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Overlooked interconversion between tetracyclines and their 4-epimers in soil and effects on soil resistome and bacterial community

Chenxi Lu [a,b,c,1], Cheng Qin [a,b,c,1], Lixia Zhao [a,b,c,*], Huike Ye [a,b,c], Mohan Bai [a,b,c], Yang Sun [a,b,c], Xiaojing Li [a,b,c], Liping Weng [a,b,c,d], Yongtao Li [e]

[a] Agro-Environmental Protection Institute, Ministry of Agriculture and Rural Affairs, Tianjin 300191, China
[b] Key Laboratory of Original Agro-Environmental Pollution Prevention and Control, MARA, Tianjin 300191, China
[c] Tianjin Key Laboratory of Agro-Environment and Agro-Product Safety, Tianjin 300191, China
[d] Department of Soil Quality, Wageningen University, Wageningen 6700 HB, The Netherlands
[e] College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China

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ABSTRACT

With the widespread use of tetracycline antibiotics (TCs) and the application of manure fertilizer in farmland, TCs and their metabolites especially 4-epimers have been heavily detected in agricultural soil. However, existing studies have focused on the residual and environmental behavior of maternal TCs, and few studies have looked at the ecotoxicity of their 4-epimers in soil. In this study, the degradation and interconversion of tetracycline (TC), oxytetracycline (OTC) and their 4-epimers (4-epitetracycline, ETC; 4-epioxytetracycline, OTC) were revealed. Their effects on antibiotic resistance genes (ARGs), mobile genetic elements (MGEs) and bacterial community in soil were also investigated in comparison. The results showed that the 4-epimers could be substantially transformed to their parents and degraded as a whole. The degradation rates of four selected pollutants are followed: TC > OTC > ETC > EOTC. This indicated that when TCs entered the soil, part of TCs transformed into slower-degraded 4-epimers, and these 4-epimers could also be converted back to their antibiotic parents, causing the long-term residue of TCs in soil. When added to the soil alone, TC and OTC significantly promoted the proliferation of most ARGs and MGEs, among them, *trb-C*, *IS1247* and *IS1111* were the top three genes in abundance. ETC and EOTC had little effect at the beginning. However, as the 4-epimers continuously converted into their parents after one month of cultivation, ETC and EOTC treatments showed similar promoting effect on ARGs and MGEs, indicating that the effect of ETC and EOTC on soil resistome was lagged and mainly caused by their transformed parents. *Nocardioides*, *unclassified_Rhizobiaceae*, *norank_Sericytochromatia*, *Microlunatus*, *Solirubrobacter* and *norank_67-14* were the most frequent hosts of ARGs, Most of which belong to the phylum Actinobacteria. Due to their large transformation to TCs, slow degradation rate and potential effects on soil microbes and ARGs, the harm of TCs' 4-epimers on soil ecosystem cannot be ignored.

1. Introduction

Tetracycline antibiotics (TCs) are widely used in animal husbandry because of their ability to promote animal growth, prevent disease, and improve livestock productivity (Gaballah et al., 2021, Kim et al., 2018). Tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) are representatives of tetracycline antibiotics. TCs that are consumed by animals are often not fully digested. Studies have shown that 72 %, 21 %, and 65 % of TC, OTC, and CTC, respectively, could not be utilized by animals after medication, leading to their excretion into the environment (Awad et al., 2014, Kuppusamy et al., 2018, Wu et al., 2024). Investigation researches have shown that animal feces and their fecal-derived organic fertilizers contain large amounts of tetracycline residues at concentrations even up to hundreds of mg kg^{-1} (Zhao et al., 2010, Li et al., 2021, Wu et al., 2024). Due to the extensively use of manure fertilizer in farmland, tetracycline antibiotics and their metabolites have been widely detected in agricultural soils. It was investigated that the total concentrations of 26 antibiotic residues in the typical

* Corresponding author.
E-mail address: zhaolixia@caas.cn (L. Zhao).
[1] Both authors contributed equally to this work.

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greenhouse vegetable soils were 83.2 ~ 4238 μg·kg^{-1}, of which TCs and TCs' metabolites (MTCs) were 27.2 ~ 2390 and 23.3 ~ 1799 μg·kg^{-1}, respectively (Zhao et al., 2023). Residual antibiotics in soil have both direct and indirect effects on microbial communities. Directly, they can hamper the structure and functioning of soil microbial community (Grenni et al., 2018). Indirectly, it is a widely accepted fact that the residues of antibiotics in soil would lead to proliferation and diffusion of resistant bacteria and antibiotic resistance genes (ARGs) (Zhang et al., 2020), which has been classified as a global public health crisis (WHO, 2018). ARGs have inherent biological characteristics and could use mobile genetic elements (MGEs) to perform horizontal gene transfer (HGT) or even self-amplification among microorganisms (Liu et al., 2020, Zhang et al., 2022, Tong et al., 2019). The emergence of high abundance of ARGs and MGEs represents a new transferable resistance crisis that threatens environmental safety and human health (He et al., 2021, Hernando-Amado et al., 2019).

Isomerization, hydroxylation, dehydration, demethylation, deamination and ring-opening are main metabolic pathways of TCs (Long et al., 2020, Peng et al., 2022). Theoretically, TCs were easily epimerized at position C-4 and converted into 4-epimers by hydrolysis or photolysis, especially in acidic conditions (Loftin et al., 2008, Halling-Soerensen et al., 2002, Yuen and Sokoloski, 1977). Wu et al found out that 4-epimers are dominant transformation products in manure and manure-derived organic fertilizers (Wu et al., 2024). The monitoring results of actual farmland soil also confirmed that 4-epimers of TCs accounted more than 85 % of MTCs in soil (Zhao et al., 2023). In the wastewater treatment plants, the epimerization/isomerization and dehydration were proved to be the main degradation pathways for TC, CTC, OTC and doxycycline (DC) (Zhong et al., 2022b). In standard regulatory assessment protocols, such as the European Scope of Chemicals Regulation and the VICH Guidelines for Veterinary Medicines, explicit references to the parent compound and its stable and/or toxic metabolites in the risk assessment are required to be included (Escher and Fenner, 2011, Evgenidou et al., 2015). However, available studies have generally focused on the ecotoxicity and degradation of TCs in soil, while lacking the corresponding understanding of MTCs. The reduction of parent antibiotics is often considered as the removal of antibiotics. In fact, exposure studies using *E. coli* showed that although the tetracycline degradation product is less bacterial toxic than the parent compound, the toxicity increases with irradiation time after almost complete degradation of the parent compound in natural water, implying that the product mixture retained ecotoxicity (Peng et al., 2022, Long et al., 2020). The findings vertified the ecotoxicity and antimicrobial activity of MTCs in the aquatic environment, while their ecological risks in soil is unknown. In addition, no experiments have removed the effects of maternal antibiotics when studying the ecological hazards of the metabolites.

In this study, the 4-epimers of TC and OTC were added to soil as a separate pollution source, and the soil with separate antibiotic parent was added as a control group, which systematically studied the conversion of TCs and MTCs under short- and long- term's cultivation and their effects on soil resistome and bacterial community. The results will fill the gaps in understanding the transformation, fate and ecological risks of TCs and MTCs in soil.

2. Materials and method

2.1. Soils and chemicals

Soil samples were collected from 0 to 20 cm surface layer at a vegetable farmland in Wuqing District, northwest of Tianjin, China (geographical coordinates: 39 37 ′41″N, 117 3′ 51″E). The fresh soil is naturally dried indoors, and impurities such as dead leaves and gravels are removed over 2 mm sieve before use. The occurrence type of test soil is tidal soil, and the soil is loam (8.15 % clay, 40.81 % powder, 51.05 % sand grain). The soil properties tested include pH of 7.37, organic matter content of 22.24 g·kg^{-1}, alkali nitrogen content of 88.90 mg·kg^{-1}.

Tetracycline hydrochloride (TC, purity > 96.3 %), 4-Epitetracycline hydrochloride (ETC, purity > 95.8 %), Oxytetracycline hydrochloride (OTC, purity > 93.8 %), 4-Epioxytetracycline (EOTC, purity > 81 %) were purchased from Alta Technology Co., LTD (Tianjin, China). The structures of four targeted compounds and interconversion process between them are shown in Fig. 1. Methanol, formic acid and acetonitrile (HPLC pure) were purchased from Fisher, USA. All other reagents involved in the test are analytically pure.

