

Metagenomic analysis of effects of oxytetracycline and copper on antibiotic resistance genes and associated pathogenic hosts in swine manure compost

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Keywords: Antibiotic resistome, Composting, Metagenome, Pathogenic host, Swine manure

Posted Date: February 21st, 2020

DOI: <https://doi.org/10.21203/rs.2.24236/v1>

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Abstract

Background: The proliferation of antibiotic resistance genes (ARGs) in compost and their horizontal transfer to human pathogenic bacteria (HPB) may lead to the failure of human antibiotics. However, the antibiotic resistome in compost has not been comprehensively characterized. This study used a metagenomic approach to obtain new insights into the effects of oxytetracycline (OTC) and copper (Cu) on the antibiotic resistome during swine manure composting and the risks posed to human health.

Results: The results showed that composting reduced the abundances and diversity of ARGs and HPB in swine manure. In total, 289 ARG subtypes and 19 ARG types were detected in the samples with abundances ranging from 1.08×10^{-1} to 9.39×10^{-1} copies/16S rRNA, which mainly encoded tetracycline, aminoglycoside, and macrolide–lincosamide–streptogramin (MLS) resistance genes. The application of OTC and Cu, especially the combined application, exacerbated the compost resistome risk scores and specific ARG subtypes responded differently. Tetracycline, multidrug, and MLS resistance genes mainly affected resistance profiles of HPB throughout the composting process. HPB and *intI1* had significant positive effects on determining the ARG profiles during the composting process, and the co-selective effect of heavy metals may increase the abundances of ARGs via strong positive effects on *intI1*. In addition, the effect of mobile genetic elements on the horizontal gene transfer of ARGs should not be ignored.

Conclusions: This study of the antibiotic resistome in compost indicates the need for effective regulation of the misuse of livestock and poultry feed additives in order to minimize the spread of the antibiotic resistome in agro-ecosystems and decrease the potential risk to public health.

Keywords: Antibiotic resistome; Composting; Metagenome; Pathogenic host; Swine manure

Background

Since the beginning of the last century, large-scale intensive farming systems have become increasingly popular, and they rely on antibiotics to prevent and treat animal diseases and improve growth performance [1]. However, veterinary antibiotics are not metabolized well by animals and up to 90% of the applied antibiotics can be excreted in urine or feces as the parent compounds or metabolites [2]. These residual antibiotics exert selective pressures on the microbial community even at low concentrations, thereby inducing the enrichment and dissemination of antibiotic resistance genes (ARGs) [3]. ARGs are readily acquired by human pathogenic bacteria (HPB) to generate superbacteria. ARGs are carried in agricultural products due to the agricultural application of manure, which can eventually eliminate the efficacy of antibiotics used in the treatment and prevention of human bacterial infections [4–7]. It has been suggested that if appropriate measures are not taken to address the important health issues associated with antimicrobial resistance, then the number of deaths due to antibiotic-resistant infections will exceed 10 million by 2050 [8, 9]. At present, organic agriculture is being promoted, and thus the use of compost that connects human health and agricultural waste resources is being investigated intensively

to assess the risks associated with the presence of antibiotics and the spread of antibiotic resistance [10, 11]. In particular, oxytetracycline (OTC) is one of the most common antimicrobial agents used for the prevention and treatment of animal diseases. In the US swine industry, OTC is commonly used to treat a variety of swine bacterial diseases, including respiratory diseases caused by pathogens such as *Glässerella*, *Pasteurella*, and *Mycoplasma* [12]. Therefore, it is necessary to conduct in-depth analyses to determine the effects of OTC residues in animal feces on the distribution and diversity of ARGs as well as identifying their pathogenic hosts during composting.

Human pathogens employ similar mechanisms to those used by pathogens to resist antibiotics in the natural environment [13]. ARGs encoding defense mechanisms are readily transferred between pathogenic microorganisms through horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs), and integrons are considered to be the main contributors to the acquisition and spread of ARGs [14, 15]. Shterzer and Mizrahi [16] have reported HGT occur in the gastrointestinal tracts of livestock, and it is enhanced in the presence of antibiotics [17, 18]. In addition, both ARGs and heavy metal resistance genes (MRG) have similarly ancient origins [19]. Luo et al. [20] analyzed 5,436 complete bacterial genomes and showed that MRGs and ARGs coexist extensively in clinical and environmental bacteria. Plasmids containing both ARGs and MRGs are more likely to be conjugated and transferred between different bacterial groups [21]. It is considered that the co-selection of MRG and ARG genes is due to the selective pressure imposed by heavy metals on ARGs through cross or co-resistance [22]. Heavy metals can also be applied in animal feeds as animal growth promoters, where Cu is detected at high concentrations in livestock and poultry manure, and it is considered to have the greatest capacity for promoting the transfer of conjugated ARGs [23, 24]. Thus, it is necessary to explore the relationships between ARGs, MRGs, and MGEs in order to understand the selection, transfer, and persistence of ARGs in composting environments.

