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Bio-organic fertilizer containing *Trichoderma* reduces antibiotic resistance genes by reshaping the bacterial community and soil properties

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Abstract

Bio-organic fertilizers containing *Trichoderma* spp. play an essential role in promoting plant growth and defense responses. However, the effect of bio-organic fertilizer on the prevalence of antibiotic resistance genes (ARGs) in ~~the~~ vegetable cropping systems has been largely overlooked. Here, we investigated the effects of soil properties and biotic factors on ARG profiles by analyzing ARG and bacterial communities in vegetable copping soils with a long-term history of manure and bio-organic fertilizer application~~s~~. The abundance of ARG in the soil was significantly increased by 116% with manure application compared to synthetic NPK fertilizer application. This finding was corroborated by our meta-analysis that the longer the duration of manure application, the greater the response of increased soil ARG abundance. However, bio-organic fertilizer containing *Trichoderma* spp. significantly reduced ARG contamination by 31% compared to manure application. About half of the ARG variation was explained by changes in bacterial abundance and structure, followed by soil properties. The mitigation of ARG by *Trichoderma* spp. is achieved by altering ~~loosening~~ the bacterial community structure and weakening the close relationship between bacteria and ARG prevalence. Taken together, these findings shed light on the role of bio-organic fertilizers in mitigating ARG contamination in agricultural soils, which can help manage the ecological risk posed by ARG inputs associated with manure application~~sources~~.

Keywords: manure; *Trichoderma*; antibiotic resistance genes; food chain; ecological risk

1 Introduction

The continued increase in antibiotic resistance genes (ARGs) is becoming recognized as an emerging global crisis for ecological safety and human health due to the heavy use of antibiotics in agricultural and healthcare settings (Gothwal and Shashidhar, 2015; Qiao et al., 2018; Zhu et al., 2013). Vegetable soils are important reservoirs of ARGs in the presence of long-term manure application (Marti et al., 2013; Pu et al., 2020). Compared to chemical fertilizers, the application of livestock manure introduces large amounts of ARGs to soil and leads to changes in the soil bacterial community~~ies in the soil~~, especially for topsoil and within the rhizosphere ~~root systems~~ (Li et al., 2022; Xie et al., 2018). ~~However, the effect of the~~Further, the long-term application of organic and inorganic fertilizers increases ~~on~~ ARG abundance and diversity in greenhouse vegetable soils ~~showed that both increased the diversity and abundance of ARG~~, although each fertilizer regime results in ~~but with~~ different types of dominant ARGs (Sun et al., 2019). This is of particular interest considering that the abundance of ARGs and associated integrase genes and bacterial communities can be greatly affected by different fertilizer applications in the soil (Liu et al., 2017; Nõlvak et al., 2016). Although many studies have revealed the distribution of ARGs in fertilized soils, the variability of ARG profiles in soils with different fertilizer applications remains poorly understood.

The bacterial community and soil properties are the major drivers associated with the variance of ARGs in soil. As hosts of ARGs, the composition and structure of the bacterial community are primary contributors to the shaping of the ARG profiles in the rhizosphere ~~soil~~ (Chen et al., 2020b; Guo et al., 2021). Organic fertilizer application significantly enhanced the abundance of ARGs in the soil-vegetable system, probably attributed to the transfer of common~~typical~~ ARGs among special bacterial species (Huang et al., 2021; Wei et al., 2022). Furthermore, the long-term application of swine manure to~~on the~~ land can increase microbial diversity and reshape antibiotic resistance in the ~~receiving~~ soil~~environment~~ (He et al., 2019). Notably, it has been shown that environmental heterogeneity determines the distribution and diversity of microbial communities (Ramette and Tiedje, 2007). This implies that soil physicochemical variables shaped by different fertilizer application conditions may indirectly affect the distribution of ARGs by influencing the bacterial community. Previous studies have

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Commented [DJ2]: Include some more up to date references identified that target ARGs such as tetracycline ARGs were closely related to soil organic carbon~~matter~~ (SOC) and total nitrogen (TN) levels, factors that directly support and regulate the size of the microbial community ~~owing to the microbiological utilization of carbon and nitrogen~~ in agricultural soils (Zhang et al., 2020; Zhou et al., 2017). Acidification promoted the reduction of sulfonamide genes in manure by inhibiting the proliferation of sulfonamide-resistant bacteria and suppressing the accumulation of *intI*1 (Lin et al., 2020). Other soil quality indicators, such as ~~Soil~~ texture, also drive~~s~~ the persistence of ARGs, and in particular, the clay fraction also affects the sorption ability and longevity of antibiotics in soil (Hu et al., 2019; Macedo et al., 2020). Additionally, farmland application of manure is frequently associated ~~coexists~~ with a higher ~~–~~abundance of~~t~~ heavy metals (e.g., Zn, Cu). Heavy metals, as a component of antibiotic resistance co-selection and transmission, promote the proliferation of ARGs (Baker-Austin et al., 2006; Li et al., 2017). Hence, it is necessary to elucidate the complexity and relevance of the influence of bacterial community and soil physicochemical factors on distributions of ARGs in relation to key soil quality indicators and ~~considering the soil applied with~~under different long-term ~~different~~ fertilizer regimes~~s~~.

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Adding *Trichoderma* spp. may influence the distribution pattern of ARGs by promoting plant growth and altering the physicochemical properties and bacterial community in soils. *Trichoderma* spp. belong to a class of free-living fungi and are widely used as a safe, eco-friendly, and effective biological control agent due to their ability to antagonize plant pathogenic microorganisms and stimulate plant growth and defense responses (Contreras-Cornejo et al., 2009; Druzhinina et al., 2011; Sood et al., 2020). Inoculation with *Trichoderma* can also promote ~~be considered an effective means of using~~ fertilizer use efficiency~~s~~ in vegetable growing systems, ~~and it~~ resulting~~s~~ in increased ~~the accumulation of~~ above-and below-ground biomass ~~in both roots and above-ground parts of the plant~~ as well as improved crop quality (Ye et al., 2020; Yedidia et al., 2001). This is likely due to *Trichoderma* affecting plant growth by promoting root development through numerous biologically active compounds or by inducing an increase in auxin in the early stages of plant development (Martínez-Medina et al., 2014; Vinale et al., 2008). The promotion of vegetable yields by *Trichoderma* ~~maintains stable vegetable yields~~ may also be due to enhanced soil nutrient cycling~~effectiveness~~ (Cai et al., 2015). Evidence

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suggests that inoculation with *Trichoderma* altered the chemical composition of the rhizosphere soil and regulated the soil microbial community composition (Zhang et al., 2019). Field trials have also confirmed that the application of bio-organic fertilizer with *Trichoderma* significantly changed the community structure of the dominant bacteria (Qiu et al., 2012).

