgun metagenomic sequencing allowed the assembly of an average of 124 million nucleotides per stool sample, which enabled monitoring of metagenome composition down to the species rank, including the identification of resistance genes and mobile elements. In summary, the results from this study indicate that, while cefprozil altered the microbiome of healthy individuals in a reproducible manner, it also permitted the emergence of potentially pathogenic *Enterobacteriaceae* and of rare resistance genes that were not detected prior to the treatment.

Materials and methods

Sample collection
The clinical protocol was approved by the ethical committee of the CHU de Québec–Université Laval. Informed written consent was obtained from participants. Participants exposed to the antibiotic and controls were selected following the same stringent criteria. Healthy volunteers recruited in the Quebec City region (Canada) were aged between 21 and 35 years and had normal intestinal transit. Characteristics of the participants are described in Supplementary Table S2. Subjects with any of the exclusion criteria below were not eligible for entry into the present study:

1. Working in a health-care facility or living with someone working in a health-care facility.
2. Working on a farm or household contact in the last 2 weeks.
3. Slaughterhouse worker or household contact.
4. Animal care worker or household contact.
5. Vegetarians.
6. Smokers.
7. Chronic alcohol consumption (more than one 1.5-ounce servings of 80 proof distilled spirits, five 12-ounce servings of beer or five 5-ounce servings of wine per day).
8. Antibiotic therapy or history of hospitalization (>24 h) in the past 12 months prior to the study.
9. Living with someone or an animal that has been on antibiotic therapy in the last month.
10. Any gastrointestinal or underlying pathology.
11. Any chronic illness.
12. Any infection requiring chemo/antibiotic therapy.
13. Diarrhoeal disease (World Health Organization definition) in the last 3 months prior to the study.
14. Gastro-intestinal-related medication (prescription antibiotics).
15. Immunomodulating medication such as antitumour necrosis factor or steroids.
16. Allergy to β-lactams.
17. Pregnant or lactating women.
18. Taking alimentary supplements.
19. Body mass index abnormal defined as <18.5 or >30 kg m^2.

Eighteen participants were treated twice a day with an oral dose of 500 mg cefprozil, a second-generation cephalosporin, for 7 days. The antibiotic was self-administered. Six controls who did not receive antibiotics were also enrolled. Fresh stool specimens were self-collected by participants at three time points: before the antibiotic treatment (0), at the end of the treatment (7), and 90 days after the end of the treatment (90). The stool collection procedure was explained to the participants at the time of enrolment. Participants were also given an illustrated document detailing the procedure. Samples were collected following human microbiome project protocols (Aagaard *et al.*, 2013) using stool specimen collection kits comprising: one Ziploc bag (Johnson and Son, Brantford, ON, Canada) and one kraft paper bag for transport, one GasPak EZ Anaerobe sachet (Becton, Dickinson and Company, Sparks, MD, USA), one M40 Transystem 408C transport swab (Copan Italia S.P.A., Brescia, Italy), one Commode Specimen Collection System (Biomedical Polymers, Inc., Gardner, MA, USA). All samples were brought to the laboratory within 2 h of collection and placed immediately in an anaerobic chamber for processing. Stool aspect was classified according to the Bristol scale (Lewis and Heaton, 1997). Stools were aliquoted and stored at -80 °C.

DNA extraction
Genomic DNA was extracted from 500 mg aliquots of each of the 72 frozen stool specimens using the MO BIO PowerMax Soil DNA Extraction Kit (MO BIO

Laboratories, Carlsbad, CA, USA) following a modified human microbiome project procedure (Aagaard et al., 2013). Briefly, each stool sample (500 mg) was homogenized by vortexing in 15 ml PowerBead Solution (MO BIO cell lysis buffer supplied with the DNA Extraction Kit) and centrifuged at 500 r.c.f. for 5 min at room temperature. Supernatant was transferred to collection tubes, incubated for 10 min at 65 °C and then at 95 °C for an additional 10 min. The supernatant was transferred to PowerMax bead tubes containing Garnet beads (supplied with the DNA Extraction Kit) for mechanical cell lysis and DNA extraction according to the directions of PowerMax Soil DNA Isolation Kit. All DNAs were eluted using PCR grade water and stored at 4 °C. The quantity and quality of isolated DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), agarose gel electrophoresis and a QuantiFluor dsDNA System (Promega, Madison, WI, USA).

Sequencing
Metagenomic DNA was quantified using the QuantiFluor dye (Promega). Sequencing libraries were prepared using the Nextera Sample Preparation Kit (Illumina, San Diego, CA, USA) with 50 ng of purified DNA. Dual indices were added during library preparation. Libraries were validated using Agilent Bioanalyzer 2100 high sensitivity DNA chips (Agilent Technologies, Santa Clara, CA, USA) and were quantified again with QuantiFluor. Libraries were diluted to a 2 nM concentration prior to pooling. Two libraries were pooled per HiSeq lane. Sequencing was performed on the HiSeq 1000 sequencer (Illumina) using v3 chemistry and paired-end 101 bp reads. A total of 72 metagenomes were sequenced with an average of 15 Gb per sample (Supplementary Table 1). Metagenomic reads and assemblies have been deposited in the European Nucleotide Archive database in project PRJEB8094.

Assembly, profiling and data mining
Metagenomes were assembled and profiled for taxonomy and resistance genes using the Ray Meta 2.0 assembler (Boisvert et al., 2010, 2012). The Ray Meta software first constructs a de Bruijn graph based on the sequenced reads using a defined k-mer length, in this case 31 nucleotides. Contigs are assembled by finding the best paths in the graph. Subsequently, the k-mers in the graph are coloured based on their occurrence in a reference database comprising a selection of the genomes available at National Center for Biotechnology Information (NCBI; last updated in September 2014, see Supplementary Table S3). Using the NCBI taxonomy, the abundance of k-mers is processed to determine taxa frequencies at relevant taxonomical ranks. Contig identification is based on de Bruijn

graph-based contig assignation (colouring) performed by Ray Meta. In brief, Ray Meta identifies the organisms that share k-mers with each contig in the reference genome database (Supplementary Table S3). Contigs longer than 2.5 kb are identified to taxa using a last-common ancestor algorithm that determines the taxonomic rank by calculating the sum of child nodes and selecting the taxa for which the sum comprises at least 90% of k-mers (https://github.com/plpla/laughing-nemesis).