Metagenomics methods and structured gene databases have become powerful new tools for accurately and comprehensively identifying and quantifying ARGs, MRGs, and MGE in different environments [25–27]. In addition, pathogenic bacteria affect the risk of resistance transferring to the human microbiome. Metagenomic sequencing can allow the classification of most unculturable and rare pathogenic bacteria, as well as the specific ARGs that they carry [3, 28], thereby providing a theoretical basis for further optimizing the treatment regimen to avoid the spread of antibiotic resistance.

The present study aimed to determine: (i) how the diversity and richness of ARGs, MRGs, and MGEs are affected by OTC and Cu during the composting process; (ii) the specific ARGs carried by pathogenic bacteria and their potential human health risks; and (iii) the contributions of different factors to the fates of ARGs

Results And Discussion

Antibiotic resistome determined for microbes during composting

Metagenomic analysis based on the structured SARG database was conducted to determine the antibiotic resistome for microbes during composting. The results showed that ARGs were diverse and abundant in swine feces, even when OTC or Cu was not administered in the CK treatment (Fig. 1). Aerobic composting effectively reduced the abundances and diversity of ARGs. These results are consistent with previous demonstrations [32, 36]. In total, 289 ARG subtypes and 19 ARG types were detected throughout the composting process. On day 0 of the composting process, 235 ARGs were detected but the amount decreased to 135–160 on day 10 and 106–120 on day 42. Sixty ARGs were prevalent throughout the composting process (Figs. 1a and 1b). The total abundances of ARGs were $9.39 \cdot 10^{-1}$ copies/16S rRNA on day 0, $1.44\text{--}2.41 \cdot 10^{-1}$ copies/16S rRNA on day 10, and $1.08\text{--}1.36 \cdot 10^{-1}$ copies/16S rRNA on day 42. The application of OTC or Cu increased the abundance of ARGs compared with CK, where the total abundances were 43.57%, 39.76%, and 67.17% higher in O, Cu, and OCu on day 10, respectively, and 4.66%, 8.93%, and 26.17% higher on day 42.

The detected ARGs were characterized by three major resistance mechanisms: antibiotic inactivation (37.02%), efflux pump (34.26%), and cell protection (21.45%), which are consistent with the results obtained by Zhu et al. [37] and Qian et al. [32]. The ARG subtypes with antibiotic inactivation mechanisms were the most common with up to 85, but the number decreased to 25–30 in the different treatments at the end of composting. The application of OTC and Cu inhibited the decreases in the abundance of ARGs with the three mechanisms on day 10, and strongly inhibited the removal of ARGs characterized by antibiotic inactivation mechanisms in the compost product. In particular, the abundance of ARGs with antibiotic inactivation mechanisms was 2.10 times that of CK in OCu (Fig. 1c).

The ARGs detected in compost can facilitate resistance to the major antibiotics used in humans and animals, thereby resulting in a high public health risk when employed in agriculture. The ARGs detected in this study mainly conferred resistance to three major classes of antibiotics comprising tetracycline ($1.17 \cdot 10^{-2}$ to $3.12 \cdot 10^{-1}$ copies/16S rRNA), aminoglycoside ($1.73 \cdot 10^{-2}$ to $2.01 \cdot 10^{-1}$ copies/16S rRNA) and MLS ($1.23 \cdot 10^{-2}$ to $2.20 \cdot 10^{-1}$ copies/16S rRNA) resistance genes. The relative abundances of the general ARG types comprising tetracyclines, macrolide–lincosamide–streptogramin (MLS), and aminoglycosides in pig manure compost have been previously reported, and they have also been found in other specific niches [17, 32, 37]. Composting was highly effective at removing these three types of ARGs as well as chloramphenicol and trimethoprim resistance genes, with removal rates above 90.47% (except the removal rate for aminoglycoside resistance genes was 80.47% in OCu) (Fig. 1d). However, multidrug, rifamycin, fosfomycin, and fosmidomycin genes were enriched. Compared with CK, the addition of OTC and Cu increased the abundances of tetracycline resistance genes in the compost product by 16.55% and 41.66%, respectively, and the combined addition of OTC and Cu increased the abundance of aminoglycoside resistance genes by 1.28 times.

PCA showed that during the composting process, the samples were significantly separated at the ARG subtype level, especially in the compost treated with both OTC and Cu (Fig. 2a), possibly because they had the maximum effect on ARGs with different resistance mechanisms. Figure 2b shows the profiles for