In this study, we aimed to investigate whether the long-term application of bio-organic fertilizers with or without *Trichoderma* spp. would alter ARG profiles in soils of vegetable cropping systems and to determine the underlying mechanisms. To accomplish this, we collected soil from a vegetable field with different fertilizer treatments, quantified 285 ARG subtypes using a high-throughput quantitative PCR platform, and analyzed the soil bacterial community using Illumina sequencing technology. The specific objectives of this study were (1) to investigate the ARG profile characteristics of vegetable soils with long-term organic fertilizer application, (2) to combine meta-analysis further to reveal the patterns and controlling factors of the effect of organic fertilizer application on ARGs, and (3) to elucidate the effect of bio-organic fertilizer with *Trichoderma* spp. on ARG distribution in soil compared to manure.

2 Materials and Methods

2.1 Experimental design and sample collection

The field experiment was established in 2015 at the teaching and research site of Nanjing Agricultural University in Nanjing, eastern China (31°43’N, 118°46’E). The climate is characterized by a subtropical monsoon, with hot-rainy summers and mindless rainy winters. The annual mean temperature is 15.4°~~°~~C, and the average rainfall is 1106 mm. The soil was classified as a Eutric Planosol (FAO, 1981) with 32.6% sand, 15.9% silt, 51.5% clay, and a bulk density of 1.33 g cm^{-3}. The surface soil (0-15 cm) possessed ~~contained~~ an organic C content of 13.5~~48~~ g C kg^{-1}, a total N content of 2.08 g N kg^{-1}, and a pH (1:2.5, soil/water) of 5.63. The experimental site has been in long-term continuous vegetable cultivation (~~e.g.,~~ *Brassica rapa* subsp. *chinensis* L. and *Brassica oleracea* var. *capitata* L.~~Chinese cabbage and cabbage~~).

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A randomized block design with three replicates (each plot with a size of 2.7 m × 2 m) was carried out ~~for~~ with four treatments in a plastic greenhouse (30 m × 6 m). There was a 0.6 m wide buffer row between adjacent plots. The four treatments referred to the unfertilized control (control), chemical fertilizer (NPK), organic fertilizer (OF), and bio-organic fertilizer (OF+T). The organic manure used ~~is~~ was swine manure with an ~~containing~~ organic matter content of 457~~.1~~ g kg^{-1}, total N of 14.8 g N kg^{-1}, phosphorus content of 25.2 g P_2O_5 kg^{-1}, and potassium content of 20.1 g K_2O kg^{-1}. The bio-organic fertilizer constituted~~was~~ organic manure plus 5% (mass fraction) solid *Trichoderma guizhouense* NJAU 4742 (10^9 CFU g^{-1} dry weight (DW), an antagonist of *Fusarium oxysporum*; Alabouvette et al., 2001). This treatment ~~containing~~ had an organic matter content of 445 g kg^{-1}, total N of 14.5 g N kg^{-1}, phosphorus of 10.5 g P_2O_5 kg^{-1} and potassium of 14.5 g K_2O kg^{-1}. In each growing stage, each treatment received equal amounts of NPK at rates of 222 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1}, and 180 kg K_2O ha^{-1}. The OF and OF+T treatments were applied at a rate of ~~with~~ 6 t ~~-~~ha^{-1} organic fertilizer, and the missing N, P, and K were supplemented with urea, superphosphate, and potassium sulfate, respectively. The NPK fertilizer treatment was~~is~~ applied directly ~~to~~ as a combination of urea, superphosphate, and potassium sulfate~~ of the appropriate nutrient~~. ~~According to the~~Following local farmer practice, fertilizer was broadcast~~ed~~ on the soil surface with two-thirds of the total nutrients applied as ~~–~~a basal fertilizer~~s~~ before planting and the remaining applied as topdressing. The vegetable planting density was~~is~~ 8 plants per plot (*Brassica rapa* subsp. *chinensis* L.).

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Soil samples were collected from surface layers (0-15 cm) at three points on the diagonal of each plot at the harvest of Chinese cabbage in October 2020 and mixed and passed through a 2 mm sieve to remove stones and other organic debris~~impurities~~ (e.g. roots, crop residues). The homogenized soil samples were stored at 4°~~°~~C or -80°~~°~~C for further analyses.

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2.2 Measurements of soil properties

Soil pH was measured using ~~the~~ a pH probe (PHS-3C, Shanghai, China) at ~~the~~ a soil-to-water ratio of 1:2.5 (*w*/*v*). Soil mineral N (NH_4^+, NO_3^-, and NO_2^-) was determined by extracting the soil with 1 M KCl at a~~the~~ soil-to-water ratio of 1:5 (*w*/*v*) after shaking for 1 h ~~one hour~~ on a rotary shaker. The NH_4^+, NO_3^-, and NO_2^- concentrations in the extracts ~~was~~ were measured using ~~the~~ a Skalar San Plus segmented flow analyzer (Skalar Analytical, Breda, Netherlands). Dissolved organic carbon (DOC) was extracted from soil using a 1:5 (*w*/*v*) ~~using~~ ultrapure water extract and DOC ~~and~~ determined using ~~the~~ a TOC-L analyzer (~~TOC-L,~~ Shimadzu, Kyoto, Japan). Soil total C and N were measured with ~~the~~ a Multi N/C 3100

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analyzer (AnalytikJena, Jena ~~TOC Analyzer,~~, Germany). All the extractions and digestion solutions ~~of~~ containing heavy metals were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Fisher Scientific, USA).

2.3 DNA extraction, *Illumina* sequencing, and prediction of bacterial community function

Soil DNA was extracted from 0.25 g of fresh soil using the MoBio PowerSoil™ DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocols. The concentration and quality of extracted DNA were measured with a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA).