2.2. Degradation and conversion experiment

Five treatments were set up to study the degradation and conversion of tetracycline antibiotics (TC, OTC) and their epimers (ETC, EOTC). In addition to the original natural soil (CK), we set up a total of four polluted groups which are the natural soil polluted by TC, ETC, OTC and EOTC, respectively. They are named as S1, S2, S3 and S4, accordingly. All the treatments were prepared in triplicates. Soil received a certain same amount of stock solution of antibiotics to reach final concentration of 10 mg·kg^{-1} dry soil, and then was homogenized by hand before separated into each beaker (6 cm (inner diameter)*9 cm (height)). Sterile deionized water was added to adjust the soil moisture content to 21 % (w/w), which is 60 % of water holding capacity. And then beakers were covered by tin foil cover with small holes and incubated at 25℃ in the dark for 33 days. Keep adding distilled water to the beaker every 72 h to maintain soil water content. Soil was collected at 3, 7, 15 and 33 days respectively for test.

2.3. Analysis of tetracyclines and metabolites

The method that we used to extract the tetracycline antibiotics and metabolites from soil is described in our previous study (Zhao et al., 2023). Firstly, 1 g of soil was weighed and mixed with 10 mL methanol and 10 mL buffer solution (21.01 g of citric acid, 44.78 g of Na_2HPO_4, 60.5 g of $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, and 1.625 L of H_2O) into a 50 mL centrifuge tube. The tubes were mixed for 8 min (2500 r min^{-1}) in vortex oscillator and then centrifuged for 10 min at 10,000 r min^{-1}. The supernatant was transferred to a pear-shaped flask. The extraction procedure was repeated once. Then, the combined supernatant was concentrated to approximately 20 mL with a rotary evaporator, filtered through a nylon filter (0.45 μm), diluted to approximately 80 mL using deionized water, and adjusted to pH 4 ± 0.1 with formic acid. A PEP-2 cartridge (500 mg/6 mL, Agela Technologies) was used to purify and concentrate the extract. The cartridge was sequentially activated with 6.0 mL methanol and 6.0 mL deionized water before loading the extract, and then eluted with 10 mL methanol. The eluent was dried under pure nitrogen to less than 1.0 mL and reconstituted back to 1.0 mL with of a water:methanol (1:1) solution before instrumental analysis. The targeted compounds was measured by liquid chromatography tandem mass spectrometry (SCIEX QTRAP®4500, USA) using a Phenomenex Kinetex^{TM} C18 LC column (100 mm × 2.1 mm, 2.6 μm, USA). The antibiotic content was quantified using the external standard method, where the coefficients (R^2) were all >0.99. The percentage of antibiotic and metabolite recovery in the soil was 61.56–72.86 %.

2.4. Caculation of enantiomer fraction

Enantiomer fraction (EF) is a selective evaluation parameter for enantiomers, ranging from 0 to 1. We define its calculation as follows:

$$EF_{TC} = \frac{c_{TC}}{c_{TC} + c_{ETC}}$$

$$EF_{OTC} = \frac{c_{OTC}}{c_{OTC} + c_{EOTC}}$$

In the formula, c_{TC}, c_{ETC}, c_{OTC} and c_{EOTC} stand for the concentration of

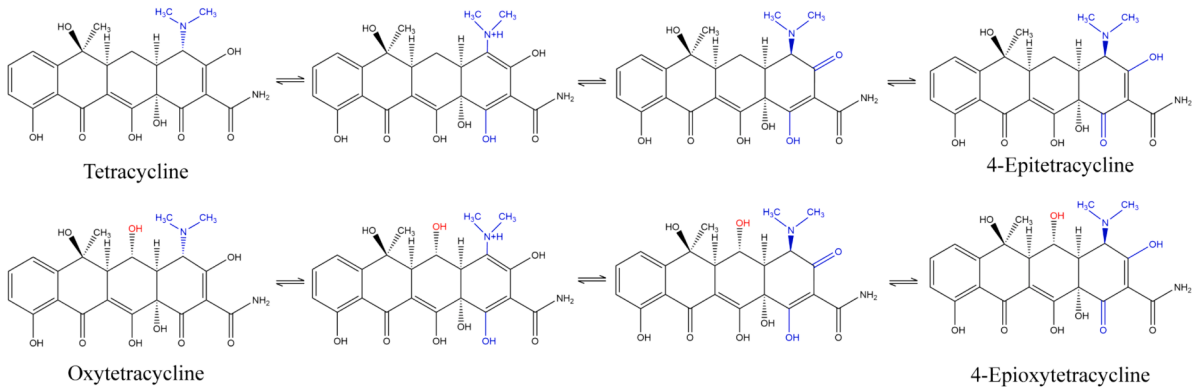

Fig. 1. The structures of four targeted compounds and interconversion process between them.

TC, OTC, ETC and EOTC, resepectively. When EF value is equal to 0.5, the enantiomer content is equal, and when EF value deviates from this value, there is enantiomer excess phenomenon.

2.5. Illumina sequencing of 16S rRNA genes

The high throughput sequencing was performed as follows. (1) DNA extraction: DNA of the tested soil was extracted using the Power Soil DNA kit (Mobio, USA) according to the manufacturer's methods for bacteria. Then the purity and concentration of the extracted DNA were tested using Nanodrop (Thermo Scientific, USA) and stored at −20 °C until further use. (2) PCR amplification: The V3-V4 regions of the 16S rRNA gene were amplified using the special primer of 338F and 806R with barcodes. Specific primers with barcode were synthesized by using the indicated sequencing regions. The materials and instruments used in PCR included TransGen AP221-02: TransStart Fastpfu DNA Polymerase and ABI GeneAmp&reg 9700. All samples were performed according to the formal experimental conditions. Triplicate PCR products for each sample were combined, purified with TIANquick Midi Purification Kit (Tiangen, China), then quantified with NanoDrop and sequenced on the Illumina Miseq platform at the Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.6. High throughput quantitative PCR

The extracted DNA was then used to detect ARGs and MGEs by WafergenSmartchip ultra-high throughput fluorescent quantitative PCR at the Differential Gene Technology Co., Ltd. (Anhui, China). A total of 96 pairs of primers were set up in this experiment, including 1 pair of 16S rRNA internal reference primers, 38 ARGs and 57 MGEs. Among them, ARGs consist of tetracycline resistance genes (TRGs) and multiple drug resistance genes (Multidrugs), and MGEs of four mechanisms include Insertional sequences, Plasmids, Integrases and Transposases. The names, classifications and primer sequence of all tested genes have been listed in the Table S1. Briefly, the PCR reaction mixture was added to the 54 (samples) × 96 (assays) mode, and then the qPCR reaction was performed on cycler. One NTC was set for each assay in each chip and three replicates per sample. The qPCR results were analyzed automatically using the instrumental qPCR software. $C_T = 31$ was set as the detection domain, and two or more of the three replicates with deviations < 20 %, and those satisfying the curve fitting analysis were judged as detection. All the samples of 16S rRNA with concentration of 10 ng·μL^{-1} were amplified, with no NTC amplified, which proved that the experimental effect was good and the results were credible. After the absolute quantification of the 16S rRNA gene, the absolute copy number of each gene can be calculated by followed:

$$\Delta CT = C_T(\text{gene}) - C_T(16S)$$

2.7. Data analysis

One-way analysis of variance was used to assess the difference between samples ($P < 0.05$, $P < 0.01$ and $P < 0.001$) and the data were organized using OriginPro 2021. The Spearman correlation analyses were conducted with the software of SPSS (version 23.0). QIIME software was employed to assess the richness (Chao1 index), diversity (Shannon index) of the bacteria community and the linear discriminant analysis effect size (LEfSe). In correlation network analysis, the absolute value of correlation coefficient is not less than 0.6, and P value is less than 0.05. The correlation network figure was drawn by Gephi (version 0.9.2).