Genes were found on contigs using Prodigal 2.6 with the metagenomic option (Hyatt et al., 2010). Putative genes were aligned to the MERGEM database using FASTA36 to identify putative resistance genes and mobile elements (Pearson, 2000). Genes were annotated as resistance genes if they had an amino-acid identity > 40% with a type gene found in MERGEM. This version of MERGEM included 1760 type genes. The abundance of a given resistance gene or mobile element in a sample was computed by summing the mode of k-mer depths for all the contigs harbouring the gene. This provides an estimation of the abundance of specific genes. This approach is less sensitive than read-based approaches but allows for more precise gene identification. The reference sequences used for resistance genes and mobile elements profiling are available in the MERGEM database (http://www.mergem.genome.ulaval.ca). Read alignment was visualized using the Integrative Genomic Viewer (Thorvaldsdóttir et al., 2013).

Statistical analysis was carried out in R 3.0.2. Taxon frequencies were processed using the Hellinger transformation (Legendre and De Cáceres, 2013) before fitting them to a mixed linear-effect model (NLME 3.1). Analysis of variance P-values were corrected for false discovery rate using the Benjamini–Hochberg–Yekutieli procedure (Benjamini and Yekutieli, 2001). Microbial diversity was computed using the VEGAN package (Dixon, 2003). Plots to visualize data distribution and density were created using the Beanplot package (Kampstra, 2008). Multiple factor analysis (MFA) was performed using FactoMineR 1.25 (Lê et al., 2008). MFA is a method conceptually similar to principal component analysis or multiple correspondence analysis. The main advantage of MFA is that it allows the integration of structured groups of variables. The current analysis included 6070 quantitative variables, which belonged to 11 variable groups. Therefore, calculation of the multidimensional distance between the samples was based on the profiling results of the class, family, genus, species, subspecies and species group taxonomical ranks, the abundance of resistance genes or mobile elements, diversity indices, sequencing statistics and Bristol stool category. The influence of each variable group on the principal components can also be estimated by the MFA. Bioinformatics and statistical analysis are detailed in Supplementary Information.

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Results

Cefprozil intake affects the microbiota
Eighteen healthy participants received the antibiotic cefprozil for 7 days. Six control participants, unexposed to antibiotics, were also enrolled to evaluate variability. Stool samples were obtained before (C0 and E0) and after the treatment (C7 and E7) and 90 days after the end of the antibiotic course (C90 and E90). These samples were then submitted to deep shotgun sequencing. The Ray Meta assembler (Boisvert *et al.*, 2012) was used to assemble and profile the taxonomic composition, gene function and resistance gene content of sequenced metagenomes. In most samples, microbiome composition at the family rank shows an overall dominance of *Bacteroidaceae* (Figure 1a). Still, diversity at the species rank highlights differences not observed at the family level. Each participant was found to harbour a specific subset of *Bacteroides* species (Figure 1b). As shown by Franzosa *et al.* (2015), the composition of the gut microbiome can serve to uniquely identify individuals. Our findings indicated that inter-individual variability at the species level was greater than the effect of the antibiotic for most participants (Supplementary Figure S1). The dominant species detected in stools were typically associated with 1 of the 18 species belonging to the *Akkermansia*, *Alistipes*, *Bacteroides*, *Dialister*, *Parabacteroides* or *Prevotella* genera (Figure 1b), with *Bacteroides* being the dominant genus for 40 out of the 72 samples. Although changes in dominant species were observed between time points, we could not link such changes to antibiotic exposure, an observation suggesting that cefprozil has a limited impact on the most abundant gut microbes, as previously observed using culture (Lode *et al.*, 1992).

A common pattern of response to the antibiotic was observed in the exposed participants. Indeed, six bacterial families were significantly decreased after exposure to antibiotics: *Bifidobacteriaceae*, *Coriobacteriaceae*, *Eubacteriaceae*, *Oxalobacteraceae*, *Pasteurellaceae*, and *Veillonellaceae* (Supplementary Figure S2, Supplementary Table S4). No bacterial family uniformly increased after antibiotic treatment, but the genera *Flavonifractor*, *Lachnoclostridium* and *Parabacteroides* increased significantly (Supplementary Figure S2). Strikingly, *Lachnoclostridium bolteae* increased in 16 out of the 18 participants exposed to cefprozil, an average rise of 20-fold, and returned to its initial (E0) level at E90 (Supplementary Figure S2). Similarly, abundance of taxa such as *Dialister invisus*, *Eubacterium rectale* and *Veillonellaceae* were drastically reduced at E7 but returned to initial E0 levels at the E90 sampling time point. In contrast, the abundance of these three taxa in all six control individuals remained stable throughout the experiment. The size of the assembled metagenomes decreased from an average of 118 million nucleotides at E0 to 97 million at E7, a 17.3% drop ($P = 0.01$). This is further supported by the N50 of E7 samples, which was 30% higher than that of the E0 assemblies ($P = 0.09$). However, the Simpson diversity of the microbiomes was only slightly reduced by 7.6% on average ($P = 0.42$).

In order to understand the interaction between antibiotic intake and the microbiome, we used MFA to integrate variable groups in a principal component analysis-like procedure (Lê *et al.*, 2008). This analysis included 11 groups of variables, including taxonomic composition at the level of four ranks, sequencing statistics and abundance of resistance genes and mobile elements. The principal component map of the MFA revealed that cefprozil had a notable effect on the microbiome of exposed participants (Figure 2a). This effect was discernible on the second axis of the MFA, whereby the ellipse representing E7 had only a slight overlap with the ellipse of non-exposed samples (E0, C0, C7 and C90). This is due to changes in bacterial composition, resistance genes and mobile elements (Supplementary Figures S3 and S4). The ellipses of controls at C0, C7 and C90 had a strong overlap (Supplementary Figure S5), indicating a lower temporal variability in controls compared with exposed participants. Hierarchical clustering based on MFA results separated samples between *Bacteroidaceae* (Clusters 1, 2 and 3) and *Prevotellaceae* (Cluster 4) dominant microbiomes (Figure 2b). The *Bacteroidaceae*-rich samples were further separated between high (Cluster 1) and low (Clusters 2 and 3) taxonomical diversity samples, in a way similar to previously described enterotypes (Arumugam *et al.*, 2011; Figure 2b). Aside from individuals P4 and P21, all individuals returned to their E0 cluster at the E90 sampling point. By contrast, the C7 stool samples from all controls remained similar to C0, with only one (P23) of six controls shifting from Cluster 1 to Cluster 3 after 90 days. It should be noted that stool samples from controls (C0, C7 and C90) are dominant in Cluster 1 (14 samples out of 18), while only 4 out of 18 E0 participants were in this cluster. By contrast, only 4 control specimens were in cluster 3, compared with 11 E0 samples. In addition, none of the control specimens were of the *Prevotellaceae* enterotype associated with our Cluster 4. This lower representation of controls outside of Cluster 1 could explain the separation between the ellipses of controls and exposed participants at E0 (Supplementary Figure S5).