the 40 ARG subtypes during the composting process with average abundances $> 1.0 \cdot 10^{-3}$ copies/16S rRNA. These ARGs mainly encoded resistance to tetracycline (tetL and tetW), MLS (ermB and ermC), aminoglycosides (aadA, aadE, and aph(3'')-I), multidrug (multidrug_transporter), sulfonamide (sul1), and chloramphenicol (chloramphenicol exporter). The genes encoding multidrug (multidrug_transporter, multidrug_ABC_transporter, acrB, mexF, and ykkC), MLS (macB and mphA), and rifamycin (rifampin monooxygenase) resistance were enriched in the compost product, whereas the abundances of other genes decreased as the composting process continued compared with day 0. The removal rates of 17 ARGs exceeded 90%, i.e., seven tetracycline, five MLS, four aminoglycoside, and one chloramphenicol resistance gene. TetL is a tetracycline efflux protein found in Gram-negative and Gram-positive bacteria and it was the most abundant ARG subtype in the compost on day 0. At the end of composting, the removal rate for tetL reached 98.44–98.79%. On day 10, the addition of either OTC or Cu alone and both together increased the abundances of all aminoglycoside and tetracycline resistance genes (except for tet44 and tetX in O and OCu) compared with CK. The ARG subtypes in the compost products varied among the different treatments. For example, compared with CK, the abundances of tetracycline resistance genes comprising tetL, tetP, tetX, and tetracycline_resistance_protein tended to increase in the treatments with added OTC and Cu, and their abundances were highest when treated with OTC and Cu combined. However, compared with CK, the abundances of most MLS resistance genes (except for lnuB) decreased in the compost product under the combined treatment, where the ermB, ermA, ermC, ermF, ermX, and ermG genes mainly encode resistance to antibiotics via cell protection.

Four universal primers for two tetracycline ARGs (tetW and tetX) and two sulfonamide ARGs (sul1 and sul2) were developed in previous studies, and their prevalence was verified by qPCR. Compared with CK, the abundances of tetracycline ARGs (tetW and tetX) and sulfonamide ARGs (sul1 and sul2) tended to increase in O, Cu, and OCu in a similar manner to the results obtained using metagenomic methods. However, the relative abundances of the genes quantified by qPCR were relatively small, probably due to the great sequence diversity covered by the macrogenes, which could only amplify specific sequences or regions of the target gene [28].

Heavy metal resistome of microbes during composting

The enrichment of ARG in various environments can be caused by co-selective pressure exerted by heavy metals. In water, soil, and fertilizer environments, significant positive correlations between the concentrations of heavy metals and the abundances of ARGs have been widely reported [38–40]. In total, 41 CRGs were detected in the compost samples (Fig. 3) and the total abundance ranged from $4.95 \cdot 10^{-2}$ to $1.29 \cdot 10^{-1}$ copies/16S rRNA. Aerobic composting effectively reduced the abundances and diversity of CRGs. In total, 41 CRGs were detected on day 0 and the number decreased to 18–24 in the composting products obtained under different treatments. The total abundance of CRGs before composting was $8.02 \cdot 10^{-2}$ copies/16S rRNA and it decreased to $4.95\text{--}6.27 \cdot 10^{-2}$ copies/16S rRNA after 42 days. Since these CRGs are often associated with ARGs, it is not surprising that their abundance is reduced [41, 42]. During the composting process, the total abundances of CRGs were always higher in Cu and OCu than CK and O, and the abundance was highest in OCu. Compared with CK, the Cu treatment increased the total

abundance of CRGs by 3.41% and 3.08% on day 10 and day 42, respectively, and OCu increased the total abundance of CRGs by 15.05% and 20.82% on day 10 and day 42. These results indicate that the combined effect of OTC and Cu was far greater than that of each single treatment.

In this study, *copA* was the most abundant CRG in the composting process. *CopA* encodes the ATPase in the Cu ion efflux pump system, which can excrete copper ions from the cytoplasm into the periplasmic space [43]. The abundance of *copA* was reduced by 27.74–40.19% in the final compost product compared with day 0. In other CRGs, the periplasmic *pco* system exists only on plasmids and it provides high resistance to Cu [44]. Composting was effective at removing *pco* genes, and the removal rates for *pco* genes reached 100% at the end of composting.

Occurrence and abundances of MGEs

The study also identified the presence of MGEs, including three important integrons and one transposon [45, 46] (Fig. 3), which play important roles in the transfer and acquisition of ARGs in various microorganisms via HGT. Integrons can capture exogenous ARG cassettes and then integrate them into their own gene cassettes through targeted recombination [47]. However, integron genes are defective due to their inability to move, but they are usually linked to transposons, which can serve as a vector for the transmission of genetic material [48, 49]. Aerobic composting reduced the total MGE abundances in the different treatments by 95.11–96.63%. The integrase gene *intI1* was the most abundant, where its abundance ranged from $1.38 \cdot 10^{-3}$ to $4.09 \cdot 10^{-2}$ copies/16S rRNA during the composting process. Following the *tnpA* transposon, the abundance range ranged from $3.11 \cdot 10^{-4}$ to $1.25 \cdot 10^{-2}$ copies/16S rRNA. The application of OTC and Cu increased the abundances of *intI1* and *tnpA* on day 10. Compared with CK, the abundance of *intI1* was 97.38%, 31.99%, and 133.42% higher in O, Cu, and OCu, respectively, and that of *tnpA* was 56.93%, 43.55%, and 107.33% higher. However, O did not increase the abundances of *intI1* and *tnpA* in the composting products, whereas Cu and OCu increased the abundance of *tnpA* by 19.07% and 25.90%, respectively. These results indicate that the presence of Cu in pig manure had a more profound impact on ARGs and it was more conducive to increasing the risk of ARGs spreading after the agricultural application of compost.