3. Results

3.1. Degradation and transformation of tetracyclines and their 4-epimers

After 33d degradation, the residual concentrations of antibiotics and metabolites in all treatments are shown in Fig. 2a, b. In S1, S2, S3, S4 treatment, the residual amounts of TC, ETC, OTC and EOTC are 0.77, 1.22, 1.01 and 1.59 mg·kg^{-1}, and the residual amounts of their 4-epimers (ETC, TC, EOTC and OTC) are 0.78, 0.74, 0.51 and 1.24 mg·kg^{-1}, respectively. Total residue of antibiotics and metabolites in each treatment reduced to less than 3 mg·kg^{-1} after 33 days of degradation, but the residue was still greater than 1 mg·kg^{-1}. The residual amount of total antibiotics in the treatments with antibiotic addition was both lower than that in the treatments with corresponding metabolites as the only source of pollution. This difference is significant between the S3 and S4 (Fig. 1b, ANOVA, P < 0.05).

Enantiomer fractions (EFs) of TC and OTC in each treatment during the experiment were calculated (Fig. 2c, d). By day 3, the proportion of ETC in the TC contaminated group (S1) was about 34 %, while the proportion of TC in the ETC contaminated group (S2) was exactly the same (Fig. 2c). A similar situation occurred in the control of OTC and EOTC contaminated soil (Fig. 2d). After 33 days of cultivation, the EF_{TC} in the TC contaminated group tended to 0.5, but the EF_{TC} in the ETC treatment was still below 0.4. In addition, the EF_{TC} in CK was similar to that in the TC contaminated group. In the comparison of OTC and its epimer (Fig. 2d), OTC can be 40 % of the total residue in the EOTC contaminated treatment (S4) on the 3rd day, but EOTC accounted for only about a quarter of the total remains in the OTC contaminated treatment (S3). After 33 days, the EF_{OTC} in the OTC contamination group dropped from 0.75 to 0.67, but the EF_{OTC} in the EOTC contamination group only went up from 0.40 to 0.44.

3.2. Variation in the total abundance of ARGs and MGEs

Of the 95 ARGs/MGEs measured, 81 genes were effectively detected. We selected the top 50 abundant genes for further analysis based on the

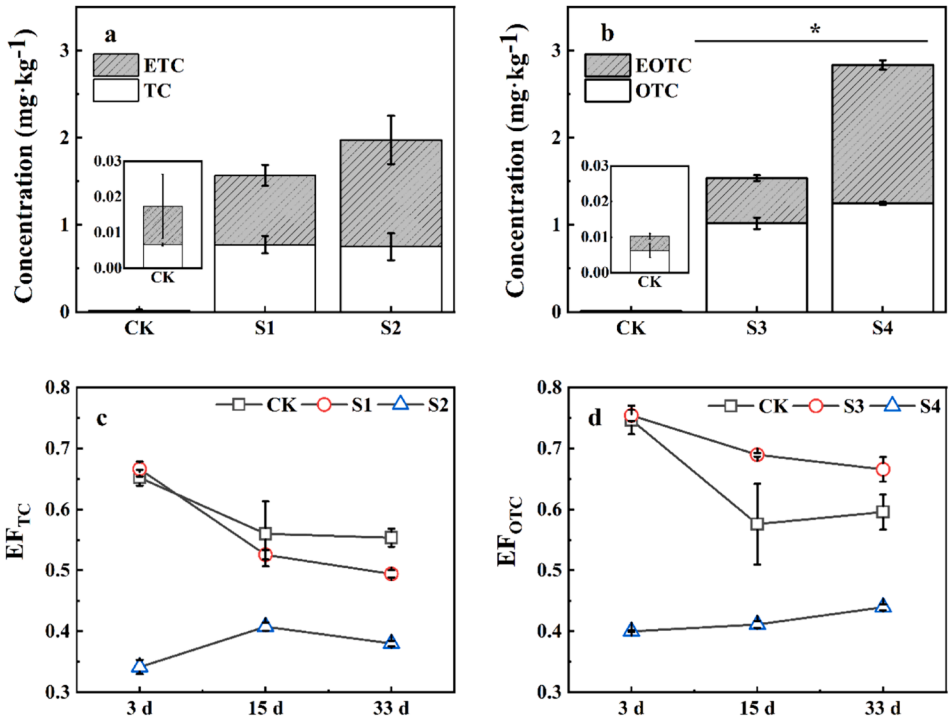

Fig. 2. Residue and interconversion of tetracycline antibiotics and their metabolites. a and b are the total amount of tetracycline antibiotics and their epimers remaining on the 33rd day of cultivation; c and d are the change of EFs over the cultivation period. CK is the natural soil; S1, S2, S3, and S4 represent the natural soil polluted only by TC, ETC, OTC and EOTC, respectively.

principle that the absolute copy number of each gene was above 10 in each treatment. Among these top 50 genes, the most detected genotype was Insertional sequence (17 species), followed by 14 TRGs, 8 Transposases, 6 Plasmids, 3 Multidrugs and 2 Integrases. The names and corresponding categories of the top 50 genes are listed in the Table S2.

In order to elucidate the impacts of targeted contaminants on ARGs and MGEs in soil, the total abundances of them in each treatment on the 3rd and 33rd day were analyzed and shown in the Fig. 3. On the third day of cultivation, the total abundance of ARGs and MGEs was significantly higher in TC and OTC supplemented soil compared to control soil (ANOVA, $P < 0.05$), and the abundance in TC treatment (S1) was the highest. However, there was no such significant rise in the soils with ETC and EOTC added compared to control soil. After 33 days of cultivation, the total abundance of ARGs and MGEs in the TC and OTC contaminated soils decreased significantly (ANOVA, $P < 0.05$), while the situation was opposite in the ETC and EOTC contaminated soils. On day 33, the total gene abundance in the ETC treatment (S2) was highest among the five treatments, significantly higher than that of control soil (ANOVA, $P < 0.01$). Except for the ETC contaminated soil, there was no significant difference in the abundance of the top 50 detected genes between other three contaminated treatments and the control group on the 33rd day.

3.3. Variation in the abundance of individual ARGs and MGEs

The heatmap of the abundances of top 50 detected genes in each treatment on the 3rd and 33rd day is shown in Fig. 4a and Fig. S1. It can be seen that on the 3rd day, except for Multidrugs, almost all kinds of ARGs and MGEs in S1 and S3 have obvious proliferation compared to the control group. Among them, the main growth in the TC contaminated soil were Insertional sequences and Plasmids, while TRGs, Transposases

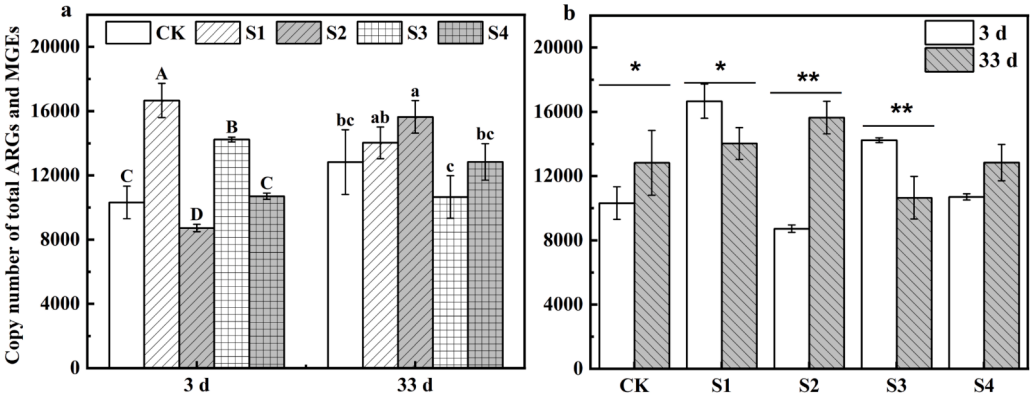

Fig. 3. The total abundance of the top 50 genes and their significance analysis. In the a, samples are classified by time, and the difference of abundances among different treatments at the same time point was analyzed. In the b, samples are classified according to the treatment, and the difference of abundances in the same treatment at the two time points was compared. CK is the natural soil; S1, S2, S3, and S4 represent the natural soil polluted only by TC, ETC, OTC and EOTC, respectively. * and ** stand for difference significance $P < 0.05$ and $P < 0.01$, respectively.