The taxonomical diversity of Cluster 1 was correlated to a higher abundance of *Rikenellaceae* and of members of the class *Clostridia*, notably *Eubacteriaceae* and *Ruminococcaceae*, compared with the other clusters (Supplementary Figure S5 and S6). Cluster 2 was associated with a higher abundance of *Enterobacter cloacae* complex bacteria, *L. bolteae* and *C. clostridioforme*. Interestingly, this cluster comprised E7 samples from six participants exposed to cefprozil, indicating a reproducible effect for the antibiotic treatment specific to these six participants. Stools of all six participants returned to
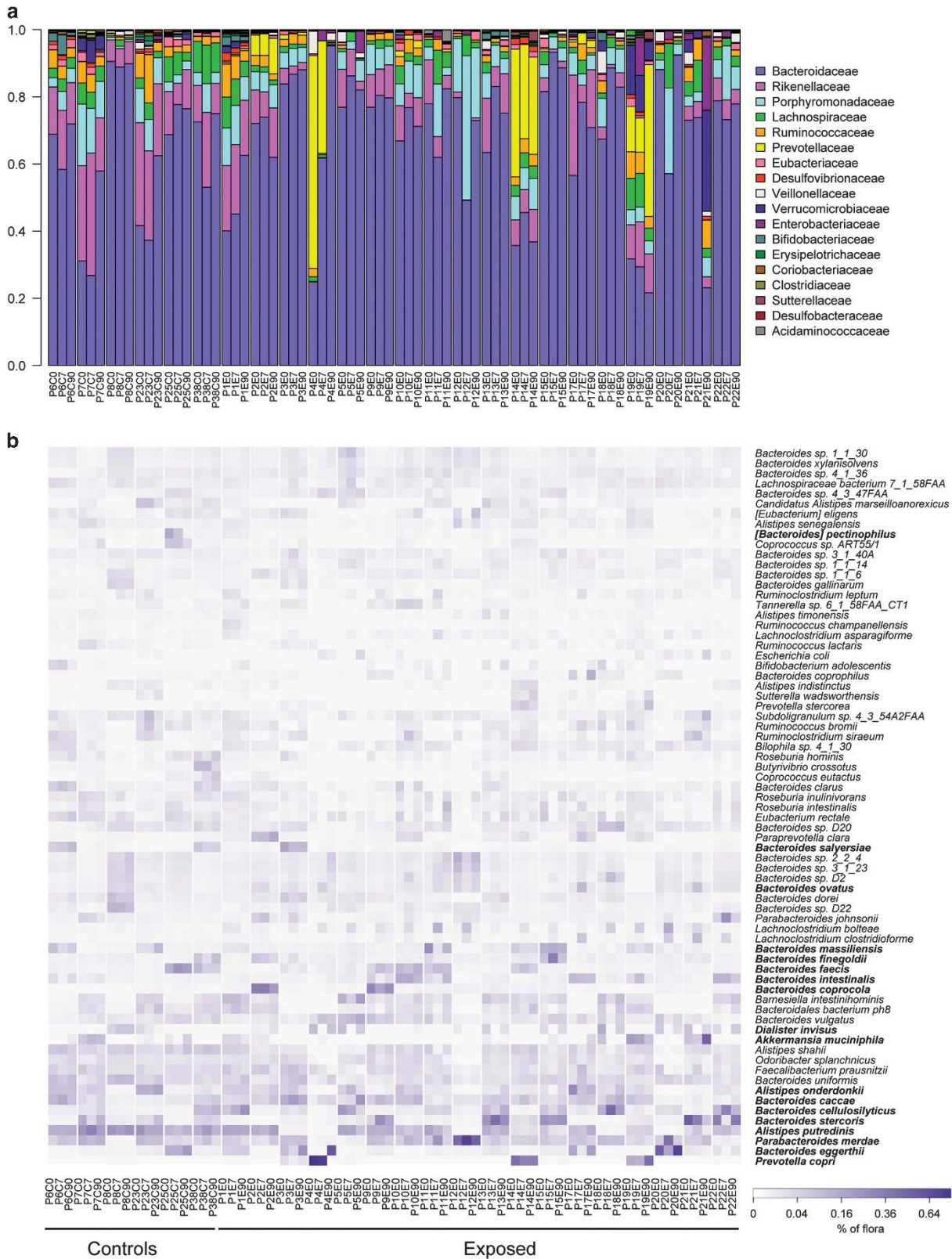
Figure 1 Composition of bacterial communities in all participants before exposure, 7 days later and 90 days after exposure to cefprozil. (**a**) Bar plot representing the relative abundance of bacterial families in controls and exposed participants. (**b**) Heat map representing the relative abundance of bacterial species that constituted >1% of the community in at least one participant. Species are grouped based on hierarchical clustering. Species in bold are the most abundant in at least one microbiome. P precedes the identification number of the participant, E indicates exposed and C indicates control.
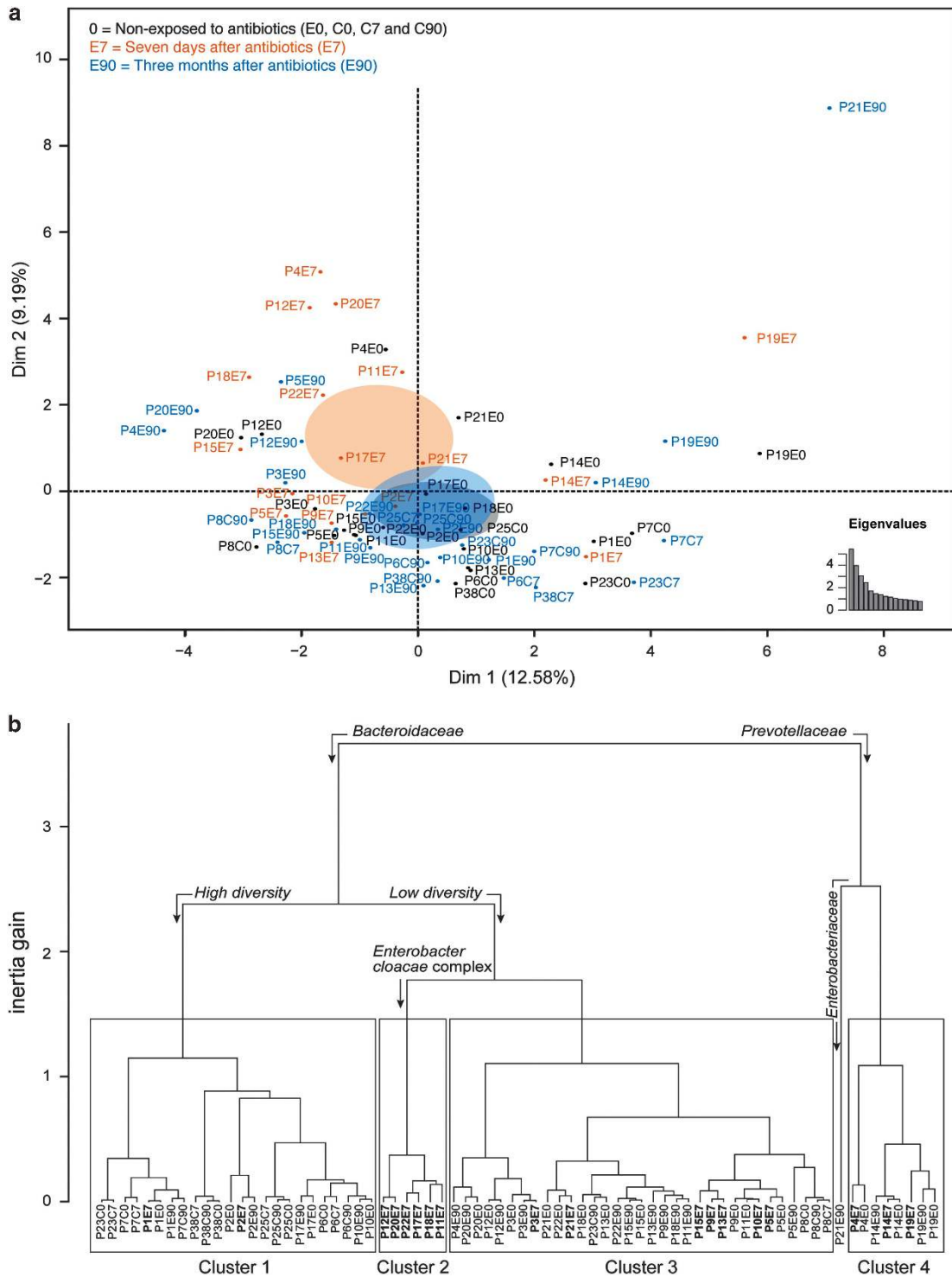
Figure 2 MFA reveals the effect of cefprozil on the microbiome of healthy individuals. MFA was performed using 11 variable groups representing data of taxonomic profiling, abundance of resistance genes or mobile elements, diversity indices, sequencing depth and assembly statistics. (**a**) The factorial map presents the impact of exposure to antibiotics on the microbial flora of participants based on the MFA. Black identifiers and ellipses indicate samples non-exposed to the antibiotic (E0, C0, C7 and C90), tan samples taken at the end of the antibiotic treatment (E7) and blue samples taken 90 days after the end of the treatment (E90). The ellipses represent the barycentre of the sample groups with 95% confidence. Eigenvalues show the significance of dimensions 1 and 2. (**b**) Hierarchical clustering based on results from the MFA. Four clusters are identified, along with a singleton sample. The quantitative variables driving the separation of samples in the clustering are indicated by bent arrows over the dendrogram. Association of microbiome characteristics with clusters was undertaken using an F-test in a one-way analysis of variance. Samples at E7 are in bold to emphasis the clustering of exposed participants.

