



Research article

A slurry microcosm study on the interaction between antibiotics and soil bacterial community

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ABSTRACT

Antibiotics released in the environment have attracted great attention. The environmental emission control of antibiotics should be based on the degree of their negative impacts on the environment and ecology. Here, we conducted a series of soil slurry microcosm experiments to investigate the interactions between antibiotics and the soil bacterial community. In the soil slurry, distinctive behaviors were observed for different antibiotics. Beta-lactams (ampicillin and ceftriaxone) experienced fast biodegradation. Kanamycin was adsorbed on soil particles soon after its addition. Nalidixic acid was stable throughout the experimental period (164 h). The main inactivation mechanism of tetracycline was deduced to be hydrolysis. Bacterial communities in slurries with or without antibiotic-treatment were profiled via high-throughput illumina sequencing of the 16S rRNA gene. Unstable (ceftriaxone) and adsorbed (kanamycin) antibiotics show minor or negligible influences on the soil bacterial community. Stable antibiotics (nalidixic acid and tetracycline) have significantly affected the structure of the bacterial community. Most of enriched bacterial genera by various antibiotics belong to the same phylum, *Proteobacteria*. Inhibited bacterial phyla by nalidixic acid are *Firmicutes* and *Bacteroidetes*, while those inhibited by tetracycline are *Firmicutes*, *Bacteroidetes* and *Cyanobacteria*. According to the PICRUSt prediction of metagenome, influence of antibiotics on overall metabolic function of the bacterial community is rather limited. This study has provided valuable information, from a phylogenetic viewpoint, about the influence of high concentration of antibiotics on soil bacterial community.

1. Introduction

Emergence and wide spread of antibiotic resistance is one of the leading medical crises of our era, especially under the background of wide application and even abuse of various antibiotics in clinic, livestock industry and aquaculture. The water and soil environment is often subjected to anthropogenic influences such as wastewater and aquaculture discharges and the application of manure or bio-solids. These human activities can disseminate antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), and result in propagation of natural antibiotic resistance due to selective pressure. It has been gradually realized that environmental bacterial communities are big reservoirs of antibiotic resistance determinants (D'Costa et al., 2011). There is evidence that antibiotic resistance can be transferred from environmental bacteria to human pathogens (Forsberg et al., 2012). The selective pressure exerted by antibiotics on the bacterial community in the environment will have a significant impact on its structure, and

may highly enrich the antibiotic-resistant bacteria therein, which can potentially threaten human health. However, due to the complexity of environmental bacterial communities, it is challenging to reveal the interaction of antibiotics and bacterial communities in water and soil environments. In order to analyze the structure of an environmental bacterial community, previous studies relied on either culture-based methods (Liu et al., 2012) or culture-independent biochemical and molecular biological methods, such as phospholipid fatty acid (PLFA) assay (Chessa et al., 2016) and denatured gradient gel electrophoresis (DGGE) (Jechalke et al., 2014a). However, those methods gave limited information on the structure shift of environmental bacterial communities due to their low resolutions.

The rapid development of next-generation gene sequencing and big data analysis technologies has brought great opportunities to understand the structure of complex bacterial communities. These technologies have been widely used in analyzing bacterial communities in human or animal gut (Qin et al., 2010; Tang et al., 2014), watershed (Yergeau et al., 2012),

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soil (Hartmann et al., 2015; Leff et al., 2015) and even the marine environment (Bizic-Ionescu et al., 2015; Sassoubre et al., 2015). At present, many studies have adopted high-throughput sequencing methods to investigate the relationship between antibiotic resistance and the bacterial community in related environments. The subjects include the soil treated with manure (Leclercq et al., 2016) or under the selective pressure of antibiotics (Cleary et al., 2016; Lin et al., 2016), and also the sewage sludge or the manure during composting (Cui et al., 2016; Su et al., 2015). The use of manure or sewage sludge, which increased antibiotic resistance in soil, made it difficult to distinguish the direct impacts of residual antibiotics from those of resistant microbiota in the manure or sludge itself (Cleary et al., 2016). On the other hand, the interaction between antibiotics and environment is mutual. According to previous studies, antibiotics released into the environment experienced different fates, such as adsorption on solid particles in manure or soil (Loke et al., 2002; Pan et al., 2012), photolysis in aqueous system (Jiao et al., 2008), hydrolysis in aqueous solution (Xuan et al., 2010), biodegradation in sludge or soil (Li and Zhang, 2010; Sun et al., 2014) or persisting stability for fairly long time in soil or manure (Jechalke et al., 2014b). The impact of an antibiotic on environmental bacterial community should depend on its environmental fate.

In this study, a series of well-designed soil slurry experiments were performed to investigate the interaction between the soil bacterial community and different kinds of antibiotics (Penicillins, Cephalosporins, Aminoglycosides, Quinolones and Tetracyclines). In order to achieve the extreme exposure condition, the soil was in direct contact with various antibiotics of fairly high dosage in a slurry mode. The behaviors of antibiotics in this simulated water and soil environment, such as adsorption, hydrolysis and biodegradation, were evaluated via experimental design. High-throughput Illumina sequencing of the 16S rRNA gene was adopted to characterize the impacts of different antibiotics on the soil bacterial community.

2. Materials and methods

2.1. Soil sampling

An “undisturbed” (non-agricultural or human impacted) soil sample collected from the Danjing Hill, Pengzhou, Sichuan, China (31°05′44.6″N and 103°49′27.7″E) was used in the slurry microcosm experiment described below. Approximately 1 kg soil sample was collected aseptically from the surficial (top 5 cm) layer of the field site. Loose litter was removed using an ethanol-cleaned trowel. Multiple soil samples were collected from a half-meter-radius circle, pooled, and placed into autoclaved plastic bottles. Soil samples were thereafter transported to the laboratory, where they were immediately homogenized and stored at 4 °C. The soil sample was identified as a clay loam (based on mechanical composition) with sand (0.05–2 mm) content of 46.5%, silt (0.002–0.05 mm) 23.2% and clay (<0.002 mm) 30.3%. Soil pH is 7.57 with total Kjeldahl nitrogen of 0.119% and total organic carbon (K₂Cr₂O₇ oxidation) of 1.6%.