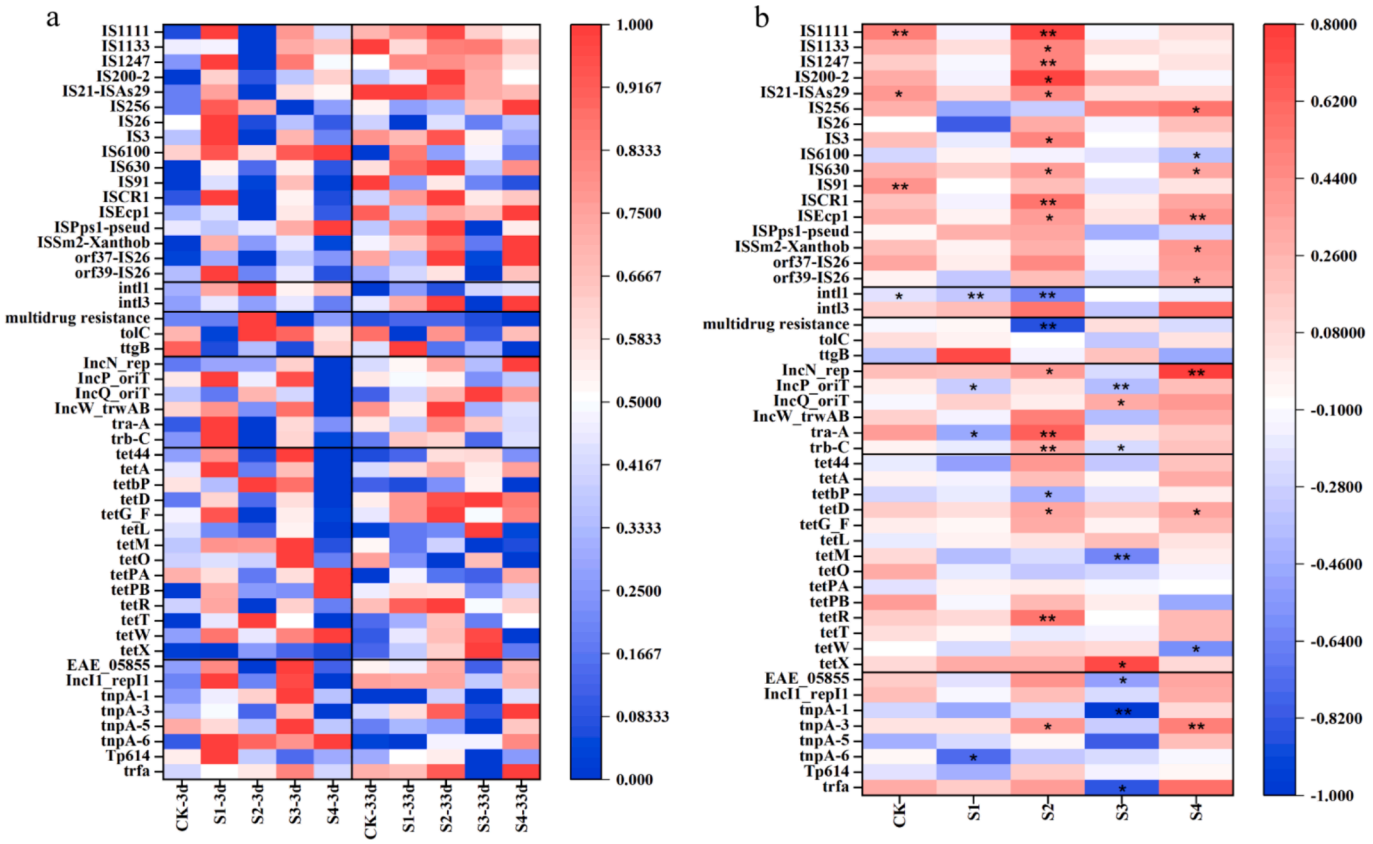

Fig. 4. Abundance and variation of individual ARGs and MGEs in the treatments. a is the heatmap of abundance of every top 50 genes in all treatments. b is the heatmap of abundance variation over time in the same treatment group. The data in b is obtained by subtracting the abundance on 3rd day from that on 33rd day and then dividing by the maximum abundance in the same gene. CK is the natural soil; S1, S2, S3, and S4 represent the natural soil polluted only by TC, ETC, OTC and EOTC, respectively. The significance of the difference between the data of 3rd day and 33rd day is indicated therein. * and ** stand for difference significance $P < 0.05$ and $P < 0.01$, respectively.

and few Insertional sequences multiplied the most in the OTC contaminated soil. To be specific, in S1, *IS1247*, *IS256*, *ISCR1* and *orf39-IS26* proliferated significantly compared to CK (ANOVA, $P < 0.05$), and *IS1111*, *trb-C*, *tra-A* and *intl1* extremely multiplied (ANOVA, $P < 0.01$). In S3, except for *IS1111* (ANOVA, $P < 0.01$), the ones that proliferated significantly were *IS1247*, *IncN_rep*, *tetM*, *tetO*, *tet44*, *tetT* and *tnpA-1* (ANOVA, $P < 0.05$). However, at the same time, less than one third of the top 50 detected genes in the ETC and EOTC contaminated groups proliferated slightly. *Intl1* showed significant proliferation in both ETC treatment (ANOVA, $P < 0.01$) and EOTC treatment (ANOVA, $P < 0.05$). In addition, only *tetT* and *multidrug resistance* significantly increased in ETC polluted soil.

On the 33rd day, more than half of the ARGs/MGEs in the TC and OTC treatments had lower abundance than in the concurrent control, while the situation was opposite in the ETC and EOTC contaminated groups (Fig. 4a). In order to compare the changes vividly, we calculated the difference and significance of gene abundances between the 3rd and 33rd days in each treatment (Fig. 4b). The abundances of ARGs/MGEs in S1 and S3 declined generally, with 4 and 6 detected genes declined significantly respectively (ANOVA, $P < 0.05$). The significantly decreased genes were mainly Plasmids and Transposases. Notably, *tetX* and *IncQ_oriT* showed a significant proliferation in S3 (ANOVA, $P < 0.05$) despite the general decline. However, in S2 and S4, 15 and 7 genes were significantly proliferated (ANOVA, $P < 0.05$), respectively. For ARGs, the abundance of *tetD* increased significantly in both S2 and S4 (ANOVA, $P < 0.05$), and *tetR* was particularly increased in S2 (ANOVA, $P < 0.01$). For MGEs, more than half of the Insertional sequences and Plasmids significantly proliferated in S2 (ANOVA, $P < 0.05$). In S4, among the 7 MGEs proliferated significantly (ANOVA, $P < 0.05$), *ISEcp1*, *IncN_rep and tnpA-3* proliferated the most (ANOVA, $P < 0.01$).

3.4. Effect of tetracyclines and their 4-epimers on soil microbial communities

According to the Alpha diversity analysis (Fig. S2), the Shannon index of all samples significantly increased after cultivation (ANOVA, $P < 0.05$), indicating that the environmental conditions of this experiment were conducive to the improvement of microbial diversity and suitable for microbial growth. However, there was no significant difference in α-diversity index between different treatment groups at the same time.

Soil bacterial community composition at the OTU level for the different treatments was analyzed using Venn diagrams (Fig. 5). On the 3rd day of cultivation (Fig. 5a and b), it can be seen that both the numbers of total OTUs and unique OTUs in different treatments follow the order of treatments TCs > treatments MTCs > CK, showing that TCs and MTCs promoted the increase of bacterial community diversity, and TCs had a stronger stimulative effect than MTCs. After one month of cultivation (Fig. 5c and d), both the numbers of total OTUs and unique OTUs in the MTCs polluted groups exceeded that in the corresponding TCs polluted groups, which is the manifestation of the mutual transformation of TCs and MTCs.