ARGs carried by HPB during composting

Composting effectively reduced the abundances of HPB in pig manure. Compared with day 0, 58.16–65.43% and 77.61–83.71% of the total HPB in compost were removed on day 10 and day 42, respectively, under different treatments. The ARGs carried by HPB comprised 37 subtypes and the variations in three ARG types mainly affected the resistance profiles of HPB during the whole composting process. Tetracycline and multidrug resistance genes were the most common ARG subtypes with 11 and 12 genes, respectively. Only seven subtypes of MLS resistance genes were detected in HPB but they were present in 9 HPB species, where *macB* was carried by nine HPB (Fig. 4).

ARGs were harbored by 20 different pathogenic hosts. These pathogenic hosts were dominated by bacteria such as *Escherichia coli*, *Clostridium botulinum*, *Enterococcus faecium*, and *Corynebacterium*

jeikeium, which belong to Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Chlamydiae. These findings are consistent with previous studies of cow feces, which found that the ARGs originated from Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria [50]. Among the HPB that carried ARGs, *Escherichia coli* was the main host bacteria and it harbored the highest diversity ARGs, including genes encoding resistance to tetracycline, MLS, multidrug, fosmidomycin, quinolone, bacitracin, and chloramphenicol. A previous survey of surface water in aquaculture areas found that the frequency of resistant *Escherichia coli* isolates increased as the distance decreased from farms [51]. *Corynebacterium* is an opportunistic pathogen that settles on human skin and it has recently been implicated as the cause of various infections [52]. *Corynebacterium* infections are resistant to multiple antibiotics [53]. However, in the present study, adding OTC and/or Cu increased the abundance of *Corynebacterium jeikeium* by 11.17%, 61.78%, and 17.90% in the composting products obtained with O, Cu, and OCu, respectively, compared with CK. *Enterococcus faecalis* and *Enterococcus faecium* are increasingly important hospital pathogens worldwide, and they are mainly related to specific multi-drug resistance clone lineages in hospital environments [54]. However, other studies found that *trcB* was located on a conjugating plasmid in *Enterococcus faecium*. The addition of the heavy metal Cu and copper tolerance may help to select for or maintain multi-drug resistant *Enterococcus* [55]. *Enterococcus* exhibited multidrug, MLS, and bacitracin resistance in the present study, and the abundances of *Enterococcus faecalis* and *Enterococcus faecium* in the compost products were 21.99% and 38.76% higher, respectively, in Cu treatment compared with CK. In addition, *Pseudomonas aeruginosa* is recognized as a well-known difficult to treat HPB and its efflux mechanism responsible for antibiotic resistance represents a great challenge for the treatment of human diseases [56]. In the present study, the addition of OTC and Cu increased the abundance of *Pseudomonas aeruginosa* in the compost products by 8.82%, 32.28%, and 2.98% in O, Cu, and OCu, respectively, and it was mainly resistant to tetracycline (*tetB* and *tet32*) and bacitracin (*bcra*).

Factors that affected ARG profiles during composting

The ranges of ARGs carried by the microorganisms were determined in this study. Various factors affected the abundances of ARG but the key factors related to the responses of the dominant ARGs to composting should be emphasized. RDA and SEMs were used to separate and order the factors related to changes in ARGs [57, 58]. The first two axes for HPB and CRGs were extracted by PCA before RDA because the number of environmental variables (related factors) cannot exceed the number of species variables (ARGs). The results showed that the factors considered in this study could explain 96.8% of the fate of ARGs (Fig. 5), where HPB_1, MRG_1, *tnpA*, *intl1*, and *intl2* were the main factors that influenced the variations in ARGs, where they explained 19.10%, 19.03%, 18.89%, 18.09%, and 17.54% of the variations in the ARG profiles, respectively. Microorganisms are carriers of genes so the reductions in the abundances of HPB during composting contributed to the reductions in the abundances of ARGs. However, the increases in the abundances of potential pathogens after composting were concerning, such as *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Bordetella bronchiseptica*. In this study, the ARGs in these HPB encoded tetracycline, bacitracin, and multidrug resistance.

SEMs were used to assess the direct and indirect effects of the five main drivers (HPB_1, MRG_1, tnpA, int11, and int12) on three major antibiotic resistance profiles (Fig. 6). HPB was the most important positive factor for shaping the ARG profiles. HPB can impose an indirect effect on ARGs by strongly affecting the abundance of MGEs and MRGs in the composting process. MGEs had strong and direct impacts on the abundances of ARGs in the composting process. HPB_1 had the highest standardized total effect on the ARG profiles (0.98–0.99), followed by int11 (0.78–0.85). In addition, the co-selective effect of MRGs on ARGs cannot be ignored. In particular, MRGs significantly increased the abundance of ARGs by positively affecting int11. Similar results were obtained in previous studies, where Rosewarne et al. [59] and Wright et al. [60] found int11 abundance was significantly increased due to the presence of heavy metals.

