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Responses of soil microbial communities to concentration gradients of antibiotic residues in typical greenhouse vegetable soils

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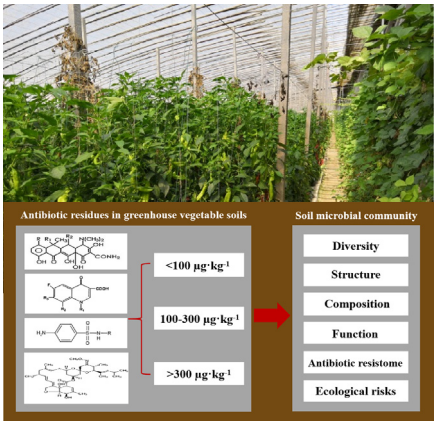
HIGHLIGHTS

- Residues of metabolites of tetracyclines in soil were almost equal to tetracyclines.
- Higher antibiotic residues reduced soil bacterial/fungal community diversity.
- Bacterial interactions first decrease and then increase as antibiotics increase in soil.
- Antibiotic resistome occurred in soil with antibiotic residues exceeding 300 μg·kg^{-1}.
- Weighted pollution index was used to assess mixed ecological risk of antibiotics.

GRAPHICAL ABSTRACT



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ABSTRACT

To explore the responses of soil microbial communities to concentration gradients of antibiotic residues in soil, 32 soil samples were collected from a typical greenhouse vegetable production base in Northern China in 2019. The total concentrations of 26 antibiotic residues in these soil samples was 83.24–4237.93 μg·kg^{-1}, of which metabolites of tetracyclines were 23.34–1798.80 μg·kg^{-1}. The total concentrations in 32 samples were clustered into three levels (L: <100 μg·kg^{-1}, M: 100–300 μg·kg^{-1}, H: >300 μg·kg^{-1}) to elucidate the impacts of antibiotic residues on the diversity, structure, composition, function and antibiotic resistome of soil microbial community. Results showed that higher concentration of antibiotic residues in soil was prone to decrease the diversity and shift the structure and composition of soil microbial community. Antibiotic resistome occurred in soils with antibiotic residues exceeding 300 μg·kg^{-1}. Interactions among soil bacteria followed the order of H > L > M, consistent with the relative abundances of mobile genetic elements. Bacteroidetes and Firmicutes were the top attributors impacting the profile of antibiotics in soil.

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According to weighted comprehensive pollution index of risk quotient, in 28.1 % of soil samples the residual antibiotics presented high ecological risk, whereas in the rest of soil samples the ecological risk is medium. The results will enrich the database and provide references for antibiotic contamination control in soils of the region and alike.

1. Introduction

Greenhouse vegetable production (GVP), which overcomes the adverse environmental conditions, is widely distributed in Northern China. At the end of 2016, the greenhouse area in China covered 13.15 million hectares, and in Tianjin municipality, where this study was carried out, it was 10.74 thousand hectares (NBS, 2017). Compared with conventional vegetable production, the demands for fertilizers and irrigations in GVP were higher. Based on the results of a survey, annually about 75–105 $t{\cdot}ha^{-1}{\cdot}year^{-1}$ manures were applied on the greenhouse vegetable farms, while 30–60 $t{\cdot}ha^{-1}$ on the open-field vegetable farms in northern China, while manure is the primary anthropogenic sources of antibiotics in agricultural soils (Zhang et al., 2022; Qiao et al., 2018). As so far, 44 antibiotics, mainly including 4 tetracyclines (TCs), 16 quinolones (QNs), 15 sulfonamides (SAs) and 9 macrolides (MLs), were detected in soil samples from China, and the antibiotic residual concentration was in the range of $\mu g{\cdot}kg^{-1}$-$mg{\cdot}kg^{-1}$ (Lyu et al., 2020). Manure-born antibiotics in soil can be partly degraded into less reactive but potentially more toxic metabolites, which may be converted to antibiotics reversely once environmental condition was suitable (Yang et al., 2012). For example, dehydration metabolites of TCs are prone to be generated under acidic conditions, and isomerization metabolites of TCs are produced under weak alkaline conditions (Li et al., 2012). Remarkably, 4-epitetracycline hydrochloride (ETC), 4-epichlortetracycline hydrochloride (ECTC) and 4-epioxytetracycline hydrochloride (EOTC) have been considered as residual markers of TCs by European Union and other countries and limits have been defined for environmental samples as well as in food (Commission Regulation, 2010). However, there is still a lack of studies on metabolites of antibiotics in GVP soils.

Excessive and aggressive land use in GVP bases would introduce antibiotics into soil and attenuate soil quality, health and function adversely, especially for the soil microbial community. Previous study had proved that antibiotic residues in soil did exert influence on the activity, composition, structure, function and antibiotic resistome of soil microbiome (Liu et al., 2018a; Liu et al., 2018b; Lin et al., 2016). For example, being exposed to antibiotics, the diversity and abundance of soil bacteria, and the use efficiency of total extractable N and microbial carbon decreased, while bioavailable C increased (Lucas et al., 2021). Sulfadiazine treatment was not only capable of altering the composition and function of soil bacterial community, but can also change the profile of soil metabolites (Qiu et al., 2021). Toth et al. (2011) reported that antibiotics at 100 and 200 $\mu g{\cdot}kg^{-1}$ could disrupt soil microbial processes, and the impact was antibiotic and process specific. Besides, fungal community is an important part of soil microbial community. There were interactions and co-occurrence between soil bacteria and fungi, and changes in the bacterial community could influence the fungal community of soil (De Menezes et al., 2017). Thus, the residual of antibiotics in soil would have impacts on soil fungal community. It was reported that the diversity of soil fungal community increased in treatment of tetracycline and decreased in treatment of sulfadiazine in soil bioelectrochemical system (Zhao et al., 2019). It is therefore necessary to explore the effects of antibiotic residues on soil fungal community. However, all these studies are based on laboratory simulation experiments, and it is unclear about the effects of concentration gradients of antibiotic residues on soil microbial community in actual farmland environment.

In addition, microbiome responses to antibiotic residues follow complex mechanisms (Ohore et al., 2022). Brandt et al. (2015) have given an overview of the possible effects of antibiotic residues on natural microbial communities, which emphasized tolerance, structure and function of microbial communities. Soil microbial communities are likely to resist interference or invasion of antibiotics to ensure the stability of its structure and function by shifting the composition of the dominant microflora, and the presence of antibiotic residues may also lead to the emergence of antibiotic resistome (Chen et al., 2017). Antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) would occur and proliferate due to antibiotic residues, which would have further impacts on agricultural soil ecosystem (Chen et al., 2022; Li et al., 2017; Heuer et al., 2011). Moreover, antibiotics and ARGs in soil could be introduced into food chain via absorption and bioaccumulation in vegetables and crops, thereby affecting human health (Hu et al., 2022; Mei et al., 2021; Zhu et al., 2019; Hu et al., 2010). What caught our attention was whether the presence of antibiotic resistance depends on the different concentration of antibiotic residues under natural condition. It is also worthwhile to further study whether there is only a simple positive or negative correlation between antibiotic resistance and antibiotic residues in soil. If not, at what concentration would a resistance gene develop.

The ecological risk of antibiotics to soil microorganisms deserves attention and this risk can be indicated with Risk quotient (RQ) approach (Menz et al., 2019; Agerstrand et al., 2015; Brandt et al., 2015). The potential ecological and human health risks of antibiotics in soil have been evaluated in Beijing-Tianjin-Hebei urban agglomeration, Yangtze River Delta, Pearl River Delta of China from north to south (Li et al., 2023; Hu et al., 2022; Gu et al., 2021; Sun et al., 2017). Based on technical guidance of European Commission (European Commission, 2003), only the potential ecological risks of TCs and QNs for soil organisms were calculated in most previous studies, while the potential risks of SAs and MLs were out of consideration. In fact, multiple antibiotics are often detected simultaneously in soil, and their effects on soil microorganisms are not independent toxicity, but mixed toxicity. Hence, it is urgent to assess the independent and mixed potential ecological risks of TCs, QNs, SAs and MLs comprehensively.