To analyze the bacterial community composition, specific V3-V4 regions of the 16S rRNA gene were amplified with the primers 515F (5'-CCTACGGGNGGCWGCAG-3') and 907R (5'-GGACTACNVGGGTATCTAAT-3'). The resulting samples were subjected to paired-end sequencing on the Illumina MiSeq300 platform (Illumina Inc., San Diego, USA). Three biological replicates were performed per set. PCR was performed using diluted genomic DNA as a template using Taq DNA Polymerase to ensure accurate and efficient amplification. Library quality control of PCR products was performed using a Fragment Analyzer (make). After the libraries passed quality control, the results of the Smear analysis (500-750 bp) were examined using the Fragment Analyzer. The mixed libraries were purified by gel cutting using the QIA quick gel recovery kit (Qiagen, Venio, The Netherlands~~IAGEN brand~~). The libraries were quality-checked and quantified using the ~~Applied Biosystems~~ Quant Studio6 real-time fluorescence quantitative PCR instrument (Applied Biosystems, Waltham, MA). The raw sequences were filtered and optimized using FLASH (Magoč and Salzberg, 2011). The data were analyzed using the QIIME pipeline (version 1.9.1) (Caporaso et al., 2010; Kemp and Aller, 2004). The sequences were clustered into operational taxonomic units (OTUs) for taxonomic classification at the 97% similarity level using UCLUST clustering (Edgar, 2013). The taxonomy of each OTU representative sequence was analyzed using the RDP Classifier (version 2.2) (Wang et al., 2007) against the 16S rRNA database (Silva database version 138). Chao1 index, Shannon index, and observed species were used to evaluate alpha diversity for each sample (Schloss et al., 2009). Illumina raw sequences for bacterial communities were deposited in the NCBI SRA under bio-project number

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To better understand the potential functional contributions of the observed shifts in microbial composition, we used the PICRUSt2 software to predict the functional potential of the bacterial community (Langille et al., 2013). PICRUSt2 uses the resulting data for predictive analysis through the Kyoto Encyclopedia of Genes and Genomes (KEGG). OTUs were normalized by dividing each known 16S rRNA gene copy number before functional prediction. The software for storing information on Clusters of Orthologous Groups of proteins (COG) and the KEGG Ortholog (KO) family was obtained by the green gene id associated with each OTU. COG and KO pathway information from the KEGG database was used to predict functional classification at three levels based on OTU abundance (Malik et al., 2018; Morrow et al., 2015).

2.4 High-throughput quantitative PCR (HT-qPCR) of ARGs

HT-qPCR was conducted to determine the composition and abundance of ARGs and mobile genetic elements (MGEs) in samples using a Wafergen SmartChip Real-time PCR system (Wafergen Inc., CA, USA). A total of 296 primer sets (Table S1) were selected, including 285 ARGs to major classes of antibiotics, 10 MGEs, and the 16S rRNA gene (Su et al., 2015; Wolters et al., 2018; Zhu et al., 2013). The SmartChip was loaded into the Wafergen SmartChip Real-Time PCR Cycler using a PCR protocol of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Wells with multiple melting peaks or amplification efficiencies beyond the acceptable range (90-110%) were discarded. A threshold cycle (CT) value of 31 was used as the detection limit. Three technical replicates were included for each sample, and ARGs with amplification in all three technical replicates were regarded as positive quantification.

2.5 Meta-analysis of the response of ARGs to manure application and soil properties

To test the generality and add additional evidence for elucidating the underlying response of manure and soil physicochemical properties affecting the variance of ARGs in vegetable soil, we performed a systematic search using the ISI Web of Science (Thomson Reuters, New York, NY, USA), Google Scholar (Google, Mountain View, CA, USA) and China National Knowledge Infrastructure (CNKI, Beijing, China). Our search terms included (“soil*” OR “vegetable soil*”) AND (“antibiotic resistance gene*” OR “ARG*” OR “resistome*”). Studies included met the following criteria: (1) both field and pot studies were selected; (2) studies had to be replicated; (3) the means, standard errors, and replication of the variables could be extracted directly from the text, tables, or digitized graphs. The following information was documented for each study: the type and abundance of ARGs, the duration of the experiment, the type of fertilizer, and soil physicochemical properties. A total of 294 observations were extracted from 15 papers (Text S1).

The data~~-~~set was analyzed using a meta-analytic technique described previously (Hedges et al., 1999). The effects of manure and soil physicochemical properties on the variance of ARGs were quantified using the natural log of response ratio (ln*R*), which was calculated as $\ln R = \ln(Xc/Xt)$, where X*c* and X*t* represent the abundance of ARGs from the control and manure-added treatments, respectively. We performed a meta-analysis using the Metawin (version 2.1) (Rosenberg et al., 2000). Similar to the previous research (van Groenigen et al., 2011), we used the number of replications for weighting. The effects of manure addition were considered significant if the confidence intervals (CIs) did not overlap with zero. Means of categorical variables were considered significantly different if their 95% CIs did not overlap. To facilitate ~~ease~~ interpretation, the results from our analyses were back-transformed and reported as the percentage change under manure addition ([ln*R*-1]×100) in the main text.

2.6 Statistical analysis

All analyses were performed in R version 4.0.3 (R Core Team, 2020). The CT measured by the WaferGen qPCR was used to calculate the copy number of genes via Copy Number = $10^{(30-CT)/(10/3)}$ (Fu et al., 2021; Stedtfeld et al., 2008). Circos and heatmap diagrams were generated to show the ARG classification in the different treatments. The differences in bacterial alpha diversity were determined using a one-way analysis of variance ANOVA followed by Tukey’s test ~~was used~~ to determine differences between individual treatments. Non-metric multidimensional scaling (NMDS) analyses based on the Bray-Curtis distance were performed to visualize the overall pattern of bacterial communities, and the permutational multivariate analysis of variance (PERMANOVA) was used to analyze the dissimilarity in soil microbial diversity based on the OTU in different samples (Legendre and Gallagher, 2001). The Mantel test and Procrustes analysis were used to determine the correlation between ARGs and the bacterial community (Delgado-Baquerizo et al., 2018; Peres-Neto and Jackson, 2001). The Hmisc package within R was used to calculate Spearman's correlation matrix between bacterial OTUs and ARGs, and the correspondences of the bacteria and ARGs were shown by network analysis on the Gephi platform (version 0.9.2) (Li et al., 2015). Redundancy analysis (RDA) and variance partitioning analysis (VPA) were applied to investigate how ARGs were related to the components of bacterial community and soil physicochemical properties.