Conclusion

The changes in the antibiotic resistome during composting were analyzed in this study, thereby providing new insights into the effects of universal additives (OTC and Cu) in feed on the risks for human health and the potential for spreading antibiotic resistance diffusion. The effects of OTC and Cu on enhancing the antibiotic resistome during composting depended on specific ARG subtypes rather than the overall ARGs at the community level. In total, 289 ARG subtypes belonging to 19 ARG types were detected with abundances ranging from $1.08 \cdot 10^{-1}$ to $9.39 \cdot 10^{-1}$ copies/16S rRNA. These ARGs were mainly tetracycline, aminoglycoside, and MLS resistance genes. The combined treatment with OTC and Cu had a much greater effect on ARGs than each separate treatment. The ARGs were harbored by 20 different HPB hosts. Three ARG types comprising tetracycline, multidrug, and MLS resistance genes significantly affected resistance by HPB throughout the composting process. In addition, HPB and int11 had the most important positive effects on the ARG profiles during composting, and the co-selective pressure imposed by heavy metals was favorable for ARG enrichment.

Materials And Methods

Experimental materials

OTC and Cu were added to swine manure compost, and the effects of OTC and Cu alone and in combination on ARGs and their pathogenic hosts were compared to determine their potential environmental risks. The amounts added were determined based on the average concentrations in swine manure [29–31]. The swine manure used in this study was collected from a medium-sized farm in Yangling, Shaanxi Province, China. Feeds containing antibiotics or heavy metals were stopped for 2 weeks before sampling. Fresh feces were transported immediately back to the laboratory and stored in a refrigerator at 4°C for future use. Wheat straw was collected from an experimental field at Northwest A&F University, China. The straw was chopped to about 1–2 cm after air drying. The moisture content and C/N ratio of the initial compost were adjusted to 60% and 30, respectively. OTC (purity > 98%, Sigma) and CuCl_2 (purity \geq 99%, China) were prepared as 10 mg/mL and 50 mg/mL stock solutions, respectively. Before the experiment, the OTC and CuCl_2 stock solutions were diluted to suitable concentrations with

sterile water and mixed with pig manure. The supplementary materials shows the basic properties of the raw composting materials.

Experimental setup and sample collection

The effective capacity of the pilot-scale compost reactor was 70 L. Each reactor was filled with 25 kg of compost mixture. Four compost mixtures were tested in triplicate, where 10 mg/kg OTC (O), 500 mg/kg Cu (Cu), and 10 mg/kg OTC + 500 mg/kg Cu (OCu) were added based on the dry weight of the swine manure, and a control group (CK) was prepared without any added OTC or Cu. The entire composting process lasted 42 days and the compost was turned over every 7 days from day 3 in order to fully mix the composting matrix and reduce edge effects. At the beginning of the composting process, the mixed and balanced raw composting materials were collected as samples on day 0, and compost samples were then collected subsequently on days 10 and 42. Finally, each sample weighing 100 g was freeze dried immediately in a freeze dryer (Beijing Songyuan, China) and pulverized to 1 mm with an ultracentrifugal mill (Retsch Z200, Germany), before storing at -80°C for DNA extraction.

Determination of OTC and diethylenetriaminepentaacetic acid (DTPA)-extractable heavy metal concentrations

The total Cu and DTPA-extractable Cu contents of compost samples were determined as described previously [32]. OTC residues were determined as described Duan et al. [33] and the full details are provided in the supplementary information.

DNA extraction, library construction, and sequencing

The 0.1000 g composting sample was weighed and the total genomic DNA was extracted in triplicate using FastDNA kit (MP Biomedicals, France). DNA from each experimental treatment group was combined and mixed well. After genomic DNA extraction, the concentration was detected with a TBS-380 system, while the purity was confirmed using a NanoDrop200 system and the integrity was detected by 1% agarose gel electrophoresis. DNA was fragmented using a Covaris M220 ultrasonicator (Gene Corporation, China) and a paired-end fragment library (approximately 300 bp) was constructed using a TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA).

Paired-end metagenomic sequencing was performed on the Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, USA) using a HiSeq 3000/4000 SBS kit and HiSeq 3000/4000 PE Cluster kit according to the manufacturer's instructions. Quality filtering of the data was conducted before subsequent biological analyses by using Seqprep (<https://github.com/jstjohn/SeqPrep>) to remove the adapter sequences at the 3' and 5' ends, and low-quality reads (length < 50 bp or with a quality value < 20 or having N bases) were removed using Sickle (<https://github.com/najoshi/sickle>). After quality control, metagenomics data were assembled using MEGAHIT (<https://github.com/voutcn/megahit>). Contigs ≥ 300 bp were selected as the final assembly results and the contigs were then used for further gene prediction and annotation.