Therefore, the objectives of this study were to (1) explore the residual characteristics of antibiotics and their metabolites in actual typical greenhouse vegetable soils; (2) elucidate the responses of soil microbial communities to antibiotic residues at difference concentrations and reveal the interactions between antibiotics, ARGs and soil microbial community in natural environment; (3) assess the ecological risks of antibiotic residues on soil ecosystems. These findings will provide more insights into ecological risks of antibiotic residues in soil, which will benefit the development of strategies to soil pollution of antibiotics.

2. Materials and methods

2.1. Sample collection

A total of 32 topsoil samples (0–20 cm) were collected from typical GVP bases in Wuqing district, Tianjin municipality, Northern China in April 2019 (Fig. S1). The primary vegetables grown were eggplant, cucumber, green pepper, tomato, beans, et al. (Table S1). At each site, five subsamples were obtained to mix into a composite soil sample. Soil samples were immediately transported to the laboratory with ice bags, and divided into three portions. About 200 g was freeze-dried, homogenized, ground and sieved through a 0.25 mm sieve for antibiotic analysis. About 5 g fresh soil was preserved at −80 °C for DNA extraction and about 200 g was stored at −20 °C as backups.

2.2. Standards and reagents

On the basis of the previous research and application of antibiotic in livestock industry (Lyu et al., 2020; Zhang et al., 2015), 57 targeted

antibiotics were screened, which included 4 TCs, 7 metabolites of TCs (MTCs), 16 QNs, 22 SAs and 8 MLs (Table S2). All the antibiotics were obtained from Alta Scientific Co., Ltd. (Tianjin, China). Acetonitrile, formic acid and methanol (HPLC grade) were offered by Fisher Co. Inc. (USA). The PEP-2 cartridge (6 mL, 500 mg) that was used for the purification of soil samples was obtained from Agela Technologies Co. Ltd. (Tianjin, China).

2.3. Antibiotic extraction and analysis

The detailed procedures of antibiotic extraction and purification were described in our previous study (Text S1) (Pan et al., 2021). Briefly, five grams of soils were treated with mixed extraction solution containing phosphate buffer (27.20 g KH_2PO_4 and 1.30 mL H_3PO_4 per L), acetonitrile and Na_2EDTA in a 50-mL polypropylene centrifuge tube. The extracts were then purified with PEP-2 cartridge. Next, the eluents were concentrated to near dryness under a gentle flow of nitrogen gas at 25 °C and redissolved with 1:1 (v/v) methanol-water to 1.00 mL. The final extracts were filtered through nylon filters (0.22 μm) before further instrumental analysis.

LC-MS/MS (Exion LC, QTRAP 4500, AB Sciex, USA) was used for quantification of antibiotics. A C_{18} column (Kinetex, 100 × 2.1 mm, 2.6 μm, F5, 100 Å, Phenomenex, USA) was used for analyte separation. Ultrapure water with 0.10 % formic acid and acetonitrile were used as mobile phases A and B, respectively (Text S2). The electrospray ionization (ESI), positive ionization mode and multiple-reaction monitoring (MRM) were used for antibiotic determination (Text S2, Table S3). The limits of detection (S/N = 3) for antibiotics were in the range of 0.01–0.50 $\mu g \cdot kg^{-1}$ (Table S4). The average recoveries of antibiotics ranged from 32.29 % to 114.53 %, with the relative standard deviation (RSD) of <20 % (Table S4). In this paper, data for antibiotics with recoveries of <60 % were corrected by soil recoveries with similar spiked concentration (10 $\mu g \cdot kg^{-1}$).

2.4. Biological analysis

Soil bacterial and fungal diversity analysis was performed by Shanghai Majorbio Bio-pharm Technology (Shanghai, China) using an Illumina MiSeq platform (San Diego, CA, USA) (Text S3). Quantitative real-time PCR (qRT-PCR) of ARGs were performed using the Wafergen Smartchip Real-Time PCR System in Anhui Microanaly Gene Technologies CO., Limited (Text S4, Table S5).

2.5. Calculation of antibiotic ecological risks

Risk quotient (RQ) approach was applied to quantify risk of individual antibiotic residues on soil microbial communities (Text S5, Table S6) (Menz et al., 2019). Moreover, the weighted comprehensive pollution index was applied to estimate the mixed ecological risk of antibiotics (Eq. (1), Zhang, 2008):

$$RQ_{sum} = \Sigma RQ_i * P_i \quad (1)$$

where RQ_i was the RQ value of the component i and P_i was the ratio of component i to all components. The RQ were classified into four levels of risk: high risk (RQ > 1), medium risk (0.1 < RQ < 1), low risk (0.01 < RQ < 0.1) and no risk (RQ < 0.01) (European Commission, 2003).

2.6. Statistical analysis

Box chart and stack column were generated in Origin 9.0. One-way ANOVA, Spearman correlation and Principal component analysis (PCA) were conducted in SPSS 23.0. Venn and Bar diagram were plotted using the R package ggplot2. Redundancy analysis (RDA) was performed using Canoco 5.0. The visualization of networks was generated with Gephi-0.9.2. R package vegan was used for nonmetric multidimensional scaling (NMDS) ordination based on the weighted-unifrac distance and variation partitioning analysis (VPA).

3. Results and discussion

3.1. Distribution of antibiotics in greenhouse vegetable soils

Antibiotics were detected in all the 32 soil samples and the detection frequency of TCs, MTCs, QNs, SAs and MLs was 100 %, 100 %, 99.55 %, 67.71 % and 73.75 %, respectively, indicating widely-spread distribution of antibiotics in the GVP soils. The concentrations of the 26 antibiotics in the 32 soil samples are shown in Fig. 1. The total concentration range of the 26 antibiotic residues in soils was 83.24–4237.93 $\mu g \cdot kg^{-1}$ (n = 32), with a mean and medium value of 463.35 $\mu g \cdot kg^{-1}$ and 160.36 $\mu g \cdot kg^{-1}$, respectively. The concentrations were in the range of 27.24–2390.16 $\mu g \cdot kg^{-1}$ for 4 TCs, 23.34–1798.80 $\mu g \cdot kg^{-1}$ for 4 MTCs, 28.91–106.78 $\mu g \cdot kg^{-1}$ for 7 QNs, 0.20–11.85 $\mu g \cdot kg^{-1}$ for 6 SAs and 0.38–10.90 $\mu g \cdot kg^{-1}$ for 5 MLs. Compared with previous studies (Table S7), the residual of antibiotics in GVP soils of the present study was at a median level. The discrepancy in the residual concentration of antibiotics in different studies is mainly attributed to the differences in sampling time, sampling region, frequency and amount of fertilizer application, etc. Significant differences were observed in the residual of four classes of antibiotics in soil (One-way ANOVA, P < 0.01), and the mean level of antibiotics followed the order of 248.40 $\mu g \cdot kg^{-1}$ (TCs) > 40.91 $\mu g \cdot kg^{-1}$ (QNs) > 2.21 $\mu g \cdot kg^{-1}$ (MLs) > 1.29 $\mu g \cdot kg^{-1}$ (SAs). This may be related to the chemical properties (e.g., K_d, K_{ow} and water-solubility) of antibiotics. For example, it was found that the concentration of antibiotics was correlated to the K_d values positively (Pan et al., 2021; Hu et al., 2010). Coincidently, the K_d values of antibiotics were TCs (215–3910 $L \cdot kg^{-1}$) > QNs (100–2100 $L \cdot kg^{-1}$) > MLs (8.3–128 $L \cdot kg^{-1}$) > SAs (4.20–31.8 $L \cdot kg^{-1}$) (Accinelli et al., 2007; Gao and Pedersen, 2005; Gu and Karthikeyan, 2005; Rabølle and Spliid, 2000), which was exactly consistent with the orders of antibiotic residues in soil.