3 Results

3.1 Distribution of ARGs in vegetable soil under long-term fertilization

Diverse ARGs and MGEs were detected in all samples, covering major antibiotics classes, including aminoglycoside, beta-lactamase, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), multidrug, sulfonamide, tetracycline, and vancomycin resistance genes (Fig. 1a). Aminoglycoside resistance genes were the most abundant ARG type in OF, while multidrug resistance genes were the most abundant in the NPK and OF+T treatments. The number of the major classes of ARGs ranged from 3 to 42 (Fig. 1b). Across all treatments, the number of ARGs detected was around 60 and independent of the type of fertilizer application.

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Overall, the abundance of ARGs in the soil receiving ~~applied with~~ organic fertilizer was universally higher than in the NPK and control treatments. Interestingly, we found that the addition of *Trichoderma* effectively reduced ~~prompted the alleviation of~~ the prevalence of ARGs ~~contamination in~~in the soil. For example, the OF+T treatment significantly reduced the abundance of ARGs by 30.9% overall compared to the OF treatment without *Trichoderma* ($P < 0.05$). This reduction was ~~, and in p~~particularly evident for aminoglycoside, tetracycline, sulfonamide, and beta-lactamase resistance genes which reduced by 50.5, 40.7, 35.9, and 17.2%, respectively (Fig. 1c). In contrast, OF+T increased ~~the~~ ARG abundance~~s~~ by 49.4 and 140~~39.9~~% relative to ~~compared to~~the NPK and ~~the~~ control treatments, respectively. In addition, the OF treatment contained the highest average ARG abundances which were elevated ~~and increased~~ by 116~~.2~~ and 247~~.1~~% compared to the NPK and ~~the~~ control treatments, respectively~~,~~. This

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was particularly the case for MLSB, tetracycline, sulfonamide, aminoglycoside, and beta-lactamase resistance genes. NPK fertilizer application increased ARG abundances by 60.5% compared to the control. Hierarchical clustering of their abundances demonstrated a clear division between the different fertilizer regimes~~d soils~~, and phylogenetic analysis revealed the clustering of soils with and without organic fertilizer application ~~was distinguished~~ (Fig. 1c).

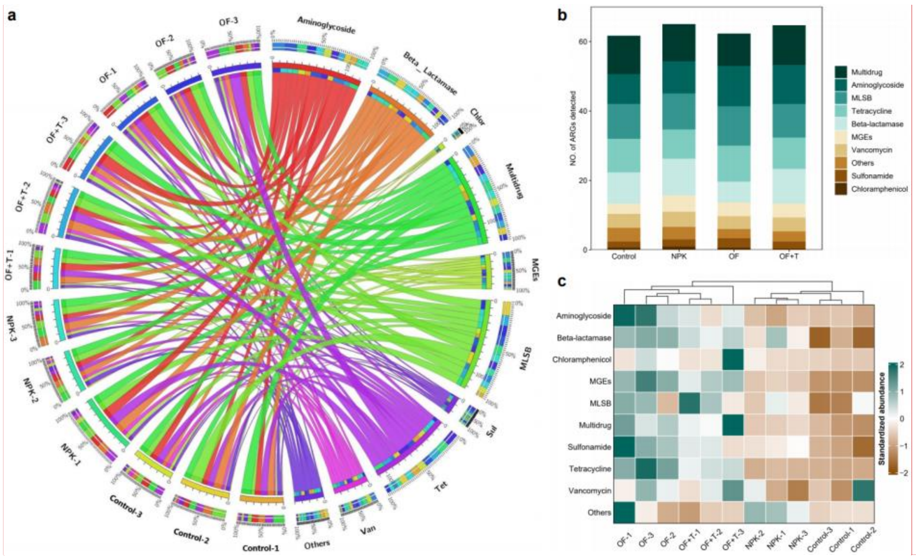

Fig. 1 ARGs detected in vegetable cropping soils under contrasting fertilizer regimes. Distribution (**a** and **c**) and number (**b**) of ARGs detected in each sample of vegetable soil under different fertilizer regimes. The length of the bars of each sample on the outer ring represents the percentage of ARGs in each sample. Sul, Tet, Van, and Chlor represent the sulfonamide, tetracycline, vancomycin, and chloramphenicol resistance genes, respectively. Samples orders are clustered using Euclidean hierarchical clustering. Color codes represent standardized abundance, from more abundant (green) to less abundant (brown, see the color scale at the right) in the heatmap. The treatments included a no-fertilizer control (control), chemical fertilizer (NPK), organic fertilizer (OF), and bio-organic (*Trichoderma*) fertilizer (OF+T).

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3.2 Meta-analysis

Based on 294 paired measurements of 15 studies, our meta-analysis showed that manure application significantly increased ARG abundance~~s~~ by an average of 51.9% in vegetable cropping soils (95% CIs: 31.0% to 77.9%; Fig. 2a). Specifically, with respect to~~on the~~ time~~-~~scale, ARG contamination was more severe in vegetable soil where manure had been applied for more than three years (161~~0.8~~%, 95% CIs: 140~~.4~~% to 187~~.1~~%). Manure application showed a significant and positive effect on aminoglycoside and sulfonamide resistance gene abundance~~s~~ (375~~.2~~%, 95% CI: 238~~.0~~ to 683~~2.7~~%; 116~~5.9~~%, 95% CI: 53~~.1~~ to 222~~.1~~%), whereas a slightly but nonsignificant increment of ARG abundances was found in other categories. Notably, fertilizer type had a significant impact on ARG abundances, especially in swine manure (263~~.2~~%, 95% CIs: 156~~.2~~% to 42~~6.8~~7%) and mixed manure (8~~7.~~8%, 95% CIs: 48~~7.7~~% to 140~~39.8~~%). Regression analysis illustrated that the changes in the manure application effect on abundance of ARGs were negatively correlated with soil pH (r = -0.073, $P < 0.01$; Fig. 2b) and soil organic C (r = -0.154, $P < 0.05$; Fig. 2d), but had a positive correlation with clay content (r = 0.235, $P < 0.01$; Fig. 2c) and soil C/N ratio~~ of soil~~ (r = 0.146, $P < 0.05$; Fig. 2e).