2.2. Soil slurry experiment

Five typical antibiotics of different antimicrobial classes were investigated in this research, including ampicillin (Penicillins), ceftriaxone (Cephalosporins), kanamycin (Aminoglycosides), nalidixic acid (Quinolones) and tetracycline (Tetracyclines). The experimental setting is illustrated in Supplementary Fig. S1. Five experimental groups were established for each antibiotic with three biological repeats. The diluted nutrient broth (dNB, 0.8 g/L) was added uniformly to support the growth of soil bacteria in the closed system. Experimental conditions for each testing group were briefly listed in Table S1. In experimental group 1, one hundred milliliters of autoclaved dNB spiked with one of five antibiotics to preset initial concentration (1024 µg/mL for ampicillin, 8 µg/mL for ceftriaxone, 256 µg/mL for kanamycin, 1024 µg/mL for nalidixic acid

and 1024 µg/mL for tetracycline) was put in a 250-mL Erlenmeyer flask to check the hydrolysis of antibiotic. Lower concentration of ceftriaxone was adopted for its high antimicrobial potency. In group 2, soil slurries were prepared with 5 g of soil sample and 100 mL of autoclaved dNB spiked with antibiotic to check the biodegradation of antibiotic by soil microorganisms. In group 3, five gram of **autoclaved** soil was mixed with 100 mL of dNB spiked with antibiotic to detect the adsorption of antibiotic on soil matrix with inactivated soil microorganisms. In group 4, five gram of soil was mixed with 100 mL of dNB to check the presence of endogenous antimicrobial compounds in the soil. In group 5, five gram of **autoclaved** soil was mixed with 100 mL of dNB to detect whether autoclaving can inactivate the endogenous antimicrobial compounds in the soil. All flasks were incubated at 30 °C in an air bath shaker incubator reciprocating at 150 rpm with lamp on. At designated time intervals, one mL of liquid (group 1) or slurry (groups 2 to 5) was aseptically sampled from each flask. The liquid samples were determined the residual antibiotic activity via bacterial inhibition tests described in the next section. The soil slurry samples were centrifuged at 16000 g for 5 min. Supernatants were also subjected to bacterial inhibition tests after being filtered through syringe filters (MILLEX® GP Filter Unit 0.22 µm, Merck Millipore Ltd. USA). The precipitates of the slurry samples from group 2 (antibiotic treated) and group 4 (without antibiotic treatment) were stored frozen at -40 °C for future DNA extraction and bacterial community analysis.

2.3. Bacterial inhibition test

Bacterial inhibition tests were used to detect the antibiotic activity in liquid-phase samples. Validity of the method was verified by examining the correlation between the size of inhibition zones and the concentration of antibiotics (calibration curves). A susceptible standard bacterial strain (*E. coli* ATCC 25922) was used as indicator bacterium in the inhibition tests. To establish the calibration curve for each antibiotic, the antibiotic stock solution was diluted into concentration series. Typically, kanamycin stock solution (25.6 mg/mL) was diluted into a concentration series of 8, 16, 32, 64, 128 and 256 µg/mL. Three aliquots of 10 µL of antibiotic solution with the same concentration were dripped apart on a LB agar plate spread with the standard strain. The agar plates dripped with antibiotic solution of different concentrations were incubated overnight at 35 °C. The size of inhibition zones was measured with a vernier caliper. Liquid-phase samples obtained from soil slurry experiments were subjected to bacterial inhibition tests following the same procedure. The size of inhibition zones reflected the change of antimicrobial activity in the liquid phase of soil slurries.

2.4. DNA sequencing

Genomic DNA was extracted from each precipitate using the FastDNA Spin Kit for Soil (MP Biomedicals, LLC. USA). Since the five antibiotics selected in this study were of four different antimicrobial classes and the two beta-lactam antibiotics (ampicillin and ceftriaxone) experienced the same fate in the slurry (refer to the section **Antimicrobial activity in the soil slurry**), precipitates obtained from the experiments of four representative antibiotics (except for ampicillin) were chosen for Illumina sequencing and bacterial community analysis. For comparison, DNA was also extracted from three individual samples of the original soil (0.5 g each), respectively. All DNA samples obtained in this study were treated in parallel during the Illumina sequencing. A modified dual-indexing approach for multiplexed 16S rRNA gene sequencing was adopted (Fadrosh et al., 2014). Bacterial 16S rRNA genes (V3-V4 hyper-variable regions) were amplified with primers 319F (ACTCCTACGGGAGGCAG-CAG) and 806R (GGACTACHVGGGTWTCTAAT) (Fadrosh et al., 2014). Both forward and reverse primers were tagged with 12 bp index sequences for pooling multiple samples into one sequencing operation. The pooled amplicons were sequenced on Illumina Miseq benchtop sequencer (2×300 bp paired-end run).

2.5. Sequencing data analysis and bacterial community profiling

For bacterial community analysis, we used the Quantitative Insights into Microbial Ecology (QIIME; version 1.9.1) pipeline to treat the Illumina sequence data following the general procedure (Navas-Molina et al., 2013). Briefly, paired Illumina reads of individual sample were firstly parsed to remove the barcode sequence. Then the forward and reverse Illumina reads were joined together with overlap of at least 55 nucleotides and mismatch less than 30% between overlapped regions. The unjoined sequences were discarded for their putatively low quality. The joined Illumina reads were thereafter de-multiplexed and quality-filtered with default parameters to obtain FASTA files of nucleotide sequences according to a constructed metadata-mapping file. The FASTA files from the same experimental run (same antibiotic) were routinely combined into a single FASTA file (namely a FNA file) according to the metadata-mapping file. The sequence reads were then clustered into operational taxonomic units (OTUs) with open-reference approach at 97% sequence identity using the UCLUST algorithm (Edgar, 2010). During this course, with default parameter setting, those OTUs with only one sequence were discarded. The sequencing data thus experienced the second round of quality filtration. Taxonomy was assigned to each OTU by comparing a representative sequence of the OTU to the Greengenes database (version 12.0). The OTUs with no match in the Greengenes database were combined together as “unassigned”. Thus the OTU table was generated, which recorded the number of times each OTU was observed in each sample. Taxonomic assignments resolved at the genus level were used in subsequent analysis, although some OTUs that could not find matches at the genus level were reported at coarser levels (family or class). Alpha diversity indexes, including Chao1 estimator, observed OTUs, Shannon-Wiener index, Simpson's index and metric phylogenetic diversity (PD) whole tree, were calculated for each sample using the OUT table. At this point, the sequencing data were subjected to a third level of quality filtration. The OTUs with a number of sequences less than 0.005% of the total number of sequences were discarded. The OTU table was subsequently rarefied at 20000 sequence reads per sample. Rarefaction curves of each group samples were generated with this rarefied OTU table.