Notably, the residue of TCs metabolites (MTCs) in soil was rarely reported previously but detected in present study (Fig. 1c). The detection frequencies of ETC, EOTC, ECTC and Demeclocycline hydrochloride (DMCTC) were all 100 % in soil, with concentrations of 6.82–146.00, 5.70–626.92, 1.08–1235.06 and 9.42–54.00 $\mu g \cdot kg^{-1}$, respectively. Furthermore, the maximum concentration of individual TCs and MTCs was 1306.02 $\mu g \cdot kg^{-1}$ (Chlortetracycline, CTC), followed by 1235.06 $\mu g \cdot kg^{-1}$ (ECTC), 680.58 $\mu g \cdot kg^{-1}$ (Oxytetracycline, OTC) and 626.92 $\mu g \cdot kg^{-1}$ (EOTC). The ratios of MTCs to corresponding TCs were 78.57 %–125.13 %, and significant correlations were observed between MTCs and TCs (P < 0.01, r = 0.509–0.991). It was concluded that the concentration of MTCs was almost equivalent to TCs, indicating that equal attentions should be paid to MTCs as well as TCs.

The composition of antibiotic residues in soil was also illustrated in Fig. 1. It was clear that TCs, MTCs and QNs accounted for 54.38 %, 37.08 % and 8.79 % of total concentration of 26 antibiotic residues in soil, respectively. TCs and MTCs were dominant antibiotic residues in soil, which may be related to the widely application of TCs in industrial livestocks (Wei et al., 2019). To be specific, the rates of TCs, MTCs and QNs to total concentration of 26 antibiotics in 32 samples were 31.12–74.91 %, 7.89–47.23 % and 0.92–40.27 %, respectively, of which CTC (25.84 %), ECTC (19.31 %), OTC (11.46 %), DOX (Doxycycline, 11.43 %), EOTC (11.18 %), TC (Tetracycline, 4.64 %), ETC (3.38 %), DMCTC (3.21 %) and CIP (Ciprofloxacin, 3.07 %) with high detection frequency were the dominant pollutants in soil.

3.2. Response of soil microbial communities under the pressure of antibiotics

In order to explore the concentration effects of antibiotics on soil microbial communities in natural environment, based on the trigger value (100 $\mu g \cdot kg^{-1}$) of antibiotics that causes ecotoxicity effects on soil organisms set by VICH (Karci and Balcioglu, 2009), the total concentration of antibiotics in the 32 samples were clustered into three levels and named as low concentration level (L: <100 $\mu g \cdot kg^{-1}$, n = 11), median concentration level (M: 100–300 $\mu g \cdot kg^{-1}$, n = 11) and high concentration level

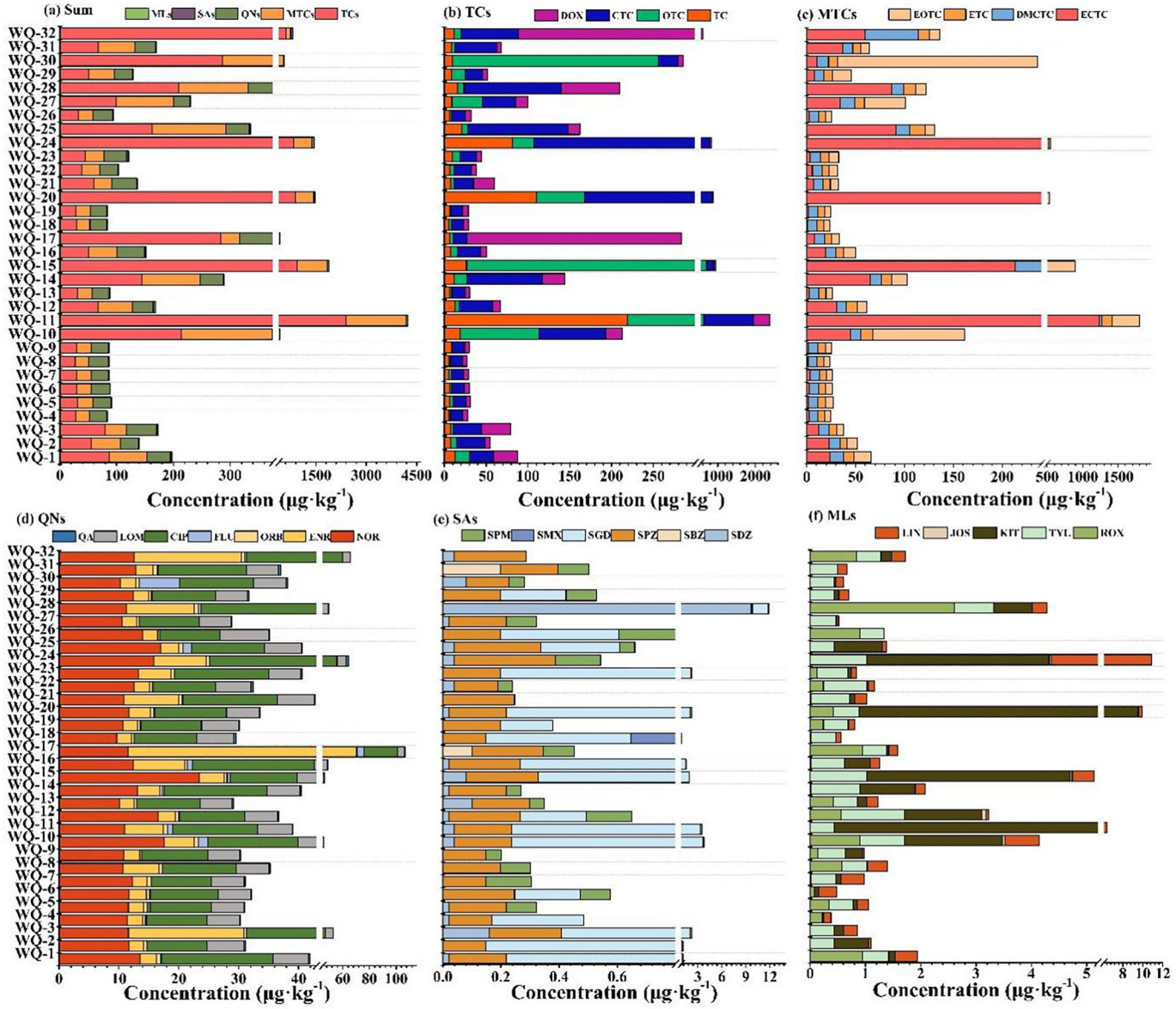

Fig. 1. The concentration of 26 antibiotics, tetracyclines (TCs), metabolites of TCs (MTCs), quinolones (QNs), sulfonamides (SAs) and macrolides (MLs) of 32 soil samples in GVP of Northern China (n = 32).

(H: >300 μg·kg^{-1}, n = 10), respectively. More specifically, the effects of different concentration levels of antibiotic residues on the diversity, structure and composition, function and antibiotic resistome of soil microbial communities were evaluated.

3.2.1. Variations of diversity in soil microbial communities

In present study, 35,854–74,768 and 30,228–60,189 high-quality sequences of bacterial and fungal communities were obtained, respectively, which were clustered into 3776 operational taxonomic units (OTUs) and 2483 OTUs correspondingly, basing 97 % similarity threshold for OTUs after eliminating low and short quality reads, replicates, singletons and chimeras. The coverage of each sample was above 96 %, indicating that the depth of sequencing was sufficient. In soil bacterial communities, the shared OTUs comprised 94.60 %, 93.95 % and 94.93 % of the total OTUs in L, M and H levels, respectively; and the number of unique OTUs was 14, 6, and 33 correspondingly (Fig. 2a). This indicated that the compositions of bacterial communities maybe similar in soils of L, M and H levels. However, it was reported that antibiotic residues at a concentration of 500 μg·L^{-1} decreased unique OTUs of bacterial communities in a constructed wetland mesocosm (Yan et al., 2017). In present study, 81.25 % of the total concentrations of 26 antibiotics were below 500 μg·kg^{-1}, which may be the main reason of the controversy. For soil fungal communities, the number of shared OTUs were 532; and the unique OTUs in soils of L, M and H levels accounted for 38.17 % (637), 31.59 % (496) and 19.53 % (184) of the total OTUs (Fig. 2b), respectively. This meant that discrepancy was likely to be observed in the compositions of fungal communities in soils of L, M and H levels and higher antibiotic residues decreased the numbers of unique OTUs of fungal communities.