LEfSe analysis between all polluted groups and contemporaneous control group was conducted to further explore the effects of different TCs and their epimers on soil microbial community composition (Fig. S3 and S4). When the pollution occurred, the growth of many microbes was significantly affected in a short time. On the 3rd day (Fig. S3), the overall abundances of Proteobacteria in S1, S3 and S4 and *Paracoccus* in all the four comtaminated treatments were significantly lower than that in CK

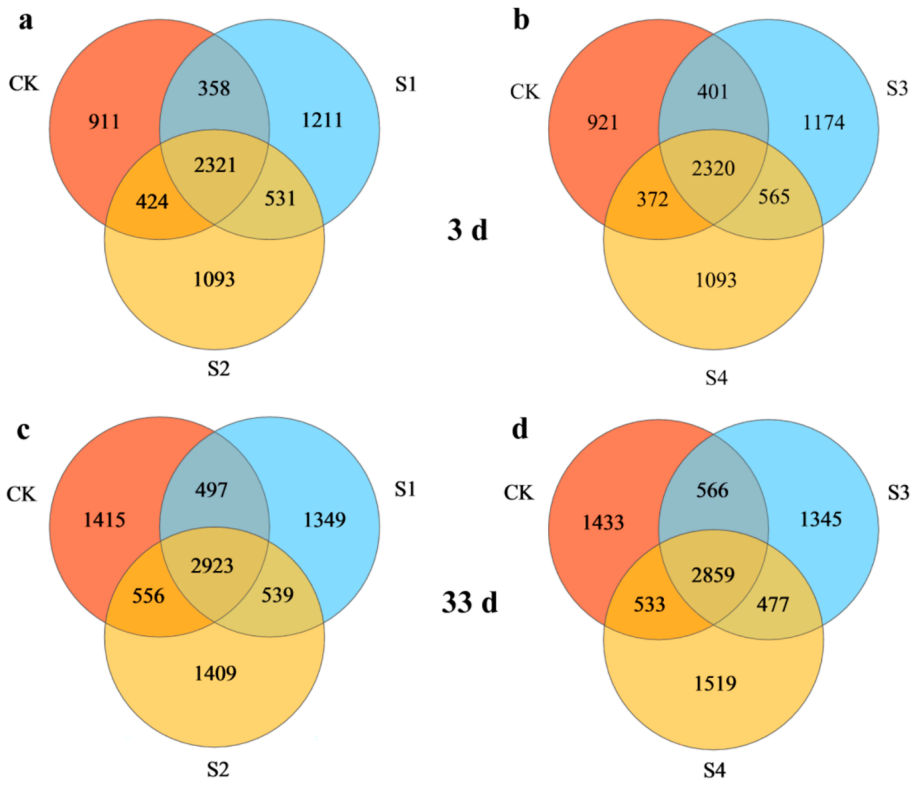

Fig. 5. Venn diagrams of species analysis at the OTU level of the different treatments. a and b are the data on the 3rd day, c and d represent the data on the 33rd day. CK is the natural soil; S1, S2, S3, and S4 represent the natural soil polluted only by TC, ETC, OTC and EOTC, respectively.

(ANOVA, $P < 0.05$). In S2 and S4, *Lysinibacillus* and *Microlunatus* were significantly promoted, while Bacteroidota, Bacteroidia and *Pedobacter* was significantly inhibited (ANOVA, $P < 0.05$). On the 33rd day (Fig. S4), it was found that there were fewer microbes with significant changes overall. However, these microbes shared more commonalities under different polluted treatments. For example, *Microbacterium* in S1 and S3, *Microlunatus* in S1 and S2, and *Solirubrobacter* in S1 and S4 all increased significantly (ANOVA, $P < 0.05$). They all belong to Actinobacteria. Besides, *Flavobacterium* and *Anaeromyxobacter* showed a significant decreasing trend in both S1 and S3 (ANOVA, $P < 0.05$). *Microvirga* had a significantly lower abundance in both S1 and S3 compared to that in the blank control (ANOVA, $P < 0.05$), but the situation was completely opposite in S2. The abundance of *Lysinibacillus*, which significantly increased in S2 and S4 on the 3rd day (ANOVA, $P < 0.05$), was significantly lower than that in CK on the 33rd day (ANOVA, $P < 0.05$). In addition, *Nitrospira* was significantly promoted in S2 by day 3 (ANOVA, $P < 0.05$), but showed a significant inhibition in S4 at the end of the study (ANOVA, $P < 0.05$).

3.5. Relationship of ARGs, MGEs and microorganisms

To explore the key bacteria affecting the proliferation and diffusion of ARGs, the correlation coefficients between the top 50 abundant genera and the 19 ARGs/MGEs with significant changes mentioned above was calculated, which is shown in the spearman correlation heatmap (Fig. 6). Correlation network analysis was attached to supplement the interactions between ARGs, MGEs and microbes in every treatment on the 3rd and 33rd day (Fig. 7). Details on the selected microbes are shown in Table S3. It can be found that TRGs, Insertional sequences and Plasmids were mainly positively correlated with the top 50 abundant microbes in soil. The microbes that interacted closely with ARGs and MGEs were mainly Actinobacteriota, Proteobacteria and Chloroflexi. On the contrary, Bacteroidata and Firmicutes were negatively correlated with most ARGs and MGEs. In addition, many MGEs share highly correlated microorganisms with TRGs. The Plasmid *trb-C* was the most abundant gene detected. It showed significant positive correlation with *Microlunatus*, *Solirubrobacter*, *Nocardioides*, Sericytochromatia and Rhizobiaceae ($P < 0.05$). *TetM*, *IS1111*, *IS1247* and *ISCR1* were very similar to the presence of *trb-C* and were likely to coexist in several of the microbes mentioned above. *Tra-A* and *IS91* showed significant positive correlation with *Peredibacter*, *Nitrospira* and Sericytochromatia ($P < 0.05$). *Multidrug resistance* only showed significant positive correlation with *Turicibacter* ($P < 0.05$) and *Paenibacillus* ($P < 0.01$). Except for *Multidrug resistance*, *tetL* ($P < 0.01$) and *tetT* ($P < 0.05$) significantly correlated with *Paenibacillus*, too. Other TRGs also shared common significantly positive correlation with some microbes. For example, *Microlunatus* with *tetL* and *tetX*, *Solirubrobacter* with *tetL* and *tet44, Streptomyces* with *tetX* and *tet44, norank_67-14* with *tetL, tet44* and *tetM,* and MB-a2-108 with *tetO* and *tetW*. Besides, *Marmoricola* showed significant positive correlation with *IS6100* and *IS256*. The Plasmid *IncN_rep* was significantly different from most of the other selected MGEs, showing an extremely positive correlation with *Ramlibacter* ($P < 0.001$).

4. Discussion

To our knowledge, this study reports the inaugural exploration about the ecotoxicity of metabolites of tetracyclines in soil. The respective addition of TC, OTC and their 4-epimers into soil as a single contaminant revealed the interconversion and overall degradation between tetracyclines and their 4-epimers. The effects of tetracycline parents and their 4-epimers on soil resistome were revealed by comparing the abundances of total and individual ARGs/MGEs in different treatments at the two time points. Meanwhile, the interferences of TCs and MTCs on soil microbial communities have also been analyzed. Ultimately, through the correlation analysis between ARGs/MGEs and microbes, the possible host bacteria of ARGs/MGEs in soil and the influence mechanism of TCs and MTCs on variation in soil resistome and bacterial community were