2.6. Statistical analysis and visualization

Canonical correlation analysis (CCA) was performed to evaluate the influences of experimental variables (antibiotic, dNB and time of incubation) on the composition of bacterial communities (abundances of bacterial phyla). P-values were calculated using non-parametric multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distances between samples. CCA and PERMANOVA were performed in PAST 3.16 (Hammer et al., 2001). Main compositions of bacterial communities in soil slurries were visualized via Circos software (<http://circos.ca/>) at both phylum and genus levels. Heat maps were generated using the R package *heatmap*. The percentages of bacterial genera in each sample were log transformed, z-scaled, and used as input data for heat map construction. Determination of statistical differences of both diversity indexes and taxonomic groups were performed by t-test in Microsoft Excel, where a P-value of less than 0.05 was considered significant.

2.7. Function prediction of bacterial communities

The metabolic profiles of bacterial communities were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) according to the online protocol (<http://picrust.github.io/picrust/>). For the analysis, a ‘close-reference’ OTU picking protocol was used to generate the OTU table, where we searched sequences against the Greengenes reference OTUs (version gg_13.5_otus).

2.8. Accession numbers

Raw Illumina sequencing data were deposited to the NCBI Sequence Read Archive (SRA) under the accession number SRP118942 (BioSample accession: SAMN07692022- SAMN07692071). The nomenclature of these samples refers to the note to supplementary Table S2. As we operated the experiments for different antibiotics in parallel, the experimental condition was the same for the slurries without antibiotic treatment. The samples C4A67, C4B67, C4C67, T4A67, T4B67 and T4C67 were actually equivalent. So, we just provided the sequencing data for the experiment of tetracycline, i.e. the data for the samples of biological triplicates T4A67, T4B67 and T4C67 without antibiotic treatment. These data were also used to represent C4A67, C4B67 and C4C67, respectively, in analyzing the effect of ceftriaxone on soil bacterial community. Furthermore, the sample K4A17 was missing due to the failure experimental operation. One cannot find the sequence data for samples C4A67 (SAMN07692029), C4B67 (SAMN07692031), C4C67 (SAMN07692033) and K4A17 in the database.

3. Results

3.1. Antimicrobial activity in soil slurries

The antimicrobial activity in the liquid phase of soil slurries was evaluated using bacterial inhibition test. According to the calibration curve for each antibiotic, the area of the inhibition zone (d^2) is linearly related to the logarithm of antibiotic concentration (Supplementary Fig. S2), which agrees well with previous reports (Hultmark et al., 1982). Therefore, the size of inhibition zones well reflects the antimicrobial activity of liquid-phase samples. For the slurries of unautoclaved or autoclaved soil without antibiotic treatment (experimental group 4 and 5, respectively), no inhibition zones were observed in bacterial inhibition tests against their liquid-phase samples. It means no detectable endogenous antimicrobial compound in the soil. Different antibiotics exhibited distinctive results in their experimental groups 1 to 3. For ampicillin and ceftriaxone, the antimicrobial activity in the liquid-phase declined to zero within 43 and 19 h in the unautoclaved soil slurry, respectively (Supplementary Fig. S3 and Figure 1a). For kanamycin, the antimicrobial activity in the liquid phase became undetectable shortly after (within 3 h) its addition in either unautoclaved or autoclaved slurry (Figure 1b). The antimicrobial activity of nalidixic acid lasted for fairly long time (164 h) in all three experimental groups without significant changes (Figure 1c). The antimicrobial activity of tetracycline gently declined along with time in all three groups (Figure 1d). The fastest decline occurred in the pure dNB (group 1), and the slowest one was in the unautoclaved slurry (group 3). We repeated the incubation experiment of tetracycline in dNB solution in the dark with lighted controls. The antibiotic activity of tetracycline still declined in the dark. Under illumination, the decline rate increased by 12% (data not shown).

3.2. Alpha diversity

The number of sequencing reads for all samples ranged between 34122 and 364412, resulting in the observed number of OTUs between 1204 and 21928 (Supplementary Table S2). In most cases, the addition of antibiotics suppressed the diversity of bacterial community in soil slurries. The species richness (Chao1), diversity (Shannon-Wiener index) and evenness (Simpson's index) in antibiotic-treated slurries are generally lower than those of untreated ones (Figure 2, Supplementary Table S2). The rarefaction curves for antibiotic-treated slurries are also generally below those for untreated ones (Supplementary Fig. S4). For the treatment of nalidixic acid or tetracycline, the suppression effect on bacterial community is revealed on the significant decline of species richness ($P < 0.05$), diversity ($P < 0.01$) and evenness ($P < 0.05$) (Figure 2). However, for the treatments of kanamycin and ceftriaxone, such a shift in bacterial