their cluster of origin at E90. We observed strong alterations in the microbiome of one (P4) of the three participants dominated by *Prevotellaceae* (Cluster 4), which saw its *Bacteroidaceae* level increase from 25 to 95% at E7 (Figure 1a), although the antibiotic had limited impact on the microbiome composition of the two other participants (P14 and P19). Sample P21E90 behaved as an outlier in showing important alterations from its initial composition. Compared with E0, the microbiome of P21E90 had a fourfold increase in *Enterobacteriaceae* and a threefold increase in *Verrucomicrobiaceae* relative abundance (Figure 1a). Interestingly, this participant started taking ferrous sulphate as a treatment for anaemia during the antibiotic course. It has been reported that ferrous sulphate can alter the microbiome in rats, increasing *Enterobacteriaceae* (Dostal *et al.*, 2012).

An additional MFA analysis that focussed specifically on samples recovered prior to antibiotic exposure (E0 and C0) revealed that the six samples from Cluster 2 (Figure 2b) that were enriched in *E. cloacae* after cefprozil exposure also tightly grouped together at baseline (Figure 3). When compared with the other E0 microbiomes using a linear model-based statistical analysis (Supplementary Table S1), these E0 samples were associated with an average species diversity 29% lower than that of all other clusters combined

($P = 0.04$). They were also associated with reduced levels of lower abundance taxa (that is, $< 0.1\%$) such as the classes *Bacilli*, *Methanobacteria* and *Mollicutes*, the genera *Streptomyces* and *Xanthomonas* and species *Dorea formicigenerans*, *Gemmata obscuriglobus*, *Marvinbryantia formatexigens* and *Prevotella marshii* (Supplementary Figures S7 and S8). Levels of the *E. cloacae* complex at E0 in these Cluster 2 members did not differ from those detected in the other E0 samples.