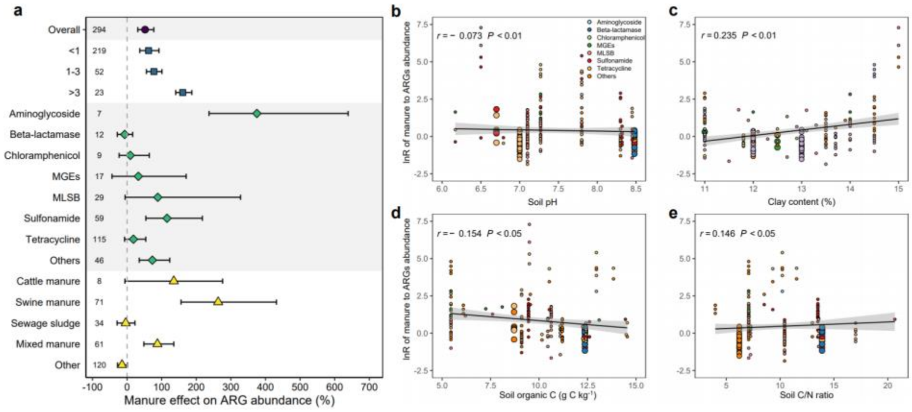

Fig. 2 Effect of manure application on ARG abundances in vegetable cropping soils. Responses of ARG abundances to manure application in soils (a) and its relationships with soil pH (b), clay content (c), organic C content (d), and C/N ratio (e). The category is divided according to the duration (year), type of fertilizers, and type of ARGs. Values are means ± 95% confidence intervals. The number of

observations is given beside each category. The vertical dashed line is drawn at zero. The sizes of the symbols grouped by ARG type are drawn proportional to the weights in the meta-regression analysis.

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In panel a, it would be better to put <1 as <1 year etc.

Others could be 'other ARGs' ?

3.3 Diversity and composition of the bacterial community in soils

A total of 1,803,910 high-quality sequences were detected across all samples, with sequences per sample ranging from 131,456 to 199,430. A total of 5518 OTUs were obtained for 16S rRNA based on a 97% similarity cut-off. Evaluation of the Specie richness, Chao1 index, and Shannon index demonstrated that the OF treatment showed the highest alpha diversity of the bacterial community, followed by the control, OF+T, and NPK (Fig. 3a-c).

The bacterial communities were classified into 18 phyla ~~from the soil~~ (Fig. 3d). *Proteobacteria* (43.3-53.9%), *Firmicutes* (6.4-15.2%), *Bacteroidetes* (7.4-10.1%), *Actinobacteria* (5.9-9.3%), and *Acidobacteria* (2.8-9.2%) were the main phylum in all treatments. *Proteobacteria* was the dominant bacterial phylum, and adding *Trichoderma* significantly increased the relative abundance of *Proteobacteria* in ~~compared~~ comparison to the OF treatment, whereas a decrease occurred in the relative abundance of *Acidobacteria* and *Planctomycetes* (all $P < 0.05$; Figs. 3d and S1a). Compared with the NPK treatment, OF+T or OF significantly increased the relative abundance of *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes* but significantly reduced the relative abundance of *Firmicutes*, *Gemmatimonadetes*, and *Candidatus Saccharibacteria* ($P < 0.05$-0.01). The application of different fertilizers did not greatly~~remarkably~~ change the relative abundances of another bacterial phylum in soil ($P > 0.05$).

The NMDS ordinations based on the Bray-Curtis dissimilarity matrices revealed that the overall patterns of the bacterial community were clustered and distinctly separated from different treatments (Fig. 3e), which was further supported by the Adonis test ($R^2 = 0.64$, $P < 0.01$). Meanwhile, the clusters of bacteria in soils applied with manure were closer to the ~~than~~ NPK treatment than to~~and~~ the control.

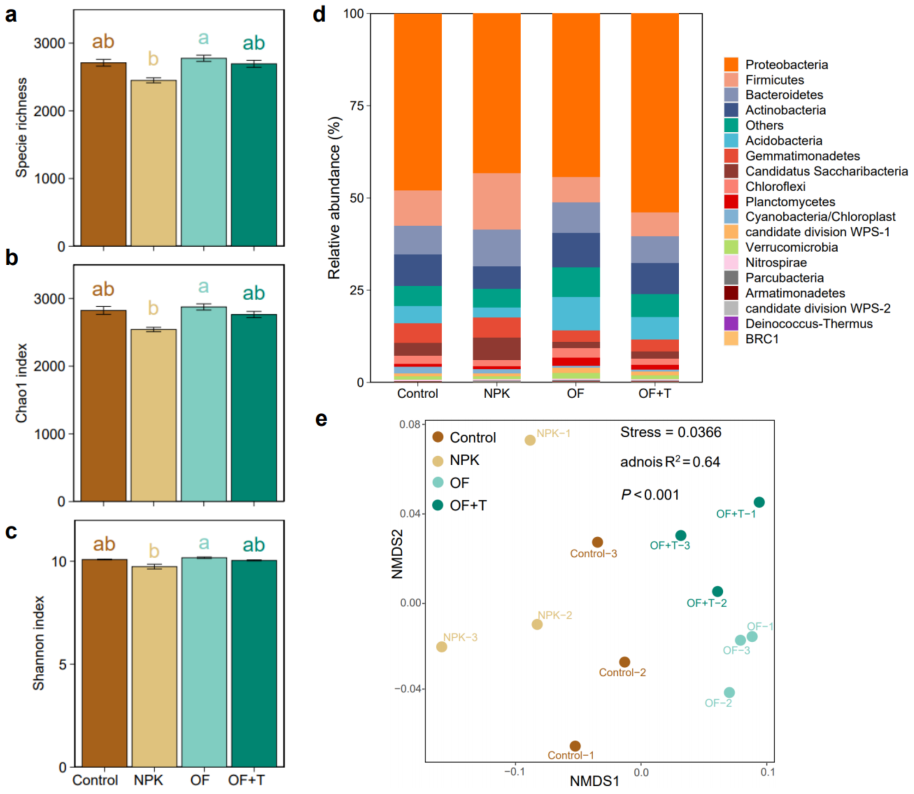

Fig. 3 Bacterial diversity and community composition in vegetable cropping soils with different fertilizer regimes. The α-diversity (Specie richness, Chao1 index, and Shannon index) (a, b, and c) and percentage of dominant phylum (d) levels of soil~~the~~ bacteria among the different fertilizer treatments ~~in soils~~. Non-metric multidimensional scaling (NMDS) ordination plot of the Bray-Curtis dissimilarity matrices between different treatments and the A~~a~~donis PERMANOVA analyses of bacterial distribution among treatments (e). The treatments included a no-fertilizer control (control), chemical fertilizer (NPK), organic fertilizer (OF), and bio-organic (*Trichoderma*) fertilizer (OF+T).