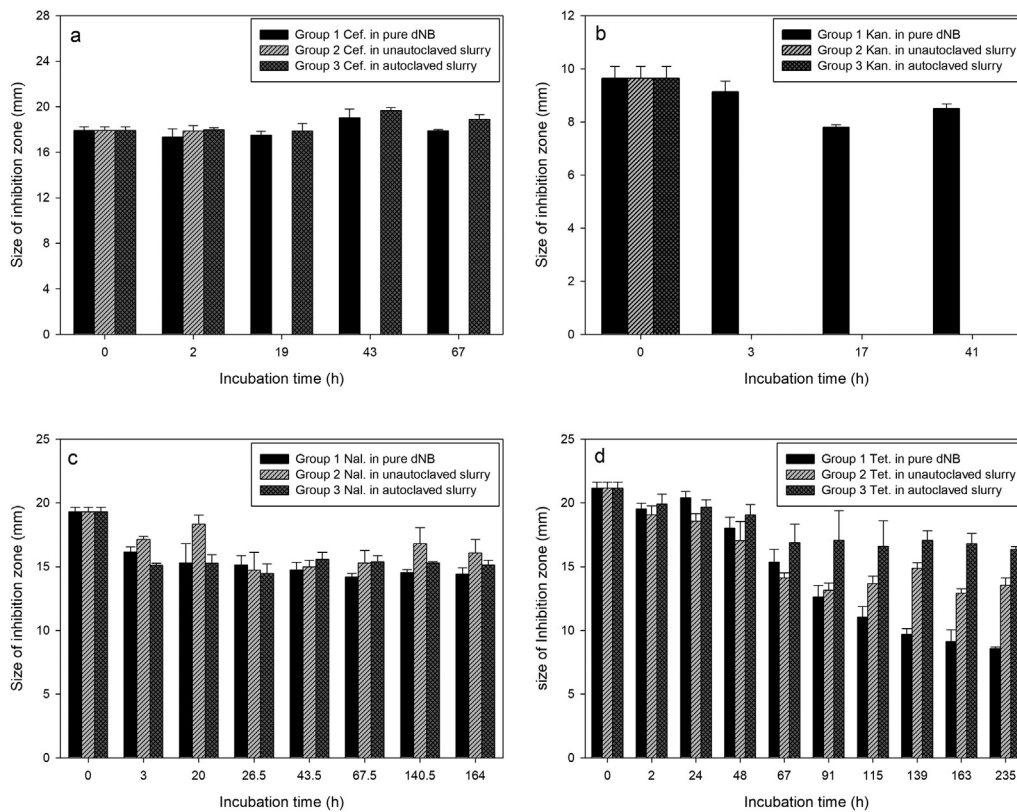


Figure 1. The data of bacterial inhibition tests illustrating the fate of ceftriaxone (a), kanamycin (b), nalidixic acid (c) and tetracycline (d) in the soil slurry. In experimental group 1, antibiotic was solved in diluted nutrient broth (dNB); group 2, antibiotic was added in unautoclaved slurry; group 3, antibiotic was added in autoclaved slurry.

community is not so significant. The significant change only occurs in the species diversity (Figure 2b, $P < 0.05$). Furthermore, the community diversity suppressed by ceftriaxone (or by nalidixic acid) was restored to some extent over time. The diversity indices of antibiotic-treated slurries at the second point are higher than those at the first point (Supplementary Table S2, $P < 0.05$), and the corresponding rarefaction curves move upwards with time (Supplementary Fig. S4a,c).

3.3. Impacts of experimental treatments on bacterial community

The slurry (or original soil) samples showed a clear separation in CCA plots (Figure 3). The three original untreated soil samples cluster together and are separated from those soil slurry samples. The points representing nalidixic acid- or tetracycline-treated slurries obviously cluster away from those of untreated slurries (Figure 3c,d). Although ceftriaxone-treated slurries are also separated from those untreated ones, the trend is not obvious, and the correlation between bacterial community structure and antibiotic treatment is weak (Figure 3a). However, the points representing the slurries with or without kanamycin treatment are distributed alternatively on the CCA plot (Figure 3b). According to the results of PERMANOVA, the preparation of soil slurry with dNB significantly affected the structure of the soil bacterial community ($P < 0.05$). The addition of nalidixic acid or tetracycline further changed the composition of bacterial community in the slurry ($P < 0.01$), while variations in incubation time did not make much difference ($P > 0.05$). Ceftriaxone and kanamycin did not significantly affect the bacterial community ($P > 0.05$). In the following discussion, we just compared the composition of bacterial community in the slurries with or without antibiotic treatment.

3.4. Variation in bacterial community structure

Nalidixic acid and tetracycline have significantly affected the composition of bacterial community at the phylum level. Relative abundances of several phyla have significantly changed under the effect of either antibiotic (Fig. S5c and d). Ceftriaxone and kanamycin give minor or negligible influences (Fig. S5a and b). More detailed shifts of the bacterial community at the genus level under the impacts of various antibiotics are visualized in heat-maps (Figure 4). Under the influence of antibiotics, the relative abundance of some bacterial genera has changed significantly (indicated by asterisk in Figure 4). If one bacterial taxon (OTU) is mostly detected in the antibiotic-treated slurries, and the reads number exceeds 95% of its total reads number from the same experiment, then the OTU has been 'highly enriched' (indicated with red dot in Figure 4) by the antibiotic. The bacterial taxon rarely detected in the antibiotic-treated slurries (with no more than 5% of its total reads) is considered as 'highly inhibited' (indicated with blue dot in Figure 4). Those significantly "enriched" or "inhibited" bacterial genera (indicated with both asterisk and dot in Figure 4) deserve more attention (also refer to Supplementary Table S3). Similarly, Nalidixic acid and tetracycline significantly affected the composition of bacterial genera in the bacterial community, while ceftriaxone and kanamycin exhibit minor or negligible influences. A set of Circos plots intuitively illustrating the source of each OTU (i.e. the reads number of each OTU from each sample) is available in the supporting information for reference (Supplementary Fig. S6). The bacterial genera highly affected by antibiotics are mainly distributed in the phyla *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Supplementary Fig. S7).

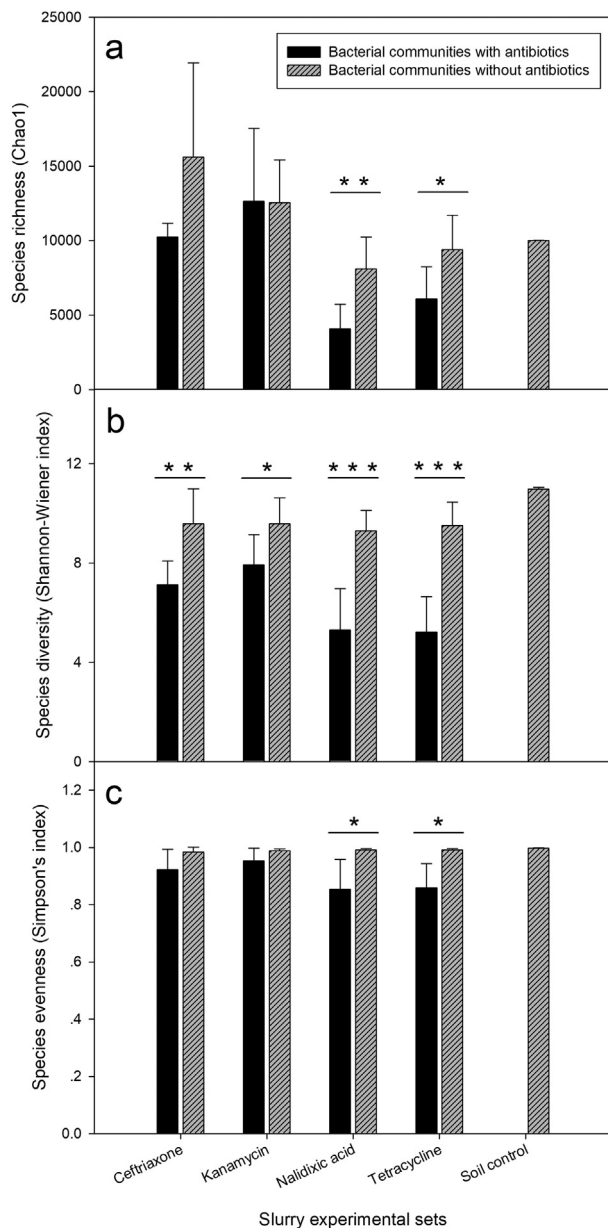


Figure 2. Alpha diversity expressed as species richness (Chao1) (a), species diversity (Shannon-Wiener index) (b), and species evenness (Simpson's index) (c) of the bacterial community in soil slurries with or without antibiotic treatment. The error bars indicate the standard errors of the indices among the samples of biological triplicates at two sampling time points (6 effective samples) or among three parallel sequencing samples of original soil. Asterisks indicate statistically significant differences. One star is $p < 0.05$, two stars is $p < 0.01$ and three stars is $p < 0.001$.