Genes altered by exposure to antibiotic
Gene ontology terms that significantly increased after exposure to antibiotics had an initial relative abundance higher than ontology terms that were decreased after drug intake. On average, the k-mer abundances of significantly increased gene ontology terms were 6.9-fold higher than significantly decreased biological processes terms (15.4-fold for molecular functions and 2.5-fold for cellular components; Supplementary Figure S9 and Supplementary Table S6). Transposition was the most abundant of the significantly increased biological process terms (93th percentile). The beta-lactam antibiotic catabolic process category was also significantly increased at E7 compared with E0 (74th percentile). Annotation of the assembled contigs allowed the
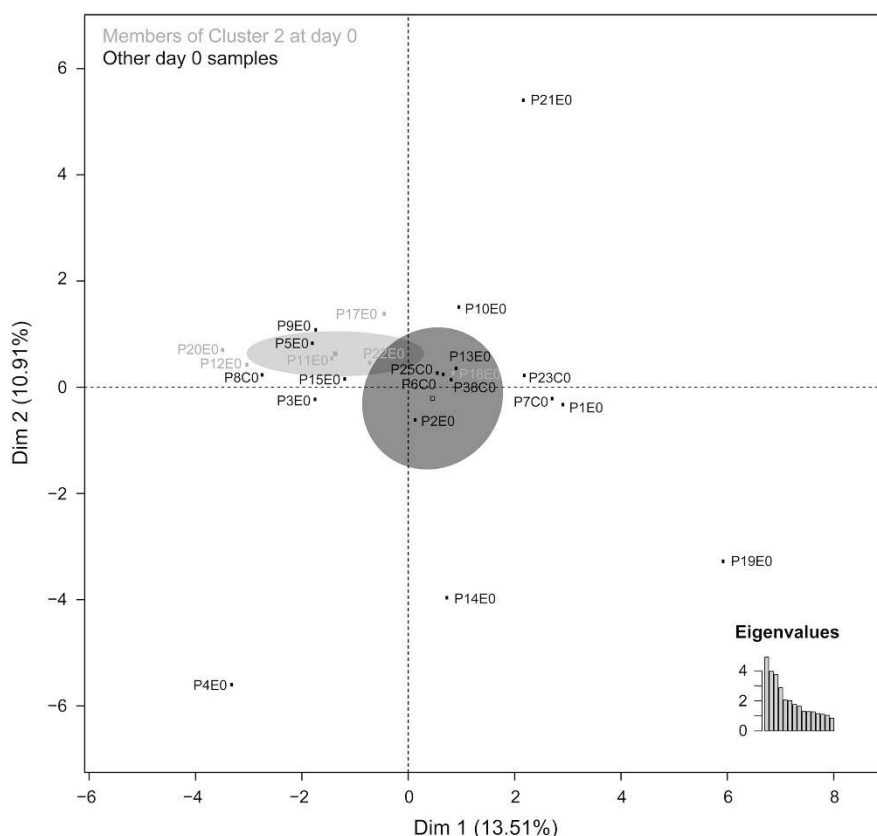

Figure 3 Participants enriched in *E. cloacae* complex after antibiotic exposure were clustered together before exposure. MFA was performed on day 0 samples only. Clustered participants enriched in the *E. cloacae* complex at E7 (Cluster 2) are represented in grey with the remainder of the samples presented in black.

detection of a total of 10.2 million putative genes, including 24 686 putative insertion sequences (IS) and 43 353 putative resistance genes. We found 2101 resistance genes located on the same contig as an IS. The sequences for 1439 putative beta-lactamases were retrieved from the assembled metagenomes.

Using the MERGEM database, we assigned these 43 353 putative resistance genes to 326 type genes classified by current resistance gene taxonomy (Liu and Pop, 2009; McArthur *et al.*, 2013). Only 21 of these 326 resistance genes types were found in all metagenomes while 47 of 326 were found in a single sample (Supplementary Table S7). Five resistance gene types were significantly increased at E7 compared with E0, notably *arr2*, bla_{CepA} and *mef*(G) (Supplementary Table S4). The β-lactamase bla_{CepA} was found in contigs that could be associated with the order *Bacteroidales.* It had several sequence variants and was present in 66 of the 72 samples. Increased abundances of *arr2* and *mef*(G) could be associated with *L. bolteae.* The *arr2*-related genes were detected in six E7 samples in contigs assigned to *L. bolteae.* They were 52% identical to the *arr2*-type sequence of MERGEM. This resistance gene functions as Rifampin ADP-ribosyl transferase, an enzyme that is widely distributed in the genomes of bacteria (Baysarowich *et al.*, 2008). Genes sharing 40–50% identity with *mef*(G) were detected in 3 E0 samples and in 13 E7 samples. One subgroup of *mef*(G) orthologs was similar to a major facilitator transporter found in a *Bacteroides* context and a second subgroup was related to a macrolide efflux pump in a *Lachnoclostridium* context.

In order to understand the relationship between antibiotic resistance genes and the modulation of taxa, we evaluated the occurrence of resistance genes in taxa that were affected by antibiotic treatment. We annotated a total of 42 resistance gene types in contigs assigned to *L. bolteae*, which is favoured by the antibiotic (Figure 4a). Hierarchical clustering of gene distribution shows a cluster of E7 samples that contained up to 26 different resistance gene types in a *L. bolteae* context. In contrast, E7 samples contained few occurrences of resistance genes associated with contigs related to *Veillonellaceae*, a family that is strongly reduced by antibiotic treatment (Figure 4b). Most E7 samples had more than 10-fold reductions of *Veillonellaceae* compared with corresponding E0 samples. However, *Veillonellaceae* in P14E7 was only reduced twofold. This participant was the only one positive for the presence of bla_{ROB-1} in a *Veillonellaceae* context.

Analysis of individual participants revealed striking modulation of beta-lactamase gene abundances following antibiotic intake. For example, we monitored a 90-fold increase in abundance of bla_{TEM-1} at E7 for participant P4 (Supplementary Figure S10). Interestingly, this bla_{TEM-1} was located between IS*26* mobile elements as previously detected on an *Escherichia coli* plasmid associated with multidrug resistance (Wang *et al.*, 2014). We also observed the

postantibiotic emergence (Figure 5a) of bla_{OXA-32} in P11E7, bla_{OXA-54} in P9E7, $bla_{OXA-192}$ in P1E7, bla_{ROB-1} in participant P14E7 (in *Veillonella*), bla_{HOA-1} in participants P15E7 (in *Akkermansia*) and P4E7, bla_{HGC-2} in P1E7, bla_{GOB-1} in P2E7, bla_{ACT-1} in P11E7 (in *Enterobacter*), bla_{ACT-7} in P18E7 (in *Enterobacter*), $bla_{AmpC-HG5}$ in P19E7 (in *E. coli*), as well as a conserved uncharacterized putative beta-lactamase in P12E7, P20E7 and P21E7, which was previously observed in *C. clostridioforme* (KC188672.1). With the exception of this last gene in P21E0, none of these beta-lactamase-coding genes were detected in the corresponding E0 samples. In contrast to beta-lactamases, resistance genes associated with vancomycin resistance were commonly detected in the samples and were not associated with E7 (Figure 5b).