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Specie in panel a should be Species

Bacterial community functions in vegetable-soil systems were predicted by PICRUSt2 using the 16S rRNA gene sequencing data to understand the differences in community potential functions between treatments. In total, 4020 KEGG orthologs with known and unknown functions involved in 407 level 3 KEGG ortholog pathways were identified in this study. These pathways were found to be involved in the seven most dominant KEGG functional categories consisting of cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems, and not included in pathway or BRITE hierarchies~~brite~~, and the metabolism was the main pathways accounting for 37% in soils (Fig. S2).

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We screened out the relevant functions involved in carbon, nitrogen metabolism, and resistance pathways (Fig. S3). Notably, the relative abundance of predicted functional genes involved in nitrogen metabolism was significantly reduced in the OF+T treatment ($P < 0.05$). Similarly, the abundance of pathways associated with carbon metabolism was the lowest but did not reach significance in the presence of *Trichoderma*. Furthermore, these results demonstrated that although the manure application maintained the resistance-related bacterial functional mostly stable, the relative abundance of functional genes assigned to beta-lactamase resistance, cationic antimicrobial peptide resistance, vancomycin resistance, antifolate resistance, and insulin resistance was found to decline in OF+T.

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3.4 Driving forces for the distribution of ARGs in vegetable soil

Procrustes analysis and Mantel test were conducted to investigate the relationship between bacterial communities and ARG profiles (Fig. 4a). Our study indicated that the structure of bacterial composition (Bray-Curtis dissimilarity of OTU tables) fitted well with ARG profiles (Bray-Curtis dissimilarity) relative ~~according~~ to the different treatments. There was a significant association between bacterial communities and ARG profiles (M^2 = 0.237~~65~~, $P < 0.05$, permutations = 999). The Mantel test further verified this finding based on Spearman's rank (r = 0.169~~3~~, $P < 0.05$, permutations = 999), indicating the bacterial structures and composition influenced the ARG profiles in the soil.

Network analysis was performed to illustrate the detailed relationships between individual ARG subtypes and the potential host bacteria at the phylum levels (Fig. 4b). There were 50 nodes and 46 edges in general, and the average degree and modularity index were 1.84 and 0.76, respectively. The density of each node indicated the co-occurrence patterns between these bacteria and ARGs in soil. The data suggests that the bacterial taxa *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes* may be the ARG hosts as~~nd~~ they were positively ~~intensively~~ correlated with multiple ARGs. Specifically, as the most pervasive taxa, *Proteobacteria* had the most edges with ARGs and were significantly related to *tet*Q (tetracycline resistance genes). It should be noted that o~~O~~ne ARG might be carried by a diverse range of ~~potential~~ bacterial hosts; the *vgb*B-02 (MLSB resistance genes) were significantly related to *Proteobacteria* and *Bacteroidetes*. Meanwhile, *Actinobacteria* and *Acidobacteria* were significantly related to *lnu*B-01 (MLSB resistance genes) and *mph*A-02 (MLSB resistance genes), respectively. *Planctomycetes* were significantly related to *mph*A-02 and *bla*$_{\mathrm{VEB}}$ (beta-lactamase resistance genes). In contrast, the relationship between other bacterial phylum and ARG types was generally weak.

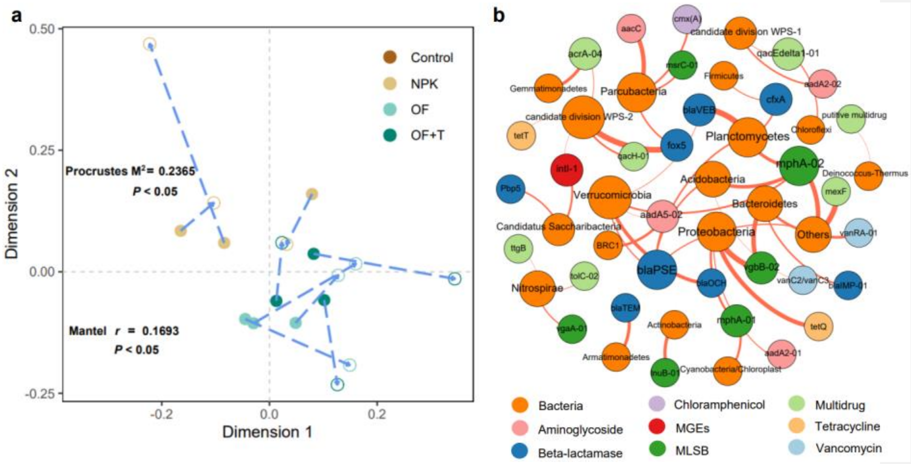

Fig. 4 Association of ARG~~s~~ distribution with the bacterial community in vegetable soils with contrasting fertilizer regimes. Procrustes analysis, Mantel test, and Network analysis were used to reveal~~unveiled the~~ significant associations between ~~the~~ ARG abundance~~s~~ and bacterial community at the phylum level~~s~~. Procrustes test between ARG profiles (Bray-Curtis) and bacterial communities using taxonomic dissimilarity metrics (Bray-Curtis), where M^2 represents the sum of the squared deviations (vector residuals) over the first two dimensions. Solid and hollow circles represent ARGs and bacterial OTUs, respectively. The connection between ARGs and bacterial taxa represents a strong (Spearman's correlation coefficient $r > 0.7$) and significant (P-value < 0.01) correlation. The nodes are colored according to bacterial and ARG types. The node size is proportional to the number of connections, and the edge thickness is proportional to the correlation coefficient. The treatments included a no-fertilizer control (control), chemical fertilizer (NPK), organic fertilizer (OF), and bio-organic (*Trichoderma*)

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fertilizer (OF+T).