3.5. Variation in metabolic profile

Metagenomic predictions showed that antibiotic treatment affected the metabolic function of the soil bacterial community. Similarly, different antibiotics had different effects. Ceftriaxone and kanamycin affected several metabolic subsystems (Figure 5a and b). Nalidixic acid is the first of synthetic quinolone antibiotics, which inhibits a subunit of DNA gyrase and topoisomerase IV and interferes bacterial DNA replication (Pommier et al., 2010). According to our result, it influenced all functions in related to genetic information processing and most functions of metabolism. Noteworthy, it significantly enhanced the membrane

transport (Figure 5c). Tetracycline works by blocking the ability of bacteria to synthesize proteins. According to the prediction, it affected the metabolism of the bacterial community and also some functions of environmental information processing (Figure 5d). Nevertheless, the basic metabolic profile of the bacterial community did not change too much in comparison with the original soil sample (Supplementary Fig. S8).

4. Discussion

Different antibiotics have experienced distinctive fates in the soil slurry. Ampicillin and ceftriaxone underwent quick bio-degradation and completely lost their antimicrobial activity. It is predictable that these two antibiotics will experience the similar fate in the environment, since they are of the same antibiotic class (β -lactam), and share the similar central chemical structure. For both antibiotics, the antimicrobial activity in the pure dNB (group 1) or autoclaved slurry (group 3) did not decrease significantly (Supplementary Fig. S3 and Figure 1a), indicating no obvious hydrolysis or soil adsorption. Their antimicrobial activity however fast declined to zero in unautoclaved slurries (group 2, Supplementary Fig. S3 and Figure 1a). Soil microorganisms in the slurry should have played an important role in the deactivation of two β -lactam antibiotics. According to a recent functional metagenomic study on soil antibiotic resistomes, β -lactamase genes are the most frequently encountered soil ARGs (Forsberg et al., 2014). Therefore, it is not surprising that both ampicillin and ceftriaxone have been inactivated fast in unautoclaved slurries. Nalidixic acid exhibits high stability in all three experimental groups (Figure 1c) throughout the experimental period (164 h). It has been reported that quinolone and fluoroquinolone antibiotics are biochemically stable in sewage treatment plant, and most of them are removed from the wastewater via sludge adsorption (Jia et al., 2012). For tetracycline, the antimicrobial activity declined gradually in all three experimental groups (Figure 1d), and the fastest deactivation occurred in the pure dNB (group 1). Control experiment showed that tetracycline also kept losing its activity in dark. Therefore, the main inactivation mechanism of tetracycline should be hydrolysis, which can be accelerated by light. Introduction of soil in the solution somehow weakened the effect of illumination. Suspended soil particles in the slurry might attenuate the energy of light by scattering and absorbing. According to a previous study, light and UV radiation play significant roles in catalytic removal of tetracycline in water (Verma et al., 2007). Intensive studies have been carried out on the photolysis mechanism of tetracycline in aqueous phase (Jiao et al., 2008; Werner et al., 2006). In addition, the antimicrobial activity declined faster in unautoclaved soil slurries (group 2) than in autoclaved ones (group 3). Biodegradation should have played a secondary role in the inactivation of tetracycline. A tetracycline-degrading *Stenotrophomonas maltophilia* strain has been ever isolated from soil (Leng et al., 2016). The strain belongs to the tetracycline-enriched genus, *Stenotrophomonas*, as indicated in Figure 4d and Supplementary Table S3. The liquid-phase activity of kanamycin dropped dramatically to zero in either unautoclaved or autoclaved soil slurries (group 2 and 3, Figure 1b), indicating a fast (within 3 h) adsorption of this antibiotic on soil matrix.

The effect of an antibiotic on the soil bacterial community depends largely on its fate in the soil slurry. Since all slurry bacterial communities have been derived from the same original soil bacterial community, alpha diversity analysis shows that the impacts of ceftriaxone and kanamycin on the soil bacterial community are negligible (not significant with $P > 0.05$). Ceftriaxone has experienced fast biodegradation and lost its full antimicrobial activity within 19 h (group 2, Figure 1b). Consequently, the influence of ceftriaxone on the soil bacterial community is limited. Only phyla *Proteobacteria* (at the second sampling time point) and *Nitrospirae* give significant changes in their relative abundance