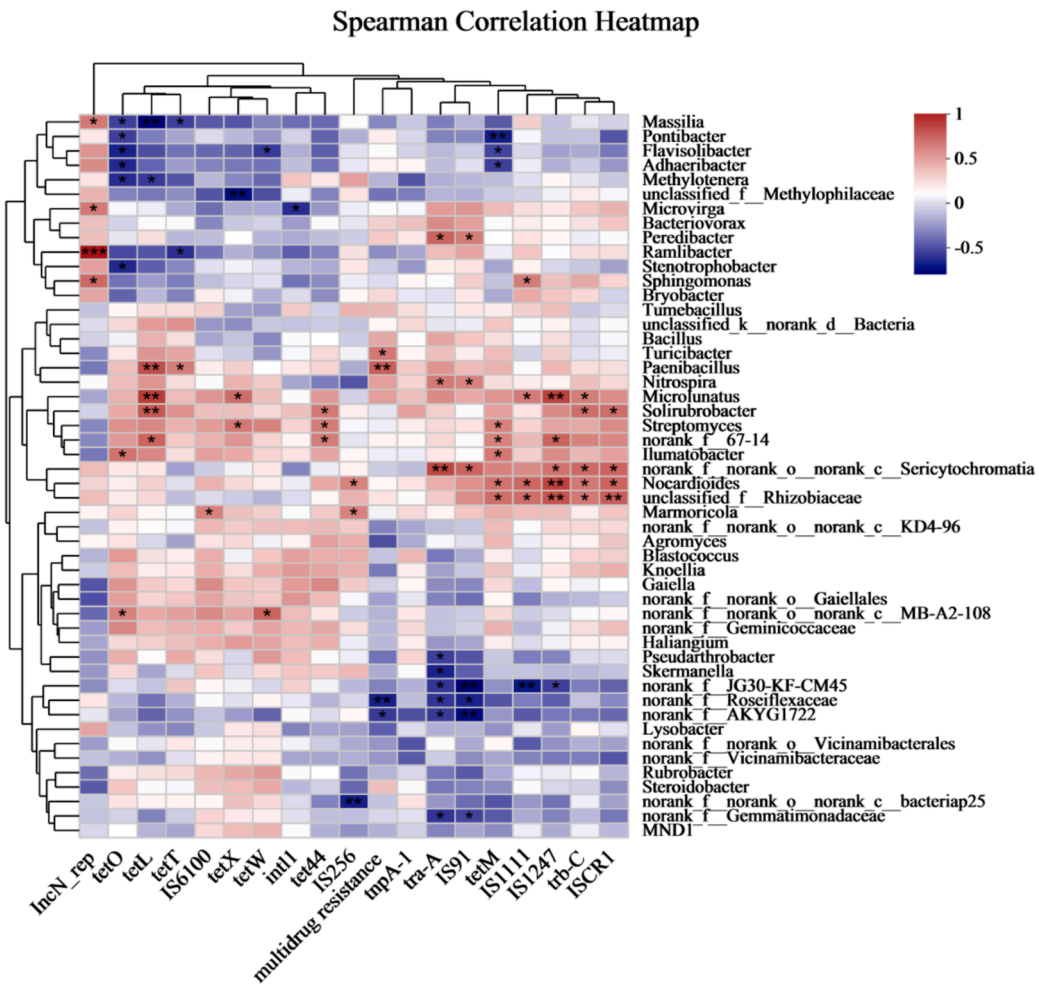

Fig. 6. Correlation analysis of ARGs, MGEs and microbes. CK is the natural soil; S1, S2, S3, and S4 represent the natural soil polluted only by TC, ETC, OTC and EOTC, respectively. *, ** and *** stand for significance $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

obtained.

The degradation rate of TCs and MTCs determines the persistence of their residues in the soil environment. Among the four contaminated treatments in this experiment, the lowest source residue was found in the TC treatment after 33 days of cultivation, followed by the OTC, ETC and EOTC treatments (Fig. 2a, b). The slower degradation rate of OTC than TC can partly explain the reason for OTC always being the highest residue among tetracycline antibiotics in soil (Zhao et al., 2023, Zeng et al., 2019). In the view of the residual level of the pollution source itself, it is worth noting that the residual amount of ETC and EOTC is much larger than that of TC and OTC ($P < 0.01$) after 33 days of cultivation (Fig. 2a, b), indicating that the degradation rate of 4-epimers of TCs is significantly lower than that of the tetracycline parent, which is a problem that few previous studies have paid attention to. In addition, it can be seen that in each treatment where antibiotic or metabolite was added alone, both the parent TC and its metabolite were detected simultaneously at the end of the experiment, which directly indicated the existence of mutual transformation in soil. Due to the slower degradation rate of ETC and EOTC and interconversion between TCs and MTCs, the total residual amount of parent and metabolite in the metabolite treatments (S2 and S4) was also higher than that in the parent treatments (S1 and S3), respectively. The difference was significant between OTC and EOTC treatment groups ($P < 0.05$). Moreover, the total residue of antibiotics and metabolites in each treatment was still greater than 1 mg·kg^{-1} at the end of cultivation, suggesting that 33 days is not enough to put soil with high levels of antibiotics out of risk. Overall, the results indicate that although ETC and EOTC are defined as metabolites, their degradation rates in soil are relatively slower than antibiotic parents (TCs), and they can be converted back to their parents under appropriate conditions, causing the long-term residue of TCs in soil, thus their impact on the soil environment and farmland ecosystem cannot be underestimated.

To clarify the conversion and proportion between TCs and their 4-epimers during cultivation, the EF value of TCs was analyzed (Fig. 2c, d). Firstly, on the third day, more than a quarter of the corresponding epimer appeared in all treatment groups, indicating that they can undergo a large degree of transformation in soil in a short time. Secondly, for TC and ETC, the EF value in CK eventually tends to 0.65, and basically didn't change in the second half of the experiment, so it can be considered as the final proportion of TC and ETC in the experimental environment. In the case of TC as a single pollution source in soil, EF_{TC} value continued to decline and the decline rate was faster in the early stage. Eventually, TC and ETC tend to be 1:1 in S1. However, when ETC is added to the soil as a single pollution source, although a large amount of TC can be generated in a short time, the highest EF_{TC} value in S3 is 0.40 on the 15th day. To sum up, the transformation from TC to ETC exceeds the opposite transform direction in both degree and speed. In the past, the research on the mutual conversion of TC and ETC was basically based on the aquatic environment. It has been proved that the conversion is more likely to occur under acidic conditions (Yuen and Sokoloski, 1977, Taylor et al., 1985), and this study demonstrates the existence of the interconversions between TC and 4-epimer in weakly alkaline soil environment and the extent of this interconversion. In the research of TCs and MTCs in three typical municipal wastewater

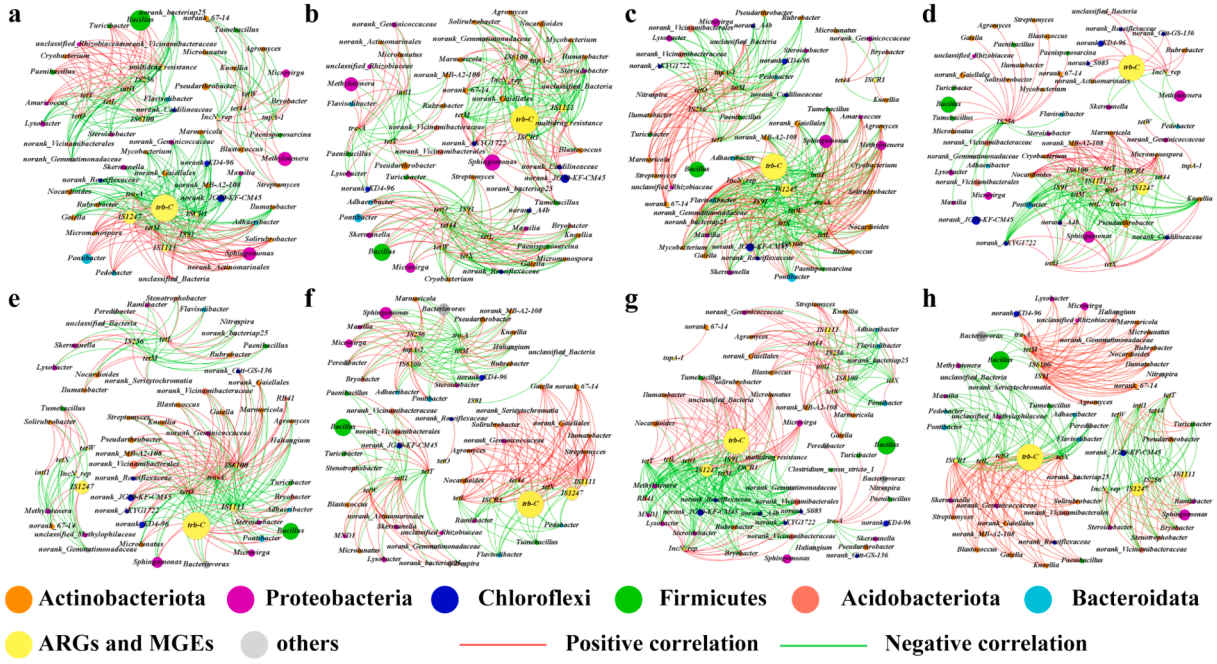

Fig. 7. Correlation network analysis of ARGs, MGEs and soil microbes, a, b, c and d represent the correlation analysis in TC, ETC, OTC and EOTC treatments on the 3rd day respectively, and e, f, g and h represent the correlation analysis in TC, ETC, OTC and EOTC treatments on the 33rd day respectively. The size of the nodes reflects the abundance, and the number and color of the edges reflect the interaction. The absolute value of correlation coefficient is not less than 0.6, and *P* value is less than 0.05.