The RDA and VPA analyses were performed to further explore ~~further~~ the linkage between ARGs with the bacterial community and soil environmental variables (Fig. 5). Overall, the results showed that the selected variables could explain 88.1% of the variance in ARGs with the first two axes of RDA (Fig. 5a). There was a positive correlation between tetracycline resistance gene abundance~~s~~ and MGEs and pH, SOC, C/N, bacterial community structure (i.e., NMDS1, NMDS2), and multiple heavy metals (e.g., Cu and Cd). Bacterial abundance and nitrogen (e.g., NH_4^+, NO_2^-, and NO_3^-) were the top two factors ~~affecting~~ associated with changes in the abundance of MLSB, sulfonamide, beta-lactamase, and vancomycin resistance genes in soil, ~~which~~ show~~ed~~ing significant~~ly~~ ~~and~~ positive~~ly~~ correlations. To differentiate the effects of the biotic and abiotic factors on the changes in the ARG profiles, VPA showed that a total of 73.5% of ARG variations could be explained by them (Fig. 5b). Specifically, the soil properties contributed 29.6% of ARG variances, and the bacterial communities, including abundance (27.3%) and structure (16.6%) contribute nearly half of ARG profiles in soil.

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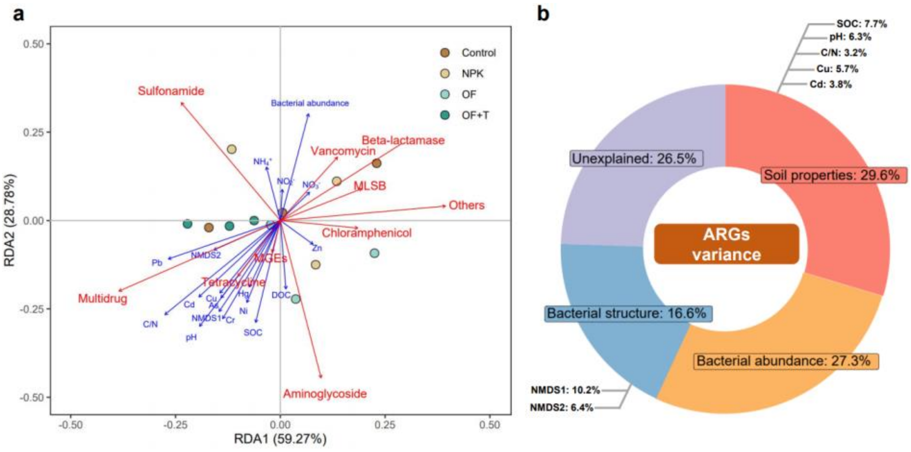

Fig. 5 Main factors driving the distribution of ARGs in vegetable soils. Redundancy analysis (RDA) (a) and variation partitioning analysis (VPA) (b) depict the contribution of biotic and abiotic factors to the variation of ARGs in soil with contrasting fertilizer regimes. Using bacterial abundance, bacterial community structure (two axes of NMDS), and soil physicochemical characteristics as explanatory variables. The blue and red arrows represent the explanatory and response variables, respectively, where

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greater lengths indicate stronger correlations between that pivotal factor and ARG distribution. The angles between arrows reveal the correlations between respective environmental parameters and individual ARGs. The factors that have a significant impact are specified in the VPA. The treatments included a no-fertilizer control (control), chemical fertilizer (NPK), organic fertilizer (OF), and bio-organic (*Trichoderma*) fertilizer (OF+T).

4 Discussion

4.1 Manure application increased the abundance of ARGs in vegetable soils

Our findings from the field experiment are in accordance with the results of the meta-analysis that showed that manure application significantly increases~~d~~ ~~the~~ ARG accumulation in soil (Figs. 1 and 2a). These results are ~~, which is~~ also consistent with previous studies (Macedo et al., 2020; Udikovic-Kolic et al., 2014). Meta-analysis revealed that ARGs were strongly enriched in soils receiving ~~applied with~~ manure and that this effect became stronger ~~continued to increase~~ over repeated applications~~time, especially over three years~~. The prolonged misuse of antibiotics in livestock production systems and subsequent ~~makes~~ accumulation ~~the continued application of~~in manure applied to soil will inevitably lead to ~~pose a great risk to~~ the widespread presence and accumulation of ARGs in agricultural soils (Liu et al., 2021; Peng et al., 2017). Notably, most studies that met our requirements for meta-analysis were short-term pot trials, so we further validated this result using a five-year field experiment with manure applications. Our field research and meta-analysis found that sulfonamide and aminoglycoside were more ~~easily~~ enriched than other ARGs, which might be related to their common use in soils (Wang et al., 2020; Zhao et al., 2018). For the type of manure, swine manure application had the most significant increase in ARG abundances, which is consistent with the OF treatment. The higher ARG contamination caused by swine manure application may be associated with the high addition of antibiotics in swine feed (Martínez-Carballo et al., 2007; Qian et al., 2018; Zhao et al., 2018).

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The changes in the distribution of ARGs may be closely related to the altered soil physicochemical characteristics after manure application. We observed that the abundance of ARGs was negatively correlated with pH and SOC and positively correlated with C/N and ~~Clay~~ clay content (Fig. 2), implying that higher ARG contamination may occur in acidic environments and soils with lower nutrient levels.

The lower TN confirms the reliability of this result in the OF compared to NPK (Table S2, P ~~$<$~~ $\leq$ 0.05), although it was not reflected in pH and SOC. An increase in pH leads to a decrease in antibiotic sorption by the soil, facilitating the removal of antibiotic residues and reducing the abundance of the corresponding ARGs (Li et al., 2020; Wang et al., 2018). Soil nutrient factors (e.g., SOC and TN) may also synergistically influence the composition of ARGs in soils (Sun et al., 2019). Meanwhile, soil texture drives the persistence of ARGs, and clay contributes the most to ARGs distribution than other properties in soils (Macedo et al., 2020; Wang et al., 2020). Animal feed additives and their subsequent presence in ~~Manure~~ manure ~~fertilization~~ could introduce heavy metals into soil (Zhao et al., 2018)~~, and~~. Here we found evidence for~~generally~~ higher levels of multiple heavy metals in the OF treatment (Table S2). Heavy metals can act as a selection pressure forcing the evolution and spread of heavy metal and antibiotic resistance, leading to the proliferation of ARGs in fertilized soil (Dickinson et al., 2019; Komijani et al., 2021; Seiler and Berendonk, 2012).