The shifts of α-diversity in microbial community were investigated. According to the analysis of the results, it was found that H level significantly reduced the Shannon index of bacterial communities compared with L and M levels (One-way ANOVA, $P < 0.05$) (Fig. 2c). It indicated that higher concentrations of antibiotic residues in soil had inhibitions on the bacterial diversity, which was in line with the previous study (Uddin et al., 2019). It was reported that under the pressure of antibiotics, the diversity of bacterial community was suppressed in soil slurries, of which Shannon-Wiener and Chao1 index were lower than those in antibiotic-untreated ones (Dong et al., 2020). However, Chen et al. (2019) found that bacterial α-diversity increased in the cornfield soil where antibiotics were introduced by application of swine manure. This finding may be related to the presence of

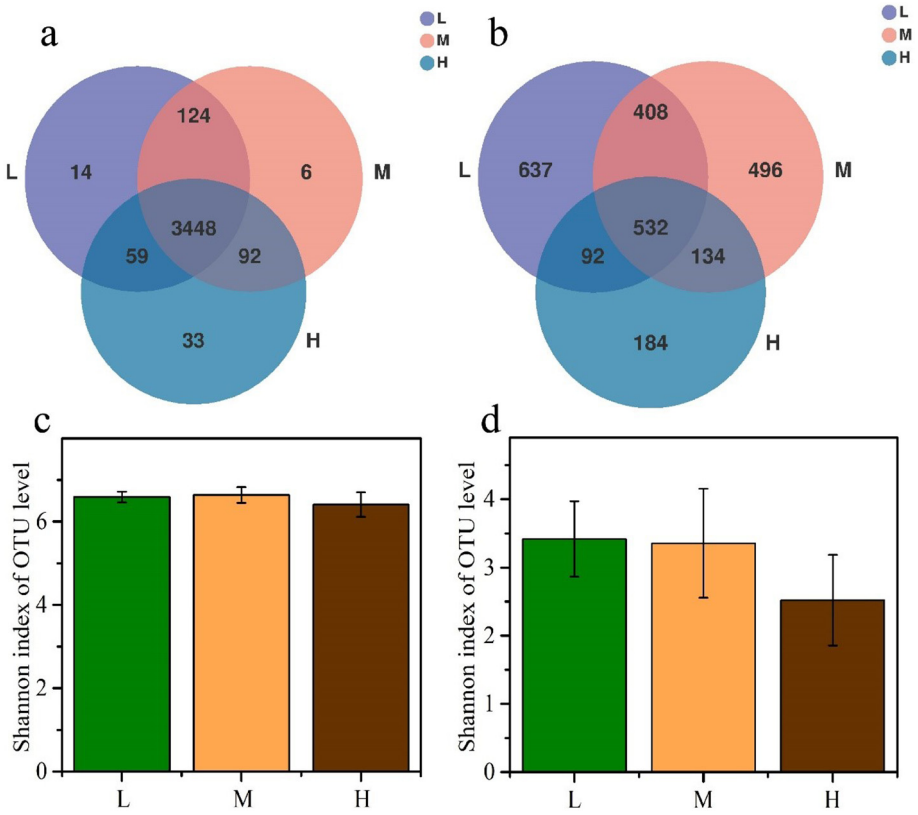

Fig. 2. Venn figures of (a) bacterial communities and (b) fungi communities with shared and unique OTUs in soils. Wilcoxon rank-sum test of Shannon index among L, M and H level of (c) bacterial communities and (d) fungi communities in soil. P values are from two-tailed Wilcoxon rank-sum test (*, $P \leq 0.05$; **, $P \leq 0.01$). L, low concentration level (n = 11), M, median concentration level (n = 11) and H, high concentration level (n = 10).

plenty of nutrients and organic matter in manure that affected the microbial community due to the evolutionary adaptations and physiological acclimation mechanisms (Urra et al., 2019; Schimel et al., 2007). Additionally, compared with L and M levels (Fig. 2d), higher concentration of antibiotic residues was inclined to affect the α-diversity of fungal communities, as well. Summarily, both bacterial and fungal diversity were decreased due to higher antibiotic residues in soil.

3.2.2. Shifts in the structure, composition and function of soil microbial community

According to the result of NMDS based on OTUs of soil samples, it was revealed that there were obvious differences in the structure of bacterial and fungal community among L, M and H levels (Fig. 3, Adonis test, $P < 0.001$). The clear separation was observed in the structure of bacterial community between L and H levels (Fig. 3a) and M level could be considered as the transition from L to H, which further illustrated that the interactions of bacteria among L, M and H were discrepant. Similar situations occurred in the structure of fungal community (Fig. 3b). This indicated that being exposed to antibiotics, the structures of both soil bacterial and fungal community shown obvious shift, which was in accordance with the previous study (Zhang et al., 2019). And the higher the level of antibiotic residues, the stronger the impacts on soil microbial community structure. This may be attributed to change of soil micro-ecological environment due to the presence of antibiotic residues, resulting in variations in the structures of microbial communities (Tang et al., 2015).

Proteobacteria (24.36–46.11 %), Actinobacteria (15.84–41.03 %), Chloroflexi (6.10–16.16 %) and Acidobacteria (3.02–21.33 %) were the most abundant phyla of bacterial community (Fig. S2a) found in the soils studied. With the concentration of antibiotics increased, the relative abundance of Actinobacteria decreased significantly (One-way ANOVA, $P < 0.05$), while the relative abundance of Bacteroidetes increased significantly (One-way ANOVA, $P < 0.05$). For example, the relative abundance of *Solirubrobacter* (Actinobacteria phylum) decreased significantly by 34.4 % in M level soils and 56.5 % in H level soils, and the relative abundance of *Microscillaceae* and *Chitinophagaceae* (Bacteroidetes phylum) increased by 28.5–43.6 % in M level and 41.5–160 % in H level soils, respectively. This may account for the variations of the relative abundance of Actinobacteria and Bacteroidetes. Similarly, manure-born antibiotics decreased the relative abundance of Actinobacteria and increased the relative abundance of Acidobacteria in field soils under various crop and manure managements (Chen et al., 2019). Actinobacteria is consisted of microbes that have the functions of recycling nutrient, decomposing complex organic matter and degrading xenobiotics (Alvarez et al., 2017), meaning that the metabolic function of microorganisms in GVP soils was inhibited by antibiotic residues. To elaborate the impacts of antibiotics on soil microbial communities, the composition of bacterial community at the genus level was also analyzed and displayed in Fig. S2b. The top 15 taxa for soil microbial community with significant differences among L, M and H levels were displayed in Fig. 3. Exactly, for soil bacterial community, as the concentration of antibiotics increased, the relative abundances of *S085*, *bacteriap25*, *MND1*, *Solirubrobacter*, *Actinobacteria*, *Gaiellales*, *KD4-96*, *Subgroup_6* decreased at the genus level (Fig. 3c), suggesting that antibiotics had inhibitory effect on these bacteria, which are also sensitive bacteria with significant differences under distinct precipitation conditions (Wang et al., 2022). However, compared with L level, *Agromyces*, *Alphaproteobacteria*, *Rhizobiaceae* and *Steroidobacter* increased in soils of M and H level significantly, indicating that these were potential resistant or degrading bacteria. Besides, it was reported that *Alphaproteobacteria* and *Rhizobiaceae* are related to nitrogen fixation and *Steroidobacter* is related to denitrification (Xun et al., 2021; Mcik et al., 2020). This indicated high levels of antibiotic residues could possibly accelerate the nitrogen cycling in soil. In general, with the increase of antibiotic residues in soil, the activity of most bacteria was inhibited, while the activity of some potential resistant or degrading bacteria was enhanced.