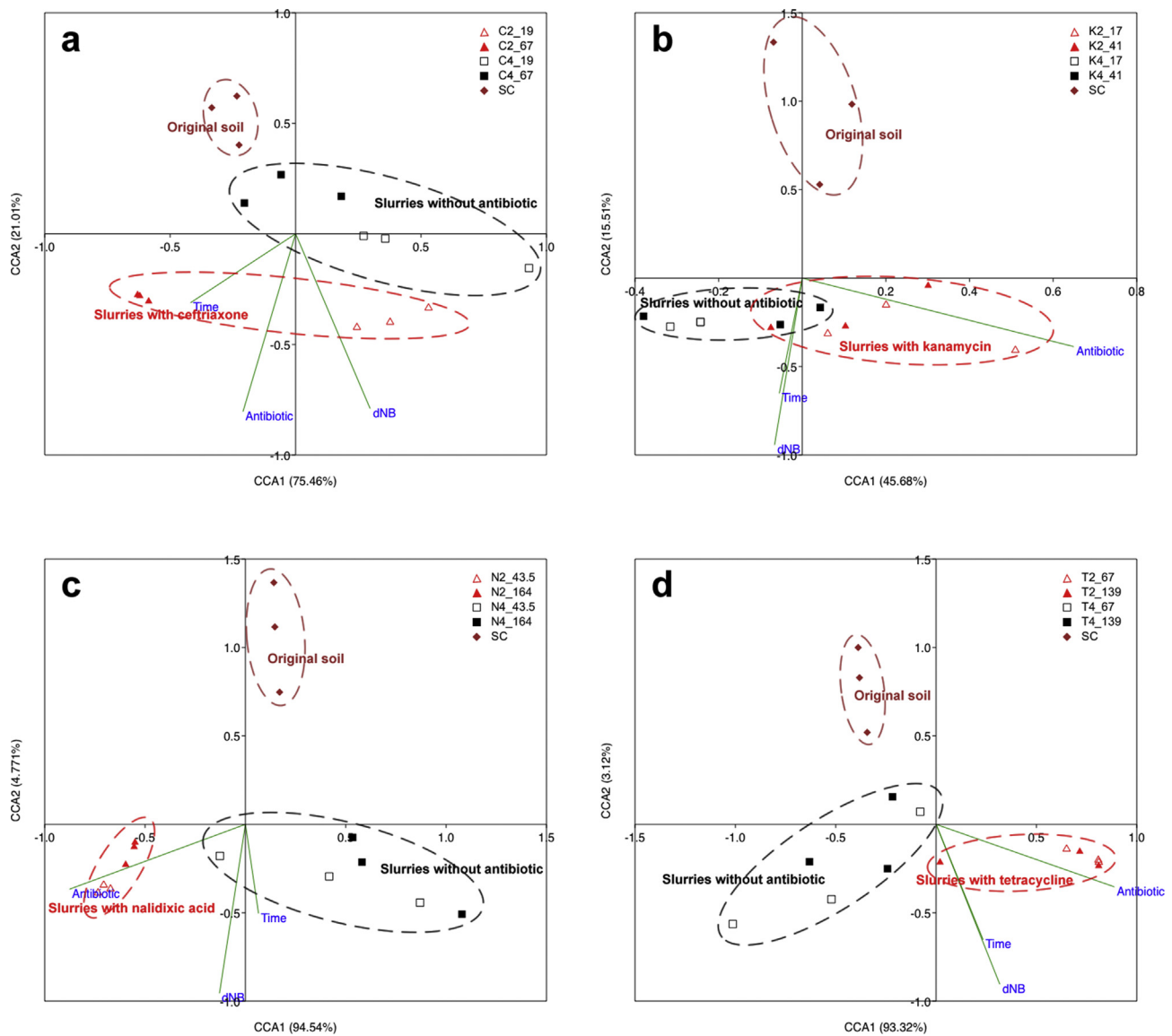


Figure 3. Canonical correspondence analysis (CCA) depicting the change of bacterial community in the slurry treated with ceftriaxone (a), kanamycin (b), nalidixic acid (c) and tetracycline (d), respectively. The impacts of experimental variables (green lines) on the abundance of bacterial phyla in samples (symbols) were illustrated. The percentage of variation explained by each axis is shown. CCA analyses were performed in PAST 3.16. Triangles indicate the samples obtained from the slurries spiked with antibiotics, and squares indicate the ones from the slurries without antibiotics. The hollow ones indicate the samples obtained at the first time point, and the solid ones are the samples from the second time point. Three diamonds in each panel indicate three parallel samples of the original soil.

(Supplementary Fig. S5a). A previous study had reported that the soil (sandy loam or clay) irrigated with ceftriaxone solution (2 $\mu\text{g}/\text{mL}$) for 14 days gave no significant changes in its bacterial community (Gatica et al., 2015). Such a low concentration of ceftriaxone should have been completely degraded in the soil at the interval of two watering (48 h). Kanamycin was quickly adsorbed on soil particles. So, its effect on the bacterial community was also fairly weak. Although the relative abundance of several bacterial taxa (especially at lower taxonomic levels) changed significantly under kanamycin treatment (Figure 4b and Supplementary Table S3), no significant changes were observed on overall structure of the bacterial community (Figure 3b), and no bacterial genera were significantly enriched or inhibited (Figure 4b). It has been reported that restricted application of streptomycin, another aminoglycoside antibiotic, in apple orchard do not adversely alter the soil bacterial community (Walsh et al., 2013). However, the applied dose is much lower (6 $\mu\text{g}/\text{mL}$ soil) in comparison with the current study (256 $\mu\text{g}/\text{mL}$). Nalidixic acid and tetracycline have significantly altered the composition of the soil bacterial community at both the genus (Figure 4c,d) and

phylum (Supplementary Fig. S5c,d) levels. Therefore, the more stable an antibiotic is in the slurry, the greater the impact it exerts on the bacterial community therein.

Three mechanisms that decrease the sensitivity of bacterial cells to quinolones includes target protection, gene mutant and efflux pump (Aldred et al., 2014). Most of the acquired tetracycline resistance genes encode energy-dependent efflux proteins or ribosomal protection proteins (Roberts, 2005). The mechanisms of resistance against both kinds of antibiotics do not affect the molecular structure of the antibiotics and do not change their concentration outside the resistant bacterial cells, which is so called the “selfish” drug resistance (Bottery et al., 2016). This may explain the stability of the two kinds of antibiotics (nalidixic acid and tetracycline) in the soil slurry and their significant influence on the bacterial community therein.

The bacterial genera highly enriched by various antibiotics are mostly of the same phylum of *Proteobacteria* and appear mainly in the classes of *Gammaproteobacteria* and *Betaproteobacteria* (Figure 4, Supplementary Table S3 and Supplementary Fig. S7). It has been revealed that the class