A unique observation from our metagenomic data was that exposure to the antibiotic selected for specific alleles of resistance genes as exemplified by the bla_{CfxA-6} variants detected in P3, P4, P14 and P19. The sequences of this gene in P3E0 and P4E0 had amino-acid identity >99%. The Y259 allele observed at E0 disappeared at E7 while the D259 allele appeared after antibiotic exposure (P3E7 and P4E7; Supplementary Table S8, Supplementary Figure S11). In both participants, the bla_{CfxA-6} gene was located near an IS*942* mobile element. Interestingly, while the total abundance of all bla_{CfxA-6} alleles was reduced sixfold at P3E7 compared with P3E0, the D259 bla_{CfxA-6} could only be detected in P3E7, where it was the dominant bla_{CfxA-6} allele. In P3E0 and P4E0, the D259 allele was at very low abundance (1 and 9 reads, respectively), whereas it became dominant in P3E7 and P4E7 (29 and 1509 reads), suggesting that it is not a *de novo* mutation but a selection of preexisting strains by the antibiotic treatment.

Discussion

We exposed 18 carefully selected young healthy volunteers to a 7-day course of cefprozil, an antibiotic known to cause minimal disturbance of the microbiota (Lode *et al.*, 1992; Wise, 1994; Edlund and Nord, 2000). Our main objective was to assess the effect of the antibiotic on the microbiome and on resistance genes. Studies by others on the effects of antibiotics on the microbiota were conducted on eight or fewer healthy volunteers and used only 16S-based metagenomics. They demonstrated that microbiota recovery following antibiotic treatment can be incomplete and that individual floras react differently to the same treatment (Dethlefsen *et al.*, 2008; Pallav *et al.*, 2014). In order to reduce variability of participants for both controls and exposed volunteers, we applied nineteen strict exclusion criteria at the enrolment stage. Exclusion criteria were devised to exclude antibiotic intake in the previous 12 months as well as to limit exposure
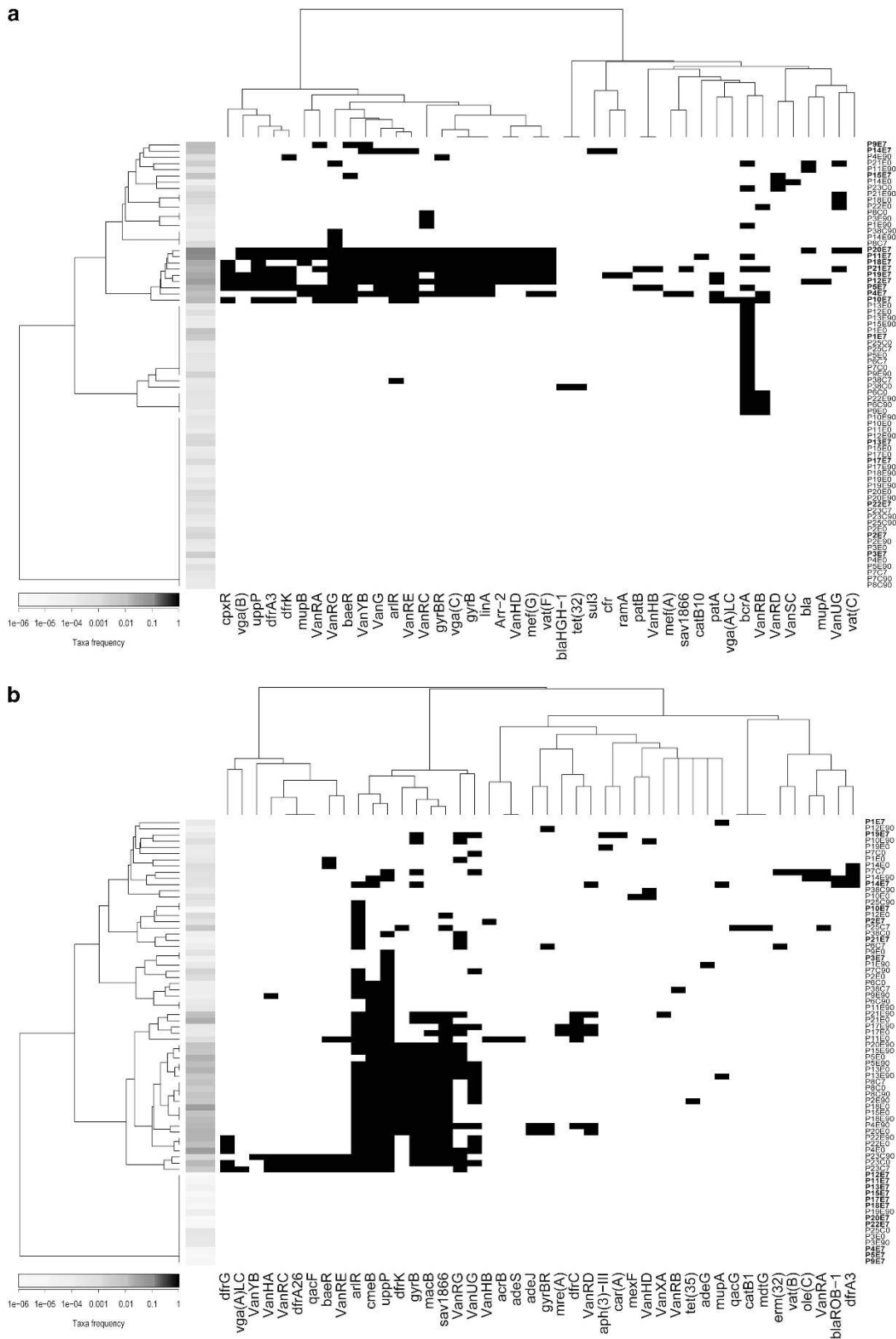
a