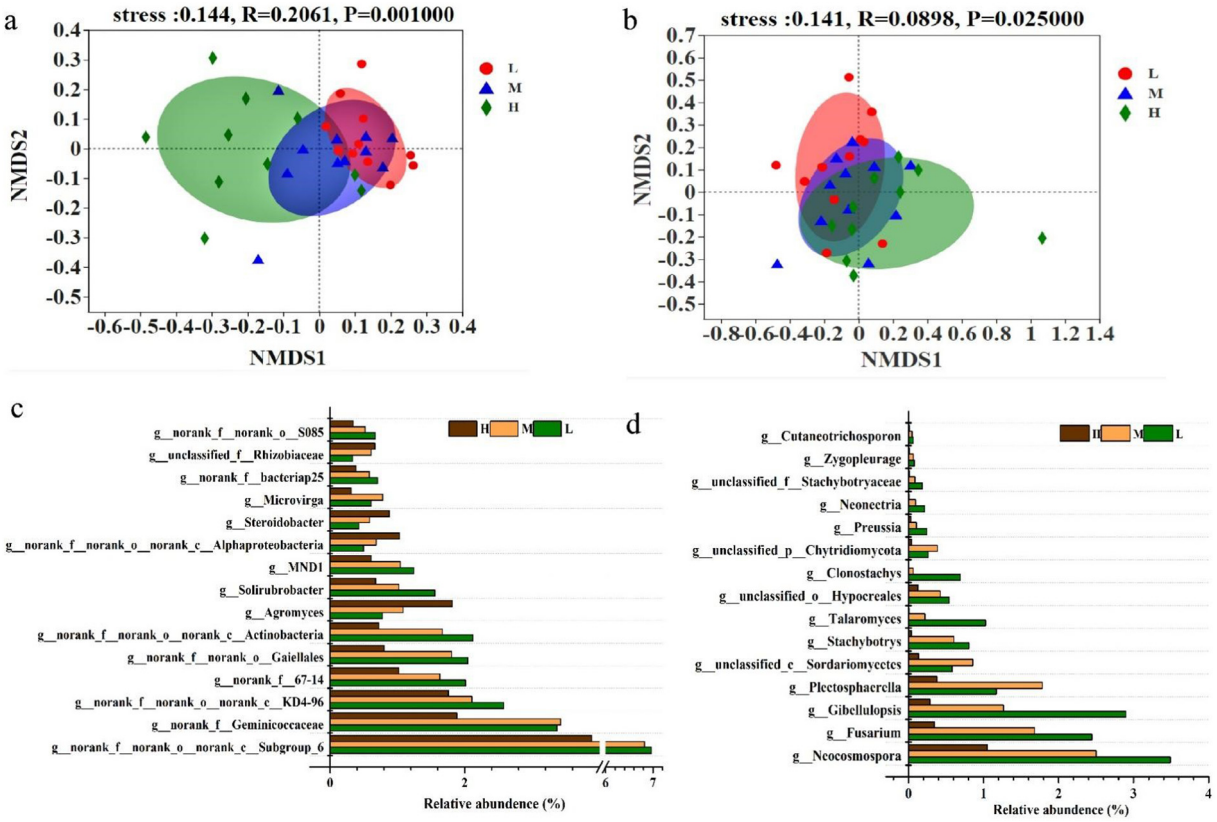

Fig. 3. NMDS plots of (a) the bacterial community structure and (b) fungi community structure among L, M and H level in soils. The 2D stress values are 0.144 and 0.141, respectively. Results of multiple groups comparative analysis of (c) bacterial communities and (d) fungal communities among L, M and H level. One-way ANOVA was used to evaluate the significance of differences between the indicated groups. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. The letters before bacterial or fungal names are abbreviations of different taxonomic levels (Kingdom-k, phylum-p, class-c, order-o, family-f, genus-g).

As for the fungal composition, Ascomycota (46.79–96.96 %), Mortierellomycota (1.27–32.68 %) and Basidiomycota (0.01–40.56 %) were the most dominant phyla in soil (Fig. S3a). There were no significant differences in soil fungal communities at the top 10 phyla among soils of L, M and H levels. This is attributed to that fungi have intact nuclei and are usually more resistant to environmental variations than bacteria. The composition of fungal community at the genus level was shown in Fig. S3b. At the genus level, with the increase of the concentration of antibiotic residues (Fig. 3d), the relative abundances of *Gibellulopsis* (0.01 %–14.66 %), *Stachybotrys* (0–2.93 %), *Talaromyces* (0–4.23 %), *Clonostachys* (0–5.48 %), *Neonectria* (0–0.49 %) and *Stachybotryaceae* (0–0.88 %) decreased by 90.13 %–99.76 %, and the relative abundances of other genera significantly decreased by 67.63 %–86.94 %. This was in accordance with the variations of fungal community diversity (Fig. 2d). It was suggested that high levels of antibiotic residues have irreversible inhibitory effects on some fungi, unlike bacteria which could be resistant to higher antibiotic residues. In conclusion, antibiotic residues significantly altered some bacterial and fungal community compositions in soil.

Furthermore, different levels of antibiotic residues could impact the function of soil microbial community (Fig. S4). It was found that soil bacterial community were mainly responsible for metabolism (69.6 %) mainly involving carbohydrate metabolism, amino acid metabolism and energy metabolism; environmental information processing (7.78 %) relating to membrane transport primarily; and genetic information processing (9.86 %) including translation, replication and repairment, which was in accordance with the previous study (Zhao et al., 2021). It was attested that it was beneficial for the degradation of organic macromolecules into easily degradable substances due to the high relative abundance of metabolism pathways (Zhao et al., 2021). Notably, the bacterial genus with the function of drug resistance took proportions of 1.06 %. Compared with soils of L level, antibiotic residues at M and H levels improved metabolic functions of cofactors and vitamins, nucleotide metabolism, glycan biosynthesis and metabolism, and suppressed amino acid metabolism, xenobiotics biodegradation and metabolism, and membrane transport, significantly (One-way ANOVA, $P < 0.001$). The prevalence of metabolism of cofactors and vitamins would accelerate carboxylation reactions, fatty acid metabolism and amino acid catabolism, of which vitamins can be served as cofactors of enzymes, which play a crucial part in maintaining metabolic homeostasis (Petri et al., 2019). The inhibition of amino acid metabolism implies that the microbial growth and metabolism would be restrained as well, which acted as energy and carbon sources for microorganisms (Lopez-Gonzalez et al., 2015). It was found that high concentrations of SDZ suppressed the function of DNA replication, metabolism of terpenoids and polyketides, amino acid metabolism and lipid metabolism remarkably and promoted carbohydrate metabolism (Qiu et al., 2021). Regardless, the metabolic functions of soil microbial community would not alter tremendously compared with the original soil (Dong et al., 2020). Despite the fact of the accuracy of PICRust functional prediction over 80 %, it was scarce to conclude that the ture situation of the microbial community in soil. More researches are needed to determine the roles of bacteria and fungi in soil using metagenomic and macrotranscriptome sequencing.

3.2.3. Impacts on the abundance of antibiotic resistome

Totally, 19 of 24 targeted ARGs were detected in the 32 soil samples, with the detected number of ARGs varied from 11 to 19. The relative abundance of detected ARGs were in the range of 1.01×10^{-7}-2.31×10^{-1} copies/16S rRNA copies. The detected ARGs mainly involved 4 resistance mechanisms: efflux pump, cellular protection, integrase and transposase. Of which, tetracycline resistance genes (*tet*, 33.01 %), sulfonamide resistance genes (*sul*, 36.39 %), and mobile genetic elements (MGEs, 29.21 %) were the dominant ARGs. The detected number of ARGs followed the order of H > L > M, and the relative abundance of ARGs was H > M > L. There were significant differences in the relative abundance of *sul* and *tet* among soils of L, M and H levels (One-way ANOVA, $P < 0.05$), and the

relative abundance of *sul* and *tet* in soils of H level (mean: *sul*: 4.35×10^{-2} copies/16S rRNA copies, *tet*: 3.05×10^{-2} copies/16S rRNA copies) was higher than those of M (mean: *sul*: 5.15×10^{-3} copies/16S rRNA copies, *tet*: 1.48×10^{-2} copies/16S rRNA copies) and L (mean: *sul*: 3.62×10^{-3} copies/16S rRNA copies, *tet*: 1.38×10^{-3} copies/16S rRNA copies) level. It was demonstrated that with the increase of antibiotic residues, the relative abundance of *sul* and *tet* increased, indicating that antibiotic resistance had occurred at the higher levels of antibiotic residues in soil. It was in accordance with previous studies that the relative abundance of ARGs were significantly correlated with the residual concentrations of antibiotics (Zhang et al., 2022; Zhu et al., 2013). Notably, it was found that the relative abundance of MGEs followed the orders: H > L > M (One-way ANOVA, $P = 0.055$).