treatment plants, ETC was found to be more easily adsorbed to sludge, with the ETC/TC ratio of 329 % (Zhong et al., 2022a). The strong adsorption capacity of ETC may be the reason for its slow degradation and transformation. In contrast, the conversion between OTC and EOTC was slower during the experiment. In CK, the EF_{OTC} eventually remained around 0.60, while the EF_{OTC} in S3 and S4 was not reached at all. Over a month, EF_{OTC} changed by only −0.08 in S3 and 0.03 in S4, and EOTC never accounted for more than a third in S3, while OTC accounted for more than 40 % in S4 on the 3rd day. This result shows that EOTC is more easily converted into OTC. There have been no previous studies on the conversion of EOTC to OTC. From the perspective of chemical structure, OTC can form hydrogen bonds between the hydroxyl group at C5 position and the dimethylamine group at C4 (Fig. 1), resulting in slower isomerization than TC. In addition, the adsorption capacity of EOTC is slightly lower than OTC (Zhong et al., 2022a), which may also lead to its easier conversion to OTC.

From the total abundance of 50 ARGs/MGEs (Fig. 3a), it can be intuitively seen that TC and OTC significantly promoted the proliferation of ARGs/MGEs on the third day ($P < 0.05$). However, there was no such significant rise in ETC and EOTC treatments (S2 and S4) compared to control soil in the short-term cultivation (Fig. 3a). Since the soil under short-term cultivation (3d) is still dominated by the set pollutant (Fig. 2c, d), the variation in the abundance of ARGs/MGEs in soil can be regarded as caused by the set pollutant itself. Therefore, it can be concluded that TC and OTC can cause the proliferation of more than 80 % of the tested ARGs/MGEs, whereas ETC and EOTC only caused a few proliferation indicating that the ecotoxicity of MTCs to soil resistome was negligible and much lower than that of TCs in a short time. The total abundance of ARGs in S2 treatment was lower than that in CK on day three, which is possibly because the production of TC in ETC treatment had certain bactericidal effect, thus the abundance of resistance genes decreased correspondingly. Studies have shown that the minimum inhibitory concentration (MIC) of ETC for *Shewanella* is much higher than that of TC, and the bactericidal activity of ETC was too weak to kill all three strains (*P. aeruginosa*, *Shewanella* and *E. coli* strains) even at 128 mg L^{-1} (Long et al., 2020). However, considering the strong adsorbability of ETC (Zhong et al., 2022a), the residue of ETC in the soil environment cannot be ignored for the future monitoring and risk assessment. In the study about the toxic effects of EOTC on Wistar rats, the results showed that EOTC had little effect on body weight or food and water consumption of rats, but EOTC can be retained and enriched in animals' organs and bodies and increase the potential risk of nephritis and hepatitis (Han et al., 2016). To sum up, the direct toxicity of MTCs might not be higher than TCs, but based on their adsorbability and accumulation and transformation, their harm cannot be ignored.

Over time, the total abundance of ARGs/MGEs in TC and OTC treatments significantly decreased, while the huge proliferation appeared in the ETC and EOTC contaminated groups after 33 days of cultivation (Fig. 3b). On the 33rd day, the abundance of ARGs/MGEs in the metabolite treatments (S2 and S4) exceeded that in the corresponding parent treatments (S1 and S3) (Fig. 3a). This can be attributed to the state and residual amount of pollutants present in soil. Since TCs and their 4-epimers can transform with each other, the variation on soil resistome in the late cultivation period can be seen as the result of the joint contamination of both the mother and the metabolites. After 33 days of cultivation, the concentrations of TC and OTC in S1 and S3 decreased from 10 $mg \cdot kg^{-1}$ to 0.77 and 1.01 $mg \cdot kg^{-1}$, while their concentrations in ETC and EOTC treated soils increased from almost 0 to 0.74 and 1.24 $mg \cdot kg^{-1}$ (Fig. 2a, b), respectively. Therefore, in S1 and S3, with the decrease of antibiotic concentration and the gradual adaptation of soil microorganisms to antibiotics, the abundance of ARGs/MGEs decreased significantly on the 33rd day ($P < 0.05$). However, in the metabolites treated soils, the gradual production of TCs stimulated the proliferation of ARGs/MGEs, thus the total abundances were relatively higher than those in the antibiotic parent treated groups at the end of experiment (Fig. 3a). Our results directly confirm the ecological risk of MTC residues in soil for the first time.

Proliferation effects of TCs and MTCs on the abundance of individual ARGs/MGEs in soil was also explored in this study. According to the results mentioned in 3.3, the MGEs of class Insertional sequence and Plasmid proliferated most significantly under the stimulation of TC, among which *trb-C*, *IS1247* and *IS1111* were the top 3 abundant MGEs.

IS1247 and *IS1111* have also been significantly promoted by OTC. Plasmid is the key to the horizontal gene transfer (HGT) of the ARGs which is a crucial mechanism through which bacteria acquire ARGs (Zhang et al., 2024b, Bai et al., 2024). A survey shows more than half of the ARGs-encoding plasmids carried mobility genes for mobilisation/conjugation (Yu et al., 2024). As a F plasmid *tra* operon gene, *trb-C* is a part of the type IV secretion system, and is essential to conjugative transfer (Maneewannakul et al., 1991, Shala-Lawrence et al., 2018). Therefore, the high abundance of *trb-C* in soil will promote the spread of other ARGs. As typical Insertional sequences, both *IS1247* and *IS1111* are compact MGEs, usually encoding only genes required for their transposition (Kanai et al., 2023). Insertional sequences can mediate various types of random genome rearrangements, and can lead to new capabilities for bacteria, such as evading the immune system and causing new infections (AlKindy and Guyeux, 2022). Besides, OTC significantly promoted the proliferation of *IncN_rep*, *tnpA-1* and TRGs. *IncN_rep* and *tnpA-1* can help ARGs transfer between nonpathogens and pathogens via MGEs upon HGT (Qi et al., 2023, Wang et al., 2022), and TRGs are clinically important ARGs, which confer bacterial resistance to all TCs (He et al., 2021, Bai et al., 2019). Most of the TRGs in this study showed similar characteristics, responding strongly to TC and OTC. Furthermore, MTCs have also been shown to promote the proliferation of some ARGs and MGEs. *Multidrug resistance* and *intl1* were the few that can be significantly promoted by ETC, and *intl1* was significantly promoted by EOTC as well. *Intl1* was detected in many researches before, and was recognized as a key factor related to the ARG dissemination (Agarwal et al., 2024, Yang et al., 2023, Xu et al., 2021, Xu et al., 2023a). Notably, the ARGs and MGEs that proliferated in the prophase of TC and OTC treatments could essentially correspond to that been proliferated later in their 4-epimer treatment groups (Fig. 3a). The ones consistent with this feature are mostly Insertional sequences and Plasmids. To be specific, *IS1111*, *IS1247*, *ICSR1*, *tra-A* and *trb-C,* all of which were significantly proliferated by TC on day 3, also showed significant proliferation in the ETC contaminated group by day 33. *IncN_rep* showed the same phenomenon in the comparison between the early OTC pollution group and the late EOTC pollution group. In addition, TRGs and Transposases showed the same trend in OTC and EOTC contaminated group, too. These phenomena indicated the proliferations of ARGs/MGEs in S2 and S4 were mostly caused by TCs and the production of TCs at later stage. To sum up, due to the conversion of metabolites to the antibiotic parent, MTCs could also lead to the proliferation of ARGs and MGEs, but the stimulation is lagged, implying the action mode of MTC residues on soil ecological risk.