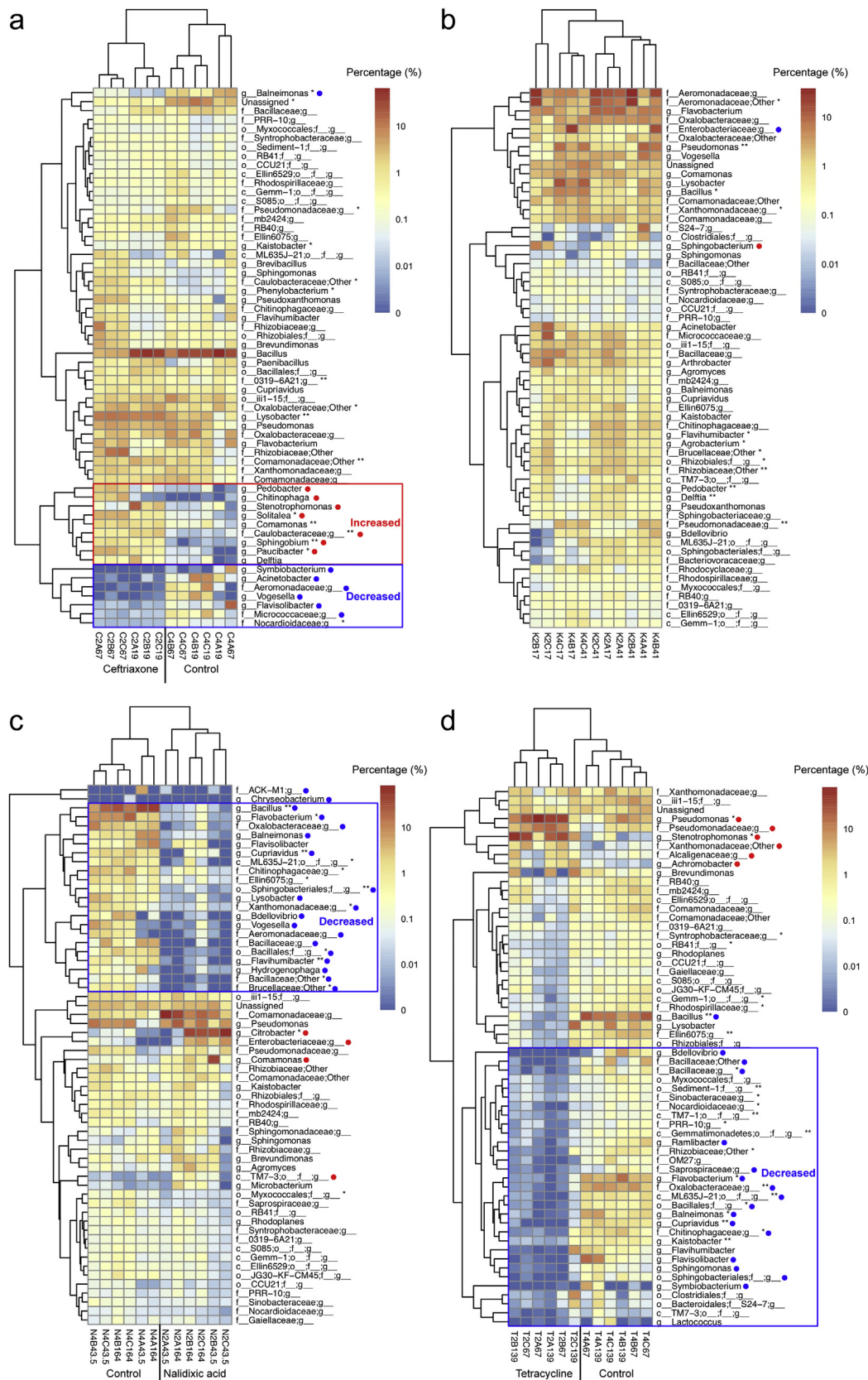


Figure 4. Heat-maps illustrating the effects of ceftriaxone (a), kanamycin (b), nalidixic acid (c), and tetracycline (d) on soil bacterial community at the genus level. The top 60 abundant genera are exhibited. The bacterial genera highly enriched (95% was detected in antibiotic-treated slurries) are indicated with red dots, and the inhibited ones (95% was detected in untreated slurries) are indicated with blue dots. Lowest levels of taxonomic assignment are exhibited for those OTUs that are not classified to genus level. Lower-case c means Class, o for Order, f for Family and g for Genus. Unassigned means those sequences not hitting any record in the GreenGenes database. Asterisks indicate statistically significant differences between antibiotic-treated and untreated slurries. One star is $p < 0.05$ and two stars is $p < 0.01$.

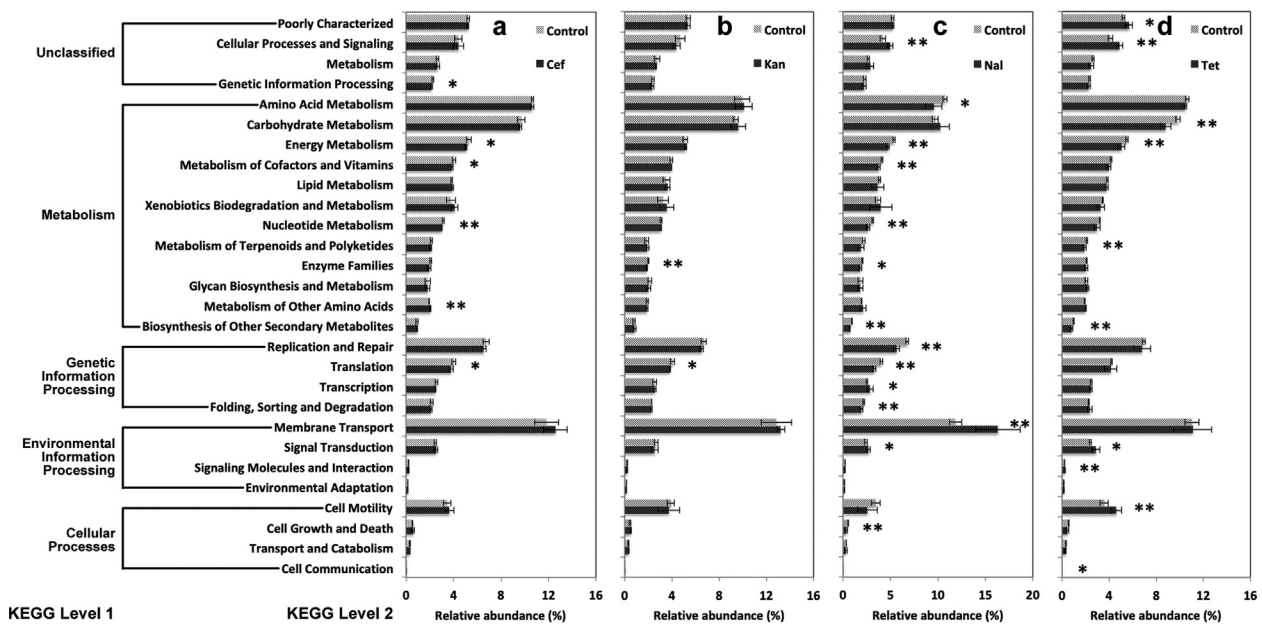


Figure 5. Relative abundance of the predicted metabolic subsystems (KEGG Level 2) in the antibiotic-treated (group 2) and control (group 4) slurries. Panel (a) is for ceftriaxone treatment, (b) kanamycin, (c) nalidixic acid and (d) tetracycline. Asterisks indicate statistically significant differences. One star is $p < 0.05$ and two stars is $p < 0.01$.