PCA (principal component analysis) further showed a strong clustering of ARGs profiles according to the classification of antibiotic residual levels in soil (Fig. 4). Antibiotic resistome in soils of H level were significantly separated from that in soils of L and M level along PC1, which accounted for 55.61 % of the total variables. This indicated that the residual concentration of antibiotics was the dominant factor for shaping the ARGs profiles in soil. Besides, Spearman correlation analysis demonstrated that the concentration of 5 classes of antibiotics (TCs, MTCs, QNs, SAs and MLs) were correlated to the relative abundances of *sul* and *tet* positively ($P < 0.05$, $r = 0.493–0.805$) (Supplementary data). In addition, the relative abundance of MGEs was related to the relative abundance of *sul* and *tet* positively ($P < 0.01$, $r = 0.385–0.397$), suggesting that *sul* and *tet* may spread to other soil organisms via MGEs. Positive correlations were also observed among *sul*, *tet* and *MLSB* (Macrolide-lincosamide-streptogramin B) ($P < 0.01$, $r = 0.585–0.764$), demonstrating cross-resistance or co-resistance would emerge in antibiotic contaminated soils.

3.2.4. Interactions among antibiotics, ARGs and microbial community in soil

Network node diagram was analyzed to illustrate the changes of interspecific relationships of soil bacterial communities under different levels of antibiotic selection pressure (Fig. 5a–c). Interestingly, it was revealed that the interactions among soil bacteria at the three levels followed the order of H (Fig. 5c) > L (Fig. 5a) > M (Fig. 5b). This indicated that the activity intensity of soil bacterial community was not simply positively or negatively correlated with antibiotic concentration. Generally speaking, bacterial activities in soil would be weakened initially when antibiotics is introduced into soil. Within a certain concentration range, the higher the concentration, the stronger the inhibition. As the concentration rises further, the antibiotic resistance emerges once soil bacteria adapt to antibiotic residues, and the activities of soil bacteria would be strengthened. Videlicet, in the study, antibiotic residues in the L and M level groups exhibited adverse impacts on soil bacterial activities, and antibiotic resistance didn't emerge or was not strong enough. In the H level group, antibiotic resistance occurred and activated the activities of soil bacteria. The result is confirmed by the consistency of the detected number of ARGs and relative abundances of MGEs in the three groups (H > L > M).

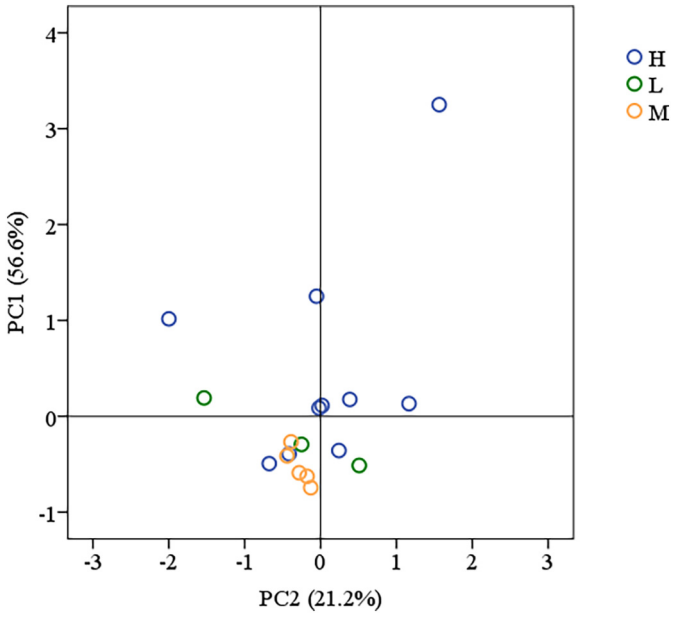

Fig. 4. The principal component analysis (PCA) of the relative abundance of ARGs in L, M and H level. The explanatory of the total variables was 77.8 %, of which the first axis explained 56.6 % and the second axis explained 21.2 % of the total variables.

Network analysis further verified that the relative abundance of ARGs in soil increased due to the increase of antibiotic residues (Fig. 5d–f). Compared with the L level (Fig. 5d), the number of relations between bacteria and ARGs in M and H level were all enhanced (Fig. 5e, f). What caught our attentionwas the question at what concentration of antibiotic residues the emergence of ARGs in soil would be triggered. It was obvious that when the total concentrations of antibiotic residues exceeded 300 $\mu g \cdot kg^{-1}$, the antibiotic resistance occurred. But it was difficult to determine whether the inflection point of antibiotic residues inducing proliferation and diffusion of antibiotic resistance is 100–300 or below 100 $\mu g \cdot kg^{-1}$. Further researches are needed to find out the accurate inflection point of emerging antibiotic resistance.

With the emergence of antibiotic resistance, antibiotic resistance bacteria (ARB) and ARGs host bacteria occurred. ARGs potential host bacteria, e.g. *Lysobacter*, *Pseudomonas*, *Flavobacterium*, *Turicibacter*, *Pedobacter*, *Cellvibrio*, *Thermomonas*, *Solibacillus*, *Acinetobacter* and *Aquamicrobium*, were screened according to previous study (Han et al., 2018). The results showed that the relative abundances of *Thermomonas* and *Aquamicrobium* in soils of H level were significantly higher than those of M and L level (One-way ANOVA, $P < 0.05$). The relative abundance of *Pseudomonas*, *Flavobacterium*, *Turicibacter*, *Pedobacter*, *Solibacillus*, *Acinetobacter* and *Aquamicrobium* had positive relations with the relative abundance of *tet* ($P < 0.05$, $r = 0.388–0.783$), which could be potential host of *tetG-01*, *tetG-02*, *tetL-02* and *tetM-01*. Similarly, *Pseudomonas*, *Pedobacter*, *Cellvibrio*, *Thermomonas* and *Aquamicrobium* were the potential host of *sul1* and *sul2* ($P < 0.05$, $r = 0.383–0.920$). In addition, spearman correlation analysis showed that *Agromyces*, *Alphaproteobacteria* and *Rhizobiaceae* were all correlated with the relative abundances of *sul1*, *sul2*, *tetA-01*, *tetA-02*, *tetD-01*, *tetD-02*, *tetG-01*, *tetG-02*, *tetL-01*, *tetL-02* and *tetM-01* positively ($P < 0.05$, $r = 0.553–0.712$). *Steroidobacter* was correlated with the relative abundance of *tetG-01* and *tetG-02* ($P < 0.05$, $r = 0.446–0.449$). *Rhizobiaceae* was correlated with the relative abundance of *tetO-01* and *tetR-02* ($P < 0.05$, $r = 0.449–0.459$). *Agromyces* was correlated with the relative abundance of *tetR-02* ($P < 0.05$, $r = 0.420$). These suggested that *Agromyces*, *Alphaproteobacteria*, *Rhizobiaceae* and *Steroidobacter* were the host bacteria of ARGs, consistent with the results shown in Fig. 3c.