b

Figure 4 Association of resistance genes with genomic context for (**a**) *L. bolteae* and (**b**) *Veillonellaceae*. The presence of a resistance gene (black rectangle) was considered positive when it was annotated on a contig originating from the taxon of interest. Ordering of the resistance genes and samples was based on the hierarchical clustering of binary distances. Abundance of taxa in samples is represented by a scale of grey on the left side of the plot. The gene identified *bla* is an uncharacterized putative beta-lactamase previously observed in *C. clostridioforme* (KC188672.1).
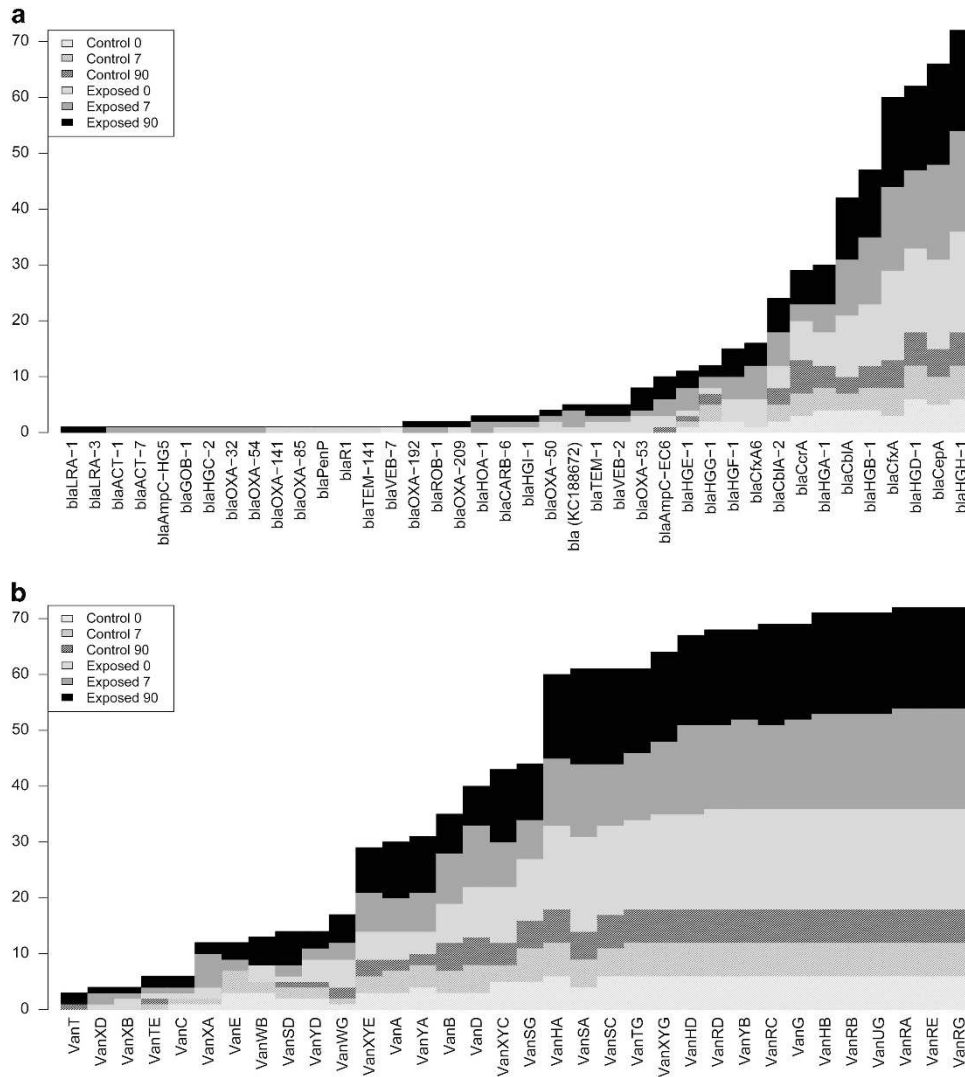
Figure 5 Number of samples positive for (**a**) beta-lactamases and (**b**) vancomycin resistance genes for antibiotic-exposed participants and controls at three time points. A stacked bar graph shows how many samples of each six categories were positive for resistance genes. The vertical axis represents the total number of reads positive for a resistance gene. Time points are coded as indicated in the upper left corner of the figure.

to microbiota potentially selected by antibiotics (occupational and household contacts). Despite careful volunteer selection, microbiome composition measured by shotgun sequencing was different between participants, with controls more homogeneous than exposed participants. Interestingly, taxa observed in the participants allowed clustering of their microbiomes into groups similar to previously described enterotypes (Arumugam *et al.*, 2011). Our use of deep shotgun sequencing allowed assembly of microbiomes and measurement of changes in gene content. Metagenomic sequences showed that the inter-individual variability of resistance genes was even higher than differences in flora, with most resistance genes observed in a limited number of participants, in some cases only after antibiotic treatment. These results show that detailed exclusion criteria are not sufficient to generate cohorts with homogenous microbiomes. Increasing the

number of participants and prescreening the microbiome of candidates could be approaches to reduce inter-individual variability in future studies.

Even though we observed a high variability between individuals, we detected that the antibiotic had a reproducible effect on microbiomes. Our results suggest that exposure to the antibiotic reduced richness as it caused a significant decrease in the level of six bacterial families (40 species; Supplementary Table S4). By contrast, only three genera systematically increased during antibiotic treatment (11 species). The total size of the assembled metagenomes was reduced at E7 compared with E0 and the N50 of assemblies increased. Microbiomes with lower richness require smaller sequencing efforts compared with richer microbiomes, yielding larger assemblies with longer contigs for the same number of reads (Rodriguez-R and Konstantinidis, 2014). On the other hand, the

Simpson diversity was only marginally reduced by treatment. It is possible that the diversity index did not reflect the reduction of bacterial richness because the reduced taxa were of low abundance. Diversity indices have been criticized for not taking into account lower abundance taxa (Li *et al.*, 2012; Haegeman *et al.*, 2013). In our study, the dominant taxa were not perceptibly affected by the antibiotic and the drug mostly affected lower abundance taxa. This resulted in a reduced abundance of k-mers associated with lower percentile gene ontology categories, for example, sporulation.