Identification of ARGs, MRGs, and MGEs

The metagenomic sequencing data obtained for each sample were compared with the structured ARG database SARG v2.0 (which integrates the sequences in the ARDB and CARD databases, as well as the latest protein sequences from the NCBI-NR database) to determine the corresponding functional annotations for ARGs [26]. MRGs were annotated by searching the BacMet database for proven MRGs (<http://bacmet.biomedicine.gu.se/index.html>). This study focused on copper resistance genes (CRGs) because Cu was applied in the experimental treatments. In addition, annotation information for MGEs was obtained from the Fungene database (<http://fungene.cme.msu.edu/>). If the best hit shared similarity with the reference sequence of not less than 80% and an alignment length of not less than 75%, then it was considered to be an like sequence. BLAST searches were conducted with a cutoff E-value $< 10^{-7}$. In order to further compare the distributions of the target gene in the microbial community, the abundance of the target gene was normalized and expressed as the target gene copy number per 16S rRNA (copy/16S rRNA).

Identification of pathogenic hosts of target genes

To further identify the hosts of the target genes, BLASTX searches were conducted for contigs with an E-value $< 10^{-10}$ to predict open reading frames (ORFs). An ORF sequence was identified as an ARG-like ORF when the optimal BLASTX hit alignment with the target gene sequence satisfied 80% similarity and 70% coverage. Hu et al. [34] suggested that this recognition method obtains high accuracy (accuracy = 99.1%). BLASTP was used to annotate the contigs carrying the target genes against the NCBI NR database for taxonomic annotation. If E-value $< 10^{-5}$, the taxonomy was determined based on a minimum similarity of 95% and a read length greater than 95%. In addition, annotation of virulence factors was conducted using BLASTP search (Version 2.2.28+) against the VFDB database (<http://www.mgc.ac.cn/VFs/>) with an E-value cutoff of 10^{-5} . Further comparisons were performed based on the HPB virulence factor database and previously published pathogen genome sequences [3, 35] to identify HPB in compost.

Real-time quantitative polymerase chain reaction(qPCR)

The partial ARG abundance of all samples was further quantified using qPCR. By normalizing the 16S rRNA genes, the units of ARG were converted into relative abundances as ARG copies of each 16S rRNA gene. The supplementary materials listed the amplification information of ARGs and 16S rRNA genes.

Statistical analysis and visualization of biological data

Principal component analysis (PCA) was performed with R 3.1.1 to distinguish the differences in ARGs between treatments during the composting process. Heat maps illustrating the changes in ARGs, MRGs, and MGEs in each sample were produced using Heml 1.0. Succession plots were obtained for pathogenic hosts of ARGs with Origin 8.5. Redundancy analysis (RDA) was performed using Canoco 5.0. Structural equation models (SEMs) were constructed in AMOS 25 using maximum likelihood estimation to isolate and order the factors related to changes in ARGs.

Abbreviations

ARG
Antibiotic resistance gene
MGE
Mobile genetic element
MRG
Heavy metal resistance gene
CRG
Copper resistance gene
HGT
Horizontal gene transfer
HPB
Human pathogenic bacteria
OTC
Oxytetracycline
Cu
Copper
MLS
macrolide–lincosamide–streptogramin
ORF
Open reading frame
DTPA
Diethylenetriaminepentaacetic acid
QPCR
Quantitative polymerase chain reaction
PCA
Principal component analysis
RDA
Redundancy analysis
SEMs
Structural equation models

Declarations

Acknowledgements

We thank Honghong Guo, Xun Qian, Wei Sun, Jing Yu, and Liusheng Lei for their assistance in field management, sampling, and lab analyses.

Authors' contributions

Honghong Guo, Jie Gu, Xiaojuan Wang, and Zilin Song designed research. Honghong Guo, Jing Yu, and Liusheng Lei performed the research. Honghong Guo, Xun Qian, and Wei Sun analyzed data. Honghong Guo wrote the paper. All authors read and approved the final manuscript.

Funding

This study was funded by the National Natural Science Foundation of China (41671474 and 41601531) and the Science and Technology Plan Key Project of Shaanxi province (2017ZDCXL-SF-03-03). We thank Dr. Duncan E. Jackson for language editing.

Availability of data and materials

The data and result supporting the findings of the study are available in this article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Nesme J, Simonet P. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. *Environ Microbiol.* 2015;17:913–930.
2. Kumar K., Gupta SC, Chander Y, Singh AK. Antibiotic use in agriculture and its impact on the terrestrial environment. *Adv Agron.* 2005;87:1–54.
3. Fang H, Wang H, Cai L, Yu Y. Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. *Environ Sci Technol.* 2015;49(2):1095.
4. Allen HK. Antibiotic resistance gene discovery in food-producing animals. *Curr Opin Microbiol.* 2014;19:25–9.
5. Ahmed W, Zhang Q, Lobos A, Senkbeil J, Sadowsky MJ, Harwood VJ, Saeidi N, Marinoni O, Ishii S. Precipitation influences pathogenic bacteria and antibiotic resistance gene abundance in storm drain outfalls in coastal sub-tropical waters. *Environ Int.* 2018;116:308–318.
6. Stachler E, Crank K, Bibby K. Co-occurrence of crAssphage with antibiotic resistance genes in an impacted urban watershed. *Env Sci Technol Lett.* 2019;6:216–221.