The qualitative correlations among antibiotics, ARGs and soil microbial community compositions (major phyla) were taken into account using redundancy analysis (RDA). RDA showed that the relative abundances of Bacteroidetes and Firmicutes were the major attributors impacting the distribution of ARGs in soil, which explained 64.33 % of the total variance (Fig. 6a). The relative abundance of Bacteroidetes was correlated to the concentration of 5 classes of antibiotics ($P < 0.05$, $r = 0.535–0.697$), and the relative abundance of MGEs (*intI-1*, $P < 0.05$, $r = 0.406$), *tet* (*tetA-02*, *tetL-02*, *tetM-01*, *tetG-01* and *tetG-02*, $P < 0.01$, $r = 0.535–0.697$) and *sul* (*sul1* and *sul2*, $P < 0.01$, $r = 0.683–0.688$). The relative abundance of Firmicutes was related to the concentration of MTCs ($P < 0.05$, $r = 0.364$), and the relative abundance of *sul* (*sul2*, $P < 0.05$, $r = 0.405$), and *tet* (*tetA-02*, *tetL-02*, *tetM-01*, *tetG-01*, *tetO-01*, and *tetG-02*, $P < 0.05$, $r = 0.362–0.635$) and *tetR-03* ($P < 0.05$, $r = -0.900$). However, there were no significant relations between antibiotics and fungal community (not shown), which is consistent with the results obtained in Section 3.2.2. On the other hand, variation partitioning analysis (VPA) was conducted to clarify the relative attributions of bacterial and fungal community to the antibiotic residues in soil. In accordance with the RDA analysis, the VPA shown

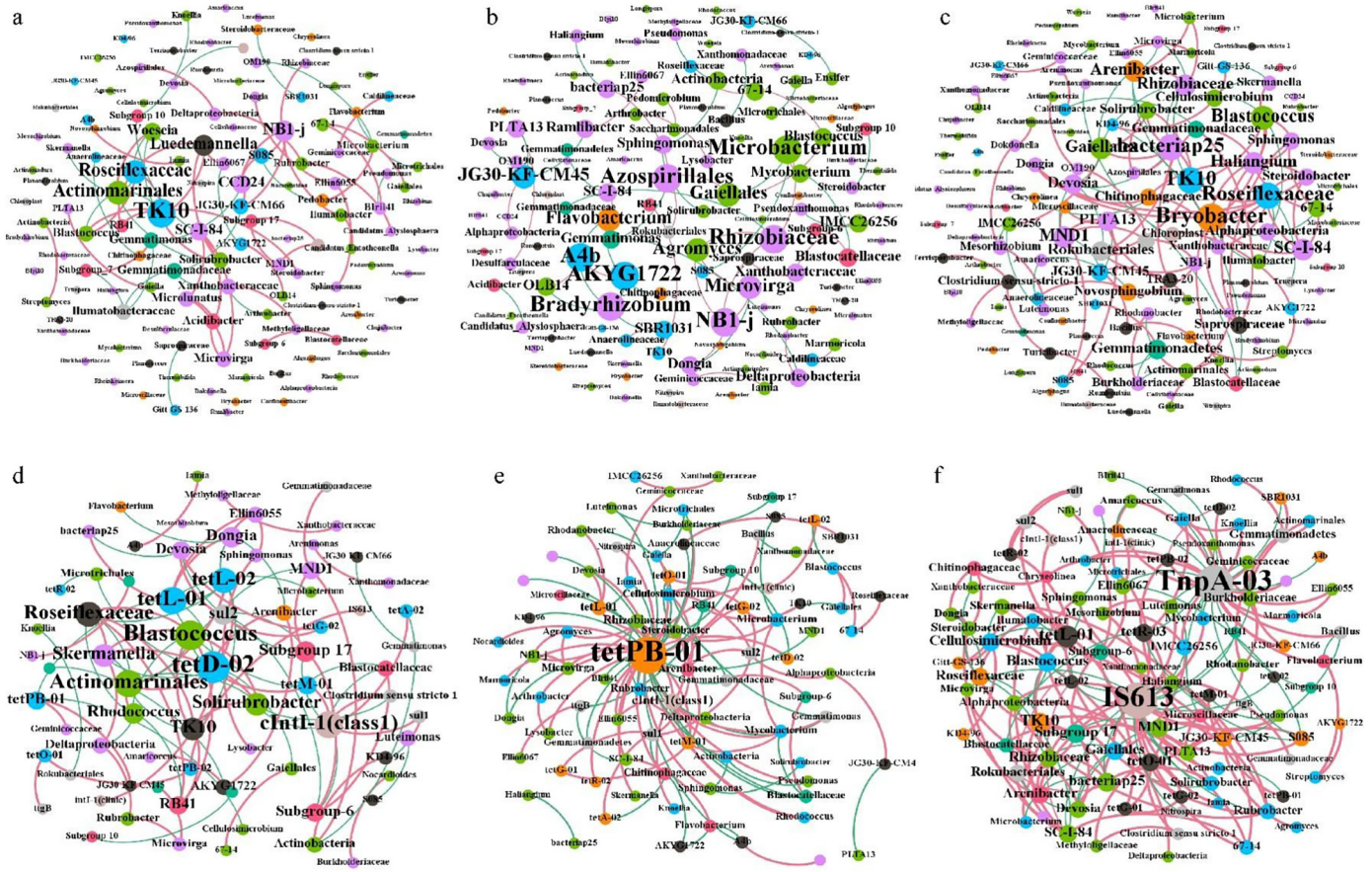

Fig. 5. The network analysis of bacterial genera in soil of L (a), M (b) and H (c) levels of antibiotic residues, and network analysis of bacterial genera and ARGs in soil of L (d), M (e) and H (f) levels of antibiotic residues. A connection shows a strong ($r > 0.8$) and significant ($p < 0.01$) correlation. The nodes with different colors represent different classes of bacterial genera. The size of each node is proportional to the number of connections. Red lines represent positive correlations, and blue lines represent negative correlations. The thickness of lines is on behalf of the correlations, and the thicker line is, the significant correlations are. (Nodes, 129(a), 127(b), 128(c); edges, 71 (a), 58 (b), 122 (c); Nodes, 73(d), 86(e), 100(f); edges, 99 (d), 123 (e), 191 (f)).

that bacterial community rather than fungal community impacted the profile of antibiotics in soil, which accounted for 10 % of the variations (Fig. 6b). Antibiotics in soil could impact prokaryotic cells by inhibiting cell envelope synthesis, protein synthesis or nucleic acid (DNA/RNA) synthesis etc., among which, bacteria are most sensitive to antibiotics (Le Page et al., 2017). The 90 % of the total variance was unexplained, which was more likely to be attributed to anthropogenic (human activities, etc.) and environmental factors (heavy metals, soil physical-chemical property, etc.) (Sun et al., 2020; Zhao et al., 2018).

3.3. Risk assessment of antibiotics on soil microbial community

Excluding the antibiotics for which the K_d (distribution coefficient) or EC_{50} (half-maximal effect concentrations) values were not available, the

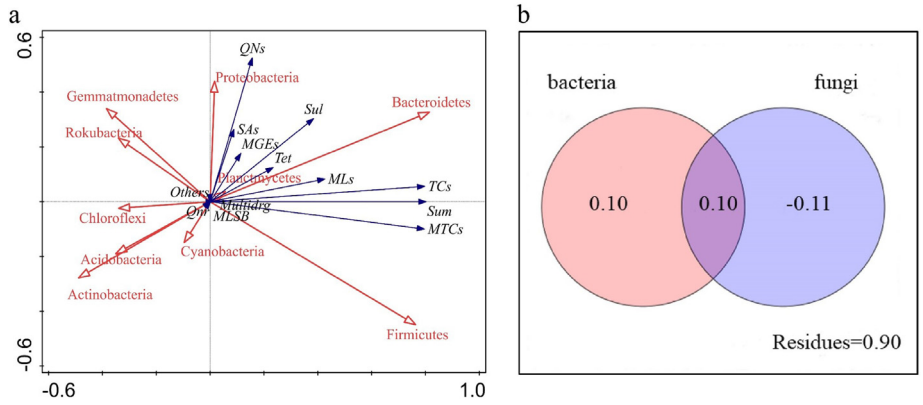

Fig. 6. The Redundancy analysis (RDA) of antibiotics, ARG and bacterial community compositions (phylum level) (a). The explanatory variables account for 64.3 % (adjusted explained variation is 24.3 %). The first axis explained 64.1 % of the total variables. (TCs, tetracyclines; MTCs, metabolites of TCs; QNs, quinolones; SAs, sulfonamides and MLs, macrolides). Variation partitioning analysis (VPA) differentiating contributions of bacterial and fungal community to the antibiotic residues in soil (b).