The most consistent effect of the antibiotic was the increase of *L. bolteae* in 16 out of the 18 cefprozil-exposed participants. In the current study, we were able to associate several resistance genes with contigs related to *L. bolteae.* In general, samples with the highest observed abundance of *L. bolteae* had more resistance genes related to this species than samples with lower abundance. However, samples presenting *L. bolteae* abundance <1% are more difficult to assemble, and this could explain the non-detection of resistance genes in these samples. The very nature of mobile elements can lead to incorrect contig identification as it is difficult to ascertain the host of such mobile genes. Nonetheless, *L. bolteae* is known to be resistant to the second-generation cephalosporin cefoxitin (Song *et al.*, 2003). This species has previously been associated with dysbiotic states, such as autism (Finegold *et al.*, 2002; Song *et al.*, 2004; Yutin and Galperin, 2013), acute rejection of liver transplant (Ren *et al.*, 2014) and with low microbiome diversity, a property associated with obesity and with a marked inflammatory phenotype (Le Chatelier *et al.*, 2013). Although the exact role of *L. bolteae* in these diverse conditions remains unknown, it may be linked to host or microbial imbalance, making it a potential marker for dysbiosis, a state also induced by antibiotics.

In addition to the response observed in all treated participants, we detected changes specific to a subset of volunteers. Six of the 18 exposed participants had their level of *E. cloacae* complex bacteria increase from an average of 0.0001 to 0.1% after a 7-day course of cefprozil. Five of these six participants were initially associated with the low diversity, *Bacteroides*-rich Cluster 3 (Figure 2b), which is related to the proposed *Bacteroides* enterotype (Arumugam *et al.*, 2011). We find the association between *E. cloacae* complex blooming and lower initial microbiota diversity reminiscent of *C. difficile* infection (Chang *et al.*, 2008). Moreover, blooms of *Enterobacteriaceae* have previously been reported after treatment with antibiotics (Buffie *et al.*, 2012). Expansion of *Enterobacter* after antibiotic treatment is not unexpected, as *Enterobacteriaceae* such as *E. cloacae* are known carriers of chromosomal beta-lactamases that confer resistance to cefprozil (Kayser, 1994; Wise, 1994). In two participants, we observed an increase of bla_{ACT} abundance in a contig closely related to an *Enterobacter* chromosome at the E7 time point. Genes related to tigecycline resistance (*ramA, acrA, acrB*) were also observed in *E. cloacae* (Veleba *et al.*, 2013). Lineages of *E. cloacae* that are considered to have epidemic potential harbour multiple beta-lactamases (Izdebski *et al.*, 2014). However, we cannot exclude that blooms of the *E. cloacae* are not only triggered by antibiotic resistance and that other fitness factors may be involved.

In all six participants, levels of the *E. cloacae* complex returned to initial levels at day 90, indicating that the antibiotic had a transient effect on this phenomenon. In a similar way, significantly modulated taxa returned to their initial abundance level at E90. The variability of the microbiome after 90 days was comparable to that of the controls (Figure 2). Only two out of 18 participants exposed to the antibiotic had a strikingly different microbiome composition 3 months after the antibiotic treatment than before exposure to cefprozil. We suspect that these two participants could have been affected by external factors. For example, P21 took iron supplements during the antibiotic treatment. We also hypothesize that the shift from a *Prevotella*-dominated microbiota to high levels of *Bacteroides* in P4 could be linked to factors other than cefprozil. Modification in diet has been shown to induce such changes in this enterotype (Wu *et al.*, 2011). However, the limited number of *Prevotella*-dominant participants enrolled in our study and the absence of data on their diet make further interpretation difficult.

We observed individual-specific changes in resistance gene abundances, confirming that potentially important resistance genes, which may be below detection levels, are present in low abundance in the microbiome and can be selected during *in vivo* exposure to antibiotics. For example, beta-lactamases such as bla_{OXA}, bla_{ACT}, bla_{AmpC} and bla_{TEM} were drastically increased at E7, whereas they could not be assembled at E0 (Figure 5a). These genes were often in proximity to mobile elements. Selection of beta-lactamase variants was also observed after antibiotic treatment, for example, the emergence of the same non-synonymous mutations in bla_{CfxA-6} was detected in two participants, a mutation that was found in very low abundance at E0. Most of these resistance genes were not detected 90 days later. Still, it remains difficult to discern with metagenomics whether resistance genes drive taxa abundance or whether modulation of specific bacteria affects resistance genes related or not to the antibiotic. For example, mutation of bla_{CfxA-6} could represent gene selection while genes found in *L. bolteae* could relate to species enrichment. Nonetheless, initially undetectable antibiotic resistance genes were selected toward higher abundance after antibiotic exposure and returned to lower levels following antibiotic withdrawal.

Overall, the impact of the antibiotic could be summarized in three categories. First, cefprozil had a reproducible effect on the microbiomes in almost all participants, leading to the increase of a limited number of specific genera and to the decrease of several bacterial families. The second effect was observed in a subset of the exposed participants, who had a bloom of a specific group of bacteria after antibiotic treatment. This increase in *E. cloacae* complex bacteria was observed in participants who initially had a *Bacteroides* enterotype with lower microbial diversity. The third effect consisted in the appearance of resistance genes at E7, in some cases revealing specific alleles, which were undetectable before the treatment. Increased abundance of resistance genes can either be modulated by gene selection or by strain enrichment. In both cases, they are the result of antibiotic treatment, a situation that could favour their transfer to pathogenic species (Beaber *et al.*, 2004; Ubeda *et al.*, 2005; Prudhomme *et al.*, 2006). Situations similar to blooms of *E. cloacae* could have clinical importance because *Enterobacteriaceae* are known for their propensity to transfer genes, especially in the presence of gut inflammation (Stecher *et al.*, 2012). For this reason, knowledge of how antibiotics or other molecules, for example iron, can affect gut microbiota would result in better treatment of at-risk patients. In conclusion, knowing how the initial state of the microbiota determines the impact of antibiotics could allow personalized treatment in order to minimize adverse effects. Moreover, we believe that the analysis of the microbiota, microbiome and resistome will become an essential part of the industrial discovery and evaluation of new antibiotics and most likely other drugs. This could guide the development of better therapeutic interventions, minimizing the impact on the microbiota and even taking advantage of the microbiome.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

Author contributions

FR contributed to experimental design, prepared sequencing libraries, sequenced samples, performed bioinformatics and data mining, analysed results and wrote the manuscript with MB. FR, AAO, NI and HG prepared sequencing libraries. EB managed stool samples. AAO and RG extracted nucleic acids. MD created and curated reference databases. MD, PLP, DKB and PHR performed bioinformatics analyses. IC and ST were in charge of the clinical study. BD, PL, JF, JLS and MCD offered technical and scientific support for the study. ST, MB, AH, PHR, MO, MGB and JC designed the study and supervised the project. All authors critically reviewed the manuscript.

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