Potential host bacteria carrying ARGs/MGEs in soil were discovered through the correlation analysis. As shown in Fig. 6, 17 ARGs/MGEs and 18 bacteria at the genus level showed significant positive correlations. *Nocardioides*, *unclassified_Rhizobiaceae*, *norank_Sericytochromatia, Microlunatus, Solirubrobacter* and *norank_67-14* were the most frequent hosts of ARGs/MGEs, carrying at least four or five ARGs/MGEs simultaneously. Most of these genera belong to the phylum Actinobacteria, which are in line with the correlation network analysis (Fig. 7) and many previous reports that Actinobacteria contains a large number of common host bacteria with resistance genes (Liu et al., 2023, Chen et al., 2018, Zhang et al., 2024a, Qi et al., 2023, Zhang et al., 2023a). *Nocardioides* and the *unclassified_Rhizobiaceae* were the common host of *IS1247*, *IS1111*, *ISCR1*, *trb-C* and *TetM*, indicating that these ARGs/MGEs could coexist in both bacteria. *Nocardioides* has been reported to be isolated from agriculture soils and activated sludge to degrade 17β-estradiol and herbicide *S*-triazin (Yu et al., 2007, Topp et al., 2000). Previous studies also showed that *Nocardioides*, *Solirubrobacter* and *norank_67-14* are host bacteria of ARGs (Yang et al., 2023, Song et al., 2024). *Microlunatus* showed strong correlation with Insertional sequences and TRGs (Fig. 6), indicating that *Microlunatus* could be the carrier of these genes and are therefore more resistant to TCs. From the extremely positive correlation ($P < 0.001$), it can be inferred that *Ramlibacter* is the host bacterium of Plasmid *IncN_rep*. Previous studies also reported that *Ramlibacter* is the most frequent host of ARGs encoding resistance to multiple antibiotics and can degrade organic pollutants such as herbicide linuron, 2,4-dichlorophenoxyacetic acid and polychlorinated biphenyl (Song et al., 2024, Lerner et al., 2020, Sul et al., 2009, Cupples and Sims, 2007). In addition, *IncN_rep* was significantly correlated with most of the ARGs (Qi et al., 2023). Therefore, the spread of *IncN_rep* and *Ramlibacter* could raise the risk of ARGs proliferating and spreading.

Bacteria are carriers and transmitters of ARGs/MGEs and are the key of contaminant degradation. The metabolic interactions dominated by antibiotic-resistant bacteria might intensify the transfer of ARGs (Xu et al., 2023b, Song et al., 2024). It can be seen from the Fig.S3 that MTCs caused more significant changes than TCs did, and there are more bacteria with common changes on the 3rd day (Fig. S3). The ability to proliferate significantly under conditions of TCs or MTCs contamination is a necessary condition for microbes to be potential degradation bacteria of them. In this study, *Lysinibacillus* showed significant proliferation in ETC and EOTC treatments on the 3rd day (Fig. S3), indicating that it may have potential degradation ability to MTCs. A previous study showed that the laccase derived from *Lysinibacillus* had promising removal efficiency towards TCs in the presence of 2′-Azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) by catalyzing ABTS to $ABTS^{+\cdot}$ and attacking the amino groups on TCs (Ouyang et al., 2022). Another study found that two strains of *Lysinibacillus* isolated from soil were resistant to TCs and did not carry ARGs, and they are probiotics that can promote the growth of beneficial intestinal microorganisms in mice (Zeng et al., 2023). *Microbacterium* has been shown to degrade enrofloxacin, nitrogen and phosphorus and has been proved to be a heavy metal-detoxifying Actinobacteriota (Zhang et al., 2023b, Wu et al., 2023). In this study, *Microbacterium* proliferated significantly in S1 and S3 on day 33, but not shown in correlation network (Fig. 7), meaning its potential to degrade TCs or MTCs. *Microlunatus* showed significant proliferation in ETC/EOTC treatments on the 3rd day and in TC/OTC treatments on the 33rd day. *Solirubrobacter* also proliferated significantly in S1 and S4 on day 33. As Actinobacteriota, *Solirubrobacter* has been reported as a potential host bacterium for ARGs in manure-fertilized soils and in phytoremediated cadmium and zinc contaminated soil (Zhang et al., 2021, Song et al., 2024). As potential host bacteria, the proliferation of these microbes in treatments of TCs and MTCs on the 33rd day indirectly reflects the possibility of proliferation and diffusion of ARGs/MGEs carried by them. In addition, TCs and MTCs exposure caused the growth of some bacteria to be inhibited. More significantly, Proteobacteria were inhibited in S1, S3 and S4 on the 3rd day, as did *Paracoccus* in all the four treatments. *Paracoccus* is a class of important aerobic bacteria with denitrifying function, which has been widely used in aquaculture, biological treatment of wastewater, degradation of organic pollutants and so on (Lu et al., 2014). The inhibition of beneficial microbes such as *Paracococcus* in soil is one of the adverse effects of TCs and MTCs on soil ecosystem.

5. Environmental implications

The degradation of parent pollutant is often considered as the complete removal of contamination. For tetracycline antibiotics, there are many transformation products especially 4-epimers that coexist with them in contaminated soils, leading to the uncertainty of environmental risks. This study focused on the interconversion between TCs and their 4-epimers and effects on soil resistome and bacterial community. Firstly, the results vertified that the degradation rate of TCs' 4-epimers is significantly slower than parent TCs, and TCs' 4-epimers could be transformed back to TCs, indicating that the long-term detection of TCs in soil is most likely due to the conversion of TCs' 4-epimers that are more difficult to degrade. Secondly, although TCs' 4-epimers themselves didn't cause ARGs/MGEs to proliferate, with the extension of time, the metabolites in soil were transformed back to the antibiotic parents, and ARGs/MGEs showed obvious proliferation, demonstrating that the residue of MTCs in soil would also lead to the proliferation of ARGs and

MGEs, thus their impact on the soil ecosystem cannot be ignored. Our findings expand the comprehensive understanding of complete removal of TCs in soil and the effects of MTCs on soil resistome.

6. Conclusions

The degradation rates of four selected pollutants are followed: TC > OTC > ETC > EOTC. It has been confirmed that TCs and their 4-epimers can be transformed into each other, and the transformation of TC is greater than that of OTC. In addition, the transformation from TC to ETC exceeds the opposite transform direction in both degree and speed, and EOTC is more easily converted into OTC than the other way. TC and OTC can induce the proliferation of more than 80 % of the ARGs/MGEs. The MGEs of class Insertional sequence and Plasmid can be significantly promoted by TC, of which *trb-C*, *IS1247* and *IS1111* were the top three abundant genes, and *tet* family genes are more easliy to be stimulated by OTC. ETC and EOTC only caused the proliferation of a few ARGs/MGEs, mainly including *intl1*, indicating that they are less ecologically toxic to soil microorganisms than TCs. However, it is worth noting that MTCs also showed significant proliferation of ARGs/MGEs after one month of degradation and transformation to their parents, and the proliferating ARGs/MGEs were consistent with those in the previous TCs treatment groups. This indicates that MTCs have a lagging influence on soil, and their effect on soil resistome mainly come from their transformed TCs. There were 17 ARGs/MGEs and 18 bacteria at the genus level showing significant positive correlations. *Nocardioides*, *unclassified_Rhizobiaceae*, *norank_Sericytochromatia, Microlunatus, Solirubrobacter* and *norank_67-14* were the most frequent hosts of ARGs, carrying at least four or five ARGs simultaneously, most of which belong to the phylum Actinobacteria. TCs and MTCs in soil both interfered with soil microbial community structure. *Lysinibacillus* was potential degradation bacteria of MTCs, and *Paracococcus* was inhibited by TCs and MTCs. The increased abundance of some host bacteria such as *Solirubrobacter* in soil would lead to the proliferation and spread of the resistance genes they carried. In summary, the results indicate that although ETC and EOTC are defined as metabolites, their degradation rates in soil are relatively slower than antibiotic parents (TCs), and they can be converted back to their parents under appropriate conditions, thus their impact on the soil environment and farmland ecosystem cannot be underestimated.

CRediT authorship contribution statement

Chenxi Lu: Writing – original draft, Methodology, Formal analysis. **Cheng Qin:** Data curation, Writing – review & editing. **Lixia Zhao:** Writing – review & editing, Methodology, Funding acquisition, Data curation, Conceptualization. **Huike Ye:** Writing – review & editing. **Mohan Bai:** Writing – review & editing. **Yang Sun:** Writing – review & editing. **Xiaojing Li:** Writing – review & editing. **Liping Weng:** Writing – review & editing, Supervision. **Yongtao Li:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108941.

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