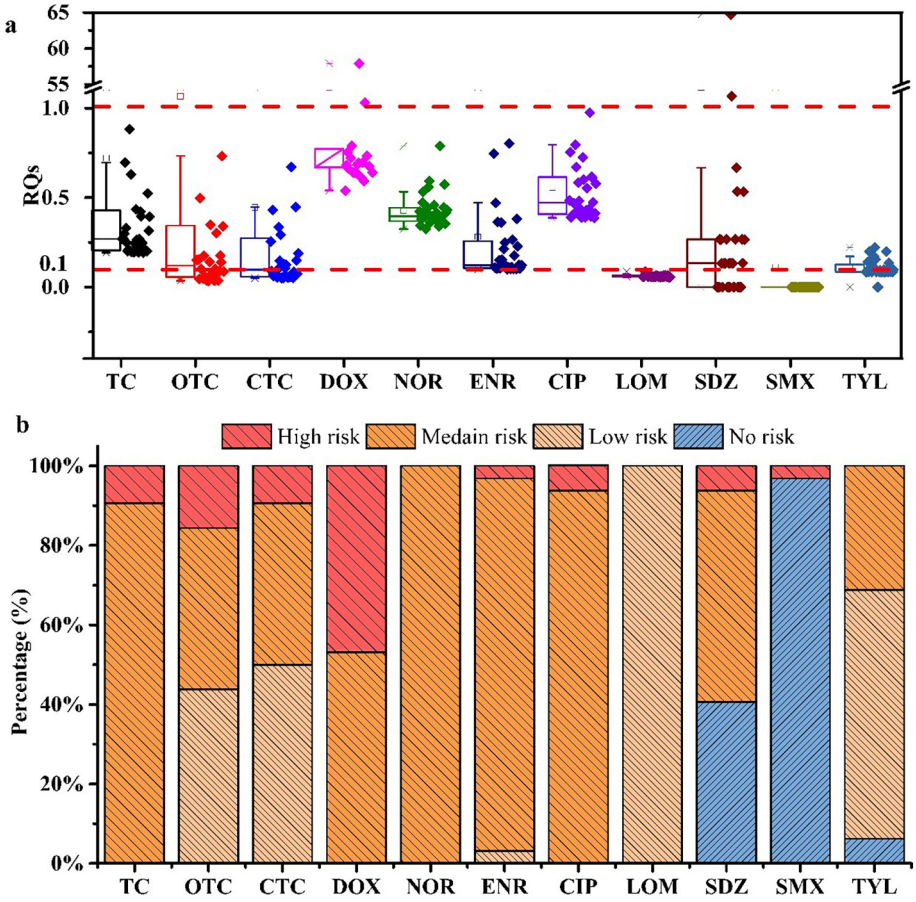

Fig. 7. Risk quotients (a) and percentages of risks (b) of 4 TCs, 4 QNs, 2 SAs and 1 MLs in soils from typical greenhouse vegetable bases of Northern China (n = 32). (High risk, RQ > 1; Medium risk, 0.1 < RQ < 1; Low risk, 0.01 < RQ < 0.1; No risk RQ < 0.01. TCs, tetracyclines; QNs, quinolones; SAs, sulfonamides and MLs, macrolides).

risk assessment of 11 antibiotics to soil microbial community were conducted conclusively (Fig. 7). In present study, DOX, OTC, TC, CTC, CIP, SDZ, ENR and SMX would pose high risks to soil organisms with the percentage of 46.88 %, 15.63 %, 9.38 %, 9.38 %, 6.25 %, 6.25 %, 3.13 % and 3.13 %, respectively. The proportions of NOR, CIP, ENR, TC, DOX, SDZ, OTC, CTC and TYL posing median risks were 100 %, 93.75 %, 93.75 %, 90.63 %, 53.13 %, 53.13 %, 40.63 %, 40.63 % and 31.25 %. LOM, TYL, CTC, OTC and ENR posed low risks with the percentage of 100 %, 62.50 %, 50.00 %, 43.75 % and 3.13 %, respectively. While it was reported that ERY, SMX and DOX would pose high risk to soil ecosystem in Beijing-Tianjin-Hebei urban agglomeration (Li et al., 2023). DOX and CIP caused the most potential ecological effects, and TC caused the lowest potential ecological risk in agricultural soils from the Yangtze River Delta (Sun et al., 2017). OTC posed the highest ecological risks among TC, CTC and OTC in vegetable fields in the Pearl River Delta (Gu et al., 2021).

With multiple antibiotics were detected simultaneously in soil, their effects on soil microorganisms are not independent toxicity, but mixed toxicity. To estimate the multiple ecological risk of antibiotics on soil microbial community, weighted comprehensive pollution index (RQ_{sum}) was tentatively applied, and the RQ_{sum} values of L, M and H levels were shown in Table S8. The values of RQ_{sum} were in range of 0.20–13.66, and multiple antibiotics in 28.1 % of soil samples posed high risk and the other samples with median risk. In the soil of H level, all samples caused high risk except one sample posing median risk (RQ_{sum} = 0.60); in soils of M and L levels, the RQ_{sum} were 0.26–0.88 and 0.20–0.23, respectively, indicating median risk. After taking antibiotic residues into account comprehensively, it was recommended that TC, CTC, OTC, DOX, ENR, NOR and CIP should be taken into the monitoring index systems of organic pollutants in the typical greenhouse vegetable production soil (He and Xu, 2020; Li et al., 2019).

Besides, without considering other co-existing pollutants, the ecological risk of antibiotics on soil organism estimated might be unreliable. Meanwhile, it's worthwhile to consider whether the current EU regulation of veterinary antibiotics needs adjustment. Menz et al. (2019) pointed out the serious defects in EU risk assessment and doubted whether the EU risk assessment keep from microbial toxicity and selection of resistant bacteria in the environment. It is suggested that the ecological risk trigger value and effect evaluation method should be improved or modified to meet the actual needs. Despite the fact of deficiency of effect assessment approach, the median and high ecological risks of antibiotic residues in soil were alarmed. Source control, end treatment, and other effective measures must be taken to eliminate the antibiotic residues in soil.

4. Conclusion

The total concentration of 26 antibiotic residues in the GVP soils studied varied from 83.24 to 4237.93 $\mu g \cdot kg^{-1}$, which was at a median level compared with previous studies. TCs and QNs were the main residual antibiotics. Metabolites of TCs were detected with the range of 23.34–1798.80 $\mu g \cdot kg^{-1}$, which was almost equivalent to the residual degree of TCs in soil and worthy of attention. With the concentrations of antibiotic residues increased, bacterial and fungal community diversity decreased significantly and structure changed. Meanwhile, the relative abundance of Actinobacteria decreased and Bacteroidetes increased, while the composition of fungal community did not change significantly at the phylum level. Bacteria could be resistant to high antibiotic residues, while high levels of antibiotic residues have irreversible inhibitory effects on some fungi. Notably, ARGs occurred in soil when antibiotic residues exceeding 300 $\mu g \cdot kg^{-1}$. With the increase of antibiotic levels, the interspecific

relationship of bacteria first decreased and then increased, consistent with the abundance of MGEs. Bacteroidetes and Firmicutes were the major attributors impacting ARGs in soil and potential ARGs host bacteria were screened in this paper. It was bacterial community not fungal community that impacted the profile of antibiotics in soil, accounting for 10 % of the variations. According to weighted comprehensive pollution index of risk quotient, 71.9 % of soil samples posed median risk on soil organisms and 28.1 % of high risk. Considering all the above, it is urgent to develop effective measures to control and eliminate the residues of antibiotics in agriculture soils. The result could provide comprehensive insights into ecological risks of antibiotic residues in agricultural soil ecosystem, which is beneficial for setting the ecological threshold of antibiotic residues in soil. In the future work, further research is needed to explain under what conditions and concentrations of antibiotic residues will cause the proliferation and spread of resistance genes in soil, and metagenomic and macrotranscriptome sequencing are required to account for changes in soil microbial function.

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CRediT authorship contribution statement

Lixia Zhao: Conceptualization, Investigation, Methodology, Writing-review & editing, Funding acquisition. Zheng Pan: Methodology, Formal analysis, Writing original draft. Baoli Sun: Investigation, Writing-review & editing. Yang Sun: Investigation, Writing-review & editing. Liping Weng: Writing-review & editing, Supervision. Xiaojing Li: Investigation, Writing-review & editing. Huike Ye: Processing data, Writing-review & editing. Jianzhi Ye: Writing-review & editing. Xiaowei Pan: Formal analysis, Processing data. Bin Zhou: Investigation, Formal analysis. Yongtao Li: Conceptualization, Methodology, Supervision, Project administration.

Data availability

Data will be made available on request.

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.

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