The association between host bacteria and ARGs may contribute to this difference in the profile of ARGs. *Proteobacteria* and *Planctomycetes* are widely distributed and abundant components of the microbial community in soil amended with manure fertilizers (Bonanomi et al., 2016; Buckley et al., 2006). As the most prevalent predicted source phylum of ARGs, the *Proteobacteria* and *Planctomycetes* were the major drivers of shifting ARG profiles in soil (Forsberg et al., 2014; Pu et al., 2020). As mentioned in the results above (Fig. 3e), the application of manure significantly increased the relative abundance of multiple bacterial phyla (e.g., *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes*). It is evident from the network that the increased abundance of ARGs in the manured soil may be due to the enhanced abundance of MLSB resistance genes carried by the above host bacteria, tetracycline resistance genes carried by *Proteobacteria*, as well as beta-lactamase resistance genes carried by *Planctomycetes* (Figs. 1 and 4), and this further supports this explanation.

4.2 Application of bio-organic fertilizers reduced ARGs by modifying biotic and abiotic factors

Bio-organic fertilizers reduced~~weakened~~ the propagation and transmission of ARGs compared to manure application alone, inferring that the change in soil physicochemical properties might be partly responsible. *Trichoderma* is very efficient in improving soil fertility and ~~the~~ promoting more

~~effectiveness~~ efficient cycling of soil nutrients (Bulluck et al., 2002; Caporale et al., 2019). Analysis based on functional predictions objectively revealed that microorganisms associated with soil carbon and nitrogen metabolism were less abundant after bio-organic fertilizer application, consistent with the results of higher SOC and TN in the OF+T (Fig. S3 and Table S2). Combined with the meta-analysis, ~~it can be~~we conclude~~ed~~ that ~~more~~ increased carbon and nitrogen ~~made less~~reduced ARG pollution (Fig. 2). Meanwhile, the co-selection mechanisms for metals and ARGs have been demonstrated (Baker-Austin et al., 2006; Li et al., 2017). There was a significant effect of Cu and Cd on the distribution of ARGs in the present study (Fig. 5), as reported by previous studies (Arya et al., 2021; Seiler and Berendonk, 2012). The concentration of microelements was significantly increased in the roots of plants planted in soil inoculated with *Trichoderma* (Yedidia et al., 2001). Hence, the reduction in heavy metal concentrations in the OF+T treatment due to plant uptake may have weakened the abundance of ARGs compared to OF (Table S2). Additionally, MGEs have a lower abundance in the OF+T treatment may also be an essential factor in attenuating ARG contamination by *Trichoderma* (Fig. 1c).

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In general, the composition and structure of bacterial communities largely determine the profile of ARGs in soils with manure application owing to the role of bacteria as the primary carriers of ARGs (Huang et al., 2021; Nõlvak et al., 2016; Xie et al., 2018). Interestingly, this study demonstrated that bio-organic fertilizer containing *Trichoderma* may affect the composition of bacterial communities and thereby reduced the presence of ARGs. This may be due to the ability of bio-organic fertilizers containing antagonistic microorganisms to alter bacterial abundance (Berg, 2009; Cai et al., 2015; Qiu et al., 2012). Evidence of long-term field experiments suggests that bio-organic fertilizers can stimulate potentially beneficial bacteria to improve crop yield and achieve disease suppression by reshaping key species in the structure and function of soil microbial communities (Qiao et al., 2019; Xiong et al., 2017). This reshaping~~improvement~~ of the microbial communities by *Trichoderma* may ~~have~~ make a significant contribution to the mitigation of ARGs. Notably, the relative abundance of *Planctomycetes* carrying beta-lactamase resistance genes was lower in the OF+T treatment, which could be one of the reasons for the low ARG content compared to manure application alone (Figs. 3d and 4b). Correspondingly, the addition of *Trichoderma* increased the relative abundance of *Proteobacteria* but reduced the tetracycline resistance genes carried by them, likely owing to the replacement of its function as host bacteria for ARGs by other microorganisms. Rare microbial taxa have been shown to have a high proportional role in biological processes in soil applied with *Trichoderma*, although *Proteobacteria* is the most abundant and diverse phylum in this study (Chen et al., 2020a; Spain et al., 2009). Bacterial function predictions further validated that the bacteria associated with resistance decreased in the soil applied with bio-organic fertilizer (Fig. S3), which coincides with the above association of bacterial abundance with the distribution of ARGs. Our findings confirm that applying bio-organic fertilizer benefits soil health by mitigating ARG pollution while ameliorating soil microorganisms.

The discrepancies in ARG profiles may also be caused by differences in bacterial structure (Guo et al., 2021). Our results indicated that the contribution of bacterial structure (16.6%) to the distribution of ARGs is slightly less than that of bacterial abundance (27.3%). The two axes of the NMDS can explain 16.6% of the ARG variance across all impact factors (Fig. 5b). Although the bacterial structural composition of OF+T was tighter compared to NPK, looser than the OF treatment (Fig. 3e), this phenomenon is also present in the relationship between bacterial communities and ARG (Fig. 4a). This suggested that the added *Trichoderma* has weakened the close relationship between bacteria and ARGs. The alteration of the bacterial structure by *Trichoderma* may derive from its competitive mechanism with other microorganisms in natural communities (Ferreira and Musumeci, 2021; Sood et al., 2020). Additionally, the effectiveness of bio-organic fertilizer in modifying the soil may be attributed to the enhancement of soil microbial activity (Ye et al., 2020). As such, we speculated the activity of microorganisms associated with the host bacteria (e.g., *Proteobacteria*) carrying ARGs might not have been enhanced in the OF+T treatment. However, further research is needed to support this hypothesis. Nevertheless, a proportion of the variance in ARG profiles remains unexplained. Overall, further follow-up studies characterizing the linkages in soil microbial community structure and composition and ARG would be beneficial for a better understanding of the distribution and dissemination of ARGs in organic fertilizer-soil-plant systems.

5 Conclusion

In summary, our field experiment and meta-analysis indicated that manure application significantly increased the abundance of ARG in vegetable soils compared to synthetic fertilizer application. However, bio-organic fertilizer with *Trichoderma* significantly reduced ARG contamination compared to manure application. The abundance and structure of the bacterial community and soil properties were the main drivers of ARG contamination. The mitigation of ARG by *Trichoderma* was accomplished by ~~loosening~~ reshaping the bacterial community structure and weakening the close relationship between bacteria and ARGs. Given the importance of ARGs to soil ecological risk, these findings deepen~~ed~~ our understanding of ARG profiles in soil under long-term fertilization and highlighted the importance of potentially controlling the prevalence of ARGs ~~by~~ using bio-organic fertilizers.

Acknowledgments

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