7. Qiao M, Ying GG, Singer AC, Zhu YG. Review of antibiotic resistance in China and its environment. *Environ Int.* 2018;110:160–172.
8. Brown ED, Wright GD. Antibacterial drug discovery in the resistance era. *Nature.* 2016;529 (7586):336.
9. O'Neill J. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Review on Antimicrobial Resistance, London. 2014.
10. Xu R, Yang ZH, Zheng Y, Wang QP, Bai Y, Liu JB, Zhang YR, Xiong WP, Lu Y, Fan CZ. Metagenomic analysis reveal the effects of oxytetracycline and copper on antibiotic resistance genes and associated pathogenic hosts in pig manure compost. *Bioresource Technol.* 2019;282:179–188.
11. Chen C, Pankowb CA, Oh M, Heath LS, Zhang L, Du P, Xia K, Pruden A. Effect of antibiotic use and composting on antibiotic resistance gene abundance and resistome risks of soils receiving manure-derived amendments. *Environ Int.* 2019;128:233–243.
12. Kathy TM, Heather KA, David PA, Julian T, Samantha JH, Johann FC, Devin BH, Steven K, Crystal LL, Susan LB. Shifts in the nasal microbiota of swine in response to different dosing regimens of oxytetracycline administration. *Vet Microbio.* 2019;237:108386.
13. Pehrsson EC, Tsukayama P, Patel S, Mejia-Bautista M, Sosa-Soto G, Navarrete KM, Calderon M, Cabrera L, Hoyos-Arango W, Bertoli MT, Berg DE, Gilman RH, Dantas G. Interconnected microbiomes and resistomes in low-income human habitats. *Nature.* 2016;533 (7602):212–216.
14. Gillings MR. Integrons: past, present, and future. *Microbiol Mol Biol R.* 2014;78:257–77.
15. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM, Wolffs PFG. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol.* 2016;7.
16. Shterzer N, Mizrahi I. The animal gut as a melting pot for horizontal gene transfer. *Can J Microbiol.* 2015;61,603–605.
17. Xiong WG, Wang YL, Sun YX, Ma LP, Zeng QL, Jiang XT, Li AD, Zeng ZL, Zhang T. Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. *Microbiome.* 2018;6:34.
18. Ubeda C, Pamer EG. Antibiotics, microbiota, and immune defense. *Trends Immunol.* 2012;33:459–66.
19. Wright GD, Poinar H. Antibiotic resistance is ancient: implications for drug discovery. *Trends Microbiol.* 2012;20:157–159.
20. Luo G, Li B, Li LG, Zhang T, Angelidaki I. Antibiotic resistance genes and correlations with microbial community and metal resistance genes in full-scale biogas reactors as revealed by metagenomic analysis. *Environ Sci Technol.* 2017;51(7):4069–4080.
21. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics.* 2015;16(1):964.
22. Imran M, Das KR, Naik MM. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: an emerging health threat. *Chemosphere*

- 2019;215:846–857.
23. Cui E, Wu Y, Jiao Y, Zuo Y, Rensing C, Chen H. The behavior of antibiotic resistance genes and arsenic influenced by biochar during different manure composting. *Environ Sci Pollut Res Int*. 2017;24:14484–14490.
 24. Zhang Y, Gu AZ, Cen T, Li X, He M, Li D, Chen J. Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. *Environ Pollut*. 2018;237:74.
 25. Yang YY, Li Z, Song WJ, Du LN, Ye C, Zhao B, Liu WZ, Deng DL, Pan YT, Lin H, Cao XH. Metagenomic insights into the abundance and composition of resistance genes in aquatic environments: Influence of stratification and geography. *Environ Int*. 2019;127:371–380.
 26. Yin XL, Jiang XT, Chai BL, Li LG, Yang Y, Cole JR, Tiedje JM, Zhang T. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics*. 2018;34 (13):2263–2270.
 27. Leplae R, Lima-Mendez G, Toussaint A. ACLAME: a classification of mobile genetic elements, update 2010. *Nucleic Acids Res*. 2010;38:D57–D61.
 28. Ma LP, Li B, Zhang T. New insights into antibiotic resistome in drinking water and management perspectives: A metagenomic based study of small-sized microbes. *Water Res*. 2019;152:191–201.
 29. Zhang SQ, Zhang FD, Liu XM, Wang YJ, Zou SW, He XS. Determination and analysis on main harmful composition in excrement of scale livestock and poultry feedlots. *Plant Nutr Fertilizer Sci*. 2005;11(6):822.
 30. Xiong X, Yanxia L, Wei L, Chunye L, Wei H, Ming Y. Copper content in animal manures and potential risk of soil copper pollution with animal manure use in agriculture. *Resour Conserv Recycl*. 2010;54:985–990.
 31. Liu N, Han H, Yin H, Han L, Huang G. Variations in the fate and risk analysis of amoxicillin and its degradation products during pig manure aerobic composting. *J Hazard Mater*. 2018;346:234.
 32. Qian X, Gu J, Sun W, Wang XJ, Stedfeld R. Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *J Hazard Mater*. 2018;344:716.
 33. Duan M, Li H, Gu J, Tuo X, Sun W, Qian X. Effects of biochar on reducing the abundance of oxytetracycline, antibiotic resistance genes, and human pathogenic bacteria in soil and lettuce. *Environ Pollut*. 2017;224:787–795.
 34. Hu YF, Yang X, Qin JJ, Lu N, Cheng G, Wu N, Pan YL, Li J, Zhu LY, Wang X, Meng ZQ, Zhao FQ, Liu D, Ma JC, Qin N, Xiang CS, Xiao YH, Li LJ, Yang HM, Wang J, Yang RF, Gao GF, Wang J, Zhu B. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat Commun*. 2013;4:2151.
 35. Cai L, Zhang T. Detecting human bacterial pathogens in wastewater treatment plants by a high-throughput shotgun sequencing technique. *Environ Sci Technol*. 2013;47(10):5433–5441.