Gammaproteobacteria is highly related to the persisting ARGs in manure-treated soil (Leclercq et al., 2016). This may explain why so many members of the class *Gammaproteobacteria* are highly enriched in soil slurries treated with various antibiotics (Supplementary Fig. S7). A big data analysis has shown that the transfer of mobile resistance genes among different bacterial species is shaped largely by phylogeny and ecological constraints. The transfer of resistance genes between bacterial species occurs mainly among four major bacterial phyla—*Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, while the mobile ARGs have been significantly enriched in the phylum of *Proteobacteria* (Hu et al., 2016). This result has somehow corroborate our observation, that antibiotic-enriched bacterial genera are mostly confined in the phylum *Proteobacteria*. In summary, although there are a few exceptions, the strong correlation between resistomes and phylogeny has been proved via functional metagenomics (Hu et al., 2016), big data analysis (Hu et al., 2016) or microcosm experiments (this study).

Some low-abundance taxa in the bacterial community of the original soil were highly enriched under the selective pressure of antibiotics. The genus *Citrobacter* (of family *Enterobacteriaceae*) was rare in original soil with reads number no more than 17. It was overall enriched in the nalidixic-acid-treated slurry. The reads number reached an impressive 11399 to 56945 in the second (N2B) and third (N2C) replicates of the slurry (genus A in the Circos plot Supplementary Fig. S6f). Early clinical studies showed that *Citrobacter* had high resistance to quinolones (Aoyama et al., 1988; Navia et al., 1999). Another low abundance genus in original soil with reads number no more than 306, genus *f_Comamonadaceae:g_* (genus C in the Circos plot Supplementary Fig. S6f) was also enriched by nalidixic acid. While, it was detected the most frequently in the first biological replicate (N2A) instead, with reads number up to 21742. Therefore, under the pressure of antibiotics, the restructuring of the soil bacterial community showed some randomness. In addition, under the stress of antibiotics, the abundance change of some bacterial genera seems to have undergone a slow process. In the experiment of tetracycline treatment, genus *Brevundimonas* (genus H in the Circos plot Supplementary Fig. S6h) is almost undetectable at 67 h in antibiotic-treated slurries with only once detection in the replicate of T2C67. While at the second time point (at 139 h), it is frequently detected in all triplicates with reads number ranged from 6092 to 11375. This phenomenon is universal for the bacterial class of *Alphaproteobacteria*

under high antibiotic pressure. No matter what kind of antibiotic is applied (ceftriaxone, nalidixic acid or tetracycline), the reads number of this bacterial class is low at early time point, and becomes significantly higher at the later time point. This can explain why the diversity of the bacterial community has recovered to some extent over time. The rarefaction curves shift upwards at latter time points (Supplementary Fig. S4a,c), and the diversity indices increase over time (Supplementary Table S2). These examples reflected the dynamic characteristics of the bacterial community under the antibiotic stress. The transformation of soil bacterial community was not followed in a greater detail in this study. The bacterial community composition was only determined at two separate time points. The dynamics of the structure shifting of the soil bacterial community under the pressure of antibiotics can be an interesting project.

The PICRUSt prediction shows that the treatment of various antibiotics has not affected the basic metabolic profile of the soil bacterial community. The relative abundance of metabolic subsystems is highly similar among slurries with or without antibiotic treatment (Figure 5) and even between slurries and the original soil sample (Supplementary Fig. S8). The metabolic function of the bacterial community is mainly shaped by the physical and chemical properties of the environment it inhabiting, while the introduction of antibiotics seems not enough to fundamentally change the properties of the simulated soil and water environment. Nevertheless, the treatment of antibiotics, especially stable ones, has significantly changed the relative abundance of a few metabolic subsystems (Figure 5c and d), and these changes seem to be closely related to the antimicrobial mechanism of antibiotics. Although the ever-reported accuracy of PICRUSt prediction for soil samples is higher than 80% (Langille et al., 2013), we can hardly declare that the predicted results have reflected the real metabolic situation of the bacterial community in the soil slurry. The ecological risk assessment of antibiotics requires further studies with multi-omics-based approaches.

5. Conclusion

A set of experiments of slurry microcosm has been carried out to study the interaction between antibiotics and the soil bacterial community. Based on the measured antimicrobial activity at different time points and comparison of different experimental conditions, most likely the beta-

lactam antibiotics (ampicillin and ceftriaxone) are quickly biodegraded, kanamycin is adsorbed to soil particles and tetracycline is subjected to slow light-promoted hydrolysis and biodegradation. In contrast, nalidixic acid is stable throughout the experimental process. Different antibiotics have exerted various impacts on the soil bacterial community according to their different fates. Antibiotics of lower stability (ceftriaxone) or adsorbed on soil particles (kanamycin) show minor or negligible effects on the soil bacterial community, while those stable ones (Nalidixic acid and tetracycline) significantly alter the structure of the bacterial community. The bacterial genera highly enriched by various antibiotics (ceftriaxone, nalidixic acid or tetracycline) are mostly of the same phylum of *Proteobacteria* and are confined in two main classes, *Gammaproteobacteria* and *Betaproteobacteria*. It indicates that antibiotic resistance determinants may be confined in certain phylogenetic boundaries. The inhibited bacterial taxa vary among different antibiotic treatments, and are distributed in different phyla. Nalidixic acid mainly inhibits bacterial phyla *Firmicutes* and *Bacteroidetes*, while tetracycline inhibits *Firmicutes*, *Bacteroidetes* and *Cyanobacteria*. The metagenomic prediction of the metabolic function of the bacterial community via PICRUSt implies that antibiotics, especially those stable ones (nalidixic acid and tetracycline) have significantly affect the abundance of some metabolic subsystems. While, the basic metabolic profile of the soil bacterial community is seldom influenced and is determined by the bacterial community in the original soil sample. The control of environmental emission of antibiotics should be based on their different fates in the environment and their negative impacts on the environment.

Declarations

Author contribution statement

Xiaohong Dong: Analyzed and interpreted the data; Wrote the paper.
Dawei Rao: Conceived and designed the experiments; Performed the experiments.

Lejin Tian: Analyzed and interpreted the data.

Qizheng Wang: Performed the experiments.

Kun Yang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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