36. Pruden A, Larsson DGJ, Amézquita A, Collignon P, Brandt KK, Graham DW. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ Health Persp.* 2013;121(8):878–885.
37. Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD. Diverse and abundant antibiotic resistance genes in chinese swine farms. *P NatL Acad Sci USA.* 2013;110(9):3435–3440.
38. Gao P, He S, Huang S, Li K, Liu Z, Xue G, Sun W. Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. *Appl Microbiol Biot.* 2015;99:3971–3980.
39. Guo H, Xue S, Nasir M, Lv J, Gu J. Role of Bentonite on the Mobility of Antibiotic Resistance Genes, and Microbial Community in Oxytetracycline and Cadmium Contaminated Soil. *Front Microbiol.* 2018;9.
40. Ji X, Shen Q, Liu F, Ma J, Xu G, Wang Y, Wu M. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. *J Hazard Mater.* 2012;235:178–185.
41. Pal C, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson DJ, Hobman JL. Metal resistance and its association with antibiotic resistance. *Adv Microb Physiol.* 2017;70:261–313.
42. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 2006;14:176–182.
43. Rensing C, Grass G. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol Rev.* 2003;27(2-3):197-213.
44. Brown N, Barrett S, Camakaris J, Lee B, Rouch D. Molecular genetics transport analysis of the copper-resistance determinant *pco* from *Escherichia coli* plasmid pRJ1004. *Mol Microbiol.* 1995;17:1153–1166.
45. Ma L, Li AD, Yin XL, Zhang T. The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. *Environ Sci Technol.* 2017;51:5721–5728.
46. van Hoek AHAM, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. Acquired antibiotic resistance genes: an overview. *Front Microbiol.* 2011;2.
47. Cury J, Jove T, Touchon M, Neron B, Rocha EPC. Identification and analysis of integrons and cassette arrays in bacterial genomes. *Nucleic Acids Res.* 2016;44 (10):4539–4550.
48. Mazel D. Integrons: agents of bacterial evolution. *Nat Rev Microbiol.* 2006;4(8):608–20.
49. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev.* 2018;31 (4).
50. Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse antibiotic resistance genes in dairy cow manure. *mBio.* 2014;5(2):e01017–3.
51. Zhang X, Li Y, Liu B, Wang J, Feng C, Gao M, Wang L. Prevalence of veterinary antibiotics and antibiotic-resistant *Escherichia coli* in the surface water of a livestock production region in northern China. *PLoS One.* 2014;9(11):e111026.

52. Wong KY, Chan YC, Wong CY. *Corynebacterium striatum* as an emerging pathogen. *J Hosp Infect.* 2010;76(4):371–372.
53. McMullen AR, Anderson N, Wallace MA, Shupe A, Burnham CAD. When good bugs go bad: epidemiology and antimicrobial resistance profiles of *Corynebacterium striatum*, an emerging multidrug resistant, opportunistic pathogen. *Antimicrob Agents Ch.* 2017;61(11).
54. Willems RJL, Top J, Van Schaik W, Leavis H, Bonten M, Siren J. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *mBio.* 2012;3(4):e00151–12.
55. Silveira E, Freitas AR, Antunes P, Barros M, Campos J, Coque TM. Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. *J Antimicrob Chemoth.* 2014;69(4):899–906.
56. Winkler ML, Papp-Wallace KM, Hujer AM, Domitrovic TN, Hujer KM, Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse antibiotic resistance genes in dairy cow manure. *mBio.* 2014;5(2):e01017–3.
57. Han XM, Hu HW, Chen QL, Yang LY, Li HL, Zhu YG, Li XZ, Ma YB. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol Biochem.* 2018;126:91–102.
58. Zhang JY, Liu JB, Lu TD, Shen PH, Zhong H, Tong J, Wei YS. Fate of antibiotic resistance genes during anaerobic digestion of sewage sludge: role of solids retention times in different configurations. *Bioresource Technol.* 2019;274:488–495.
59. Rosewarne CP, Pettigrove V, Stokes HW, Parsons YM. Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol Ecol.* 2010;72:35–46.
60. Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stokes HW, McArthur JV. Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J.* 2008;2(4):417–428.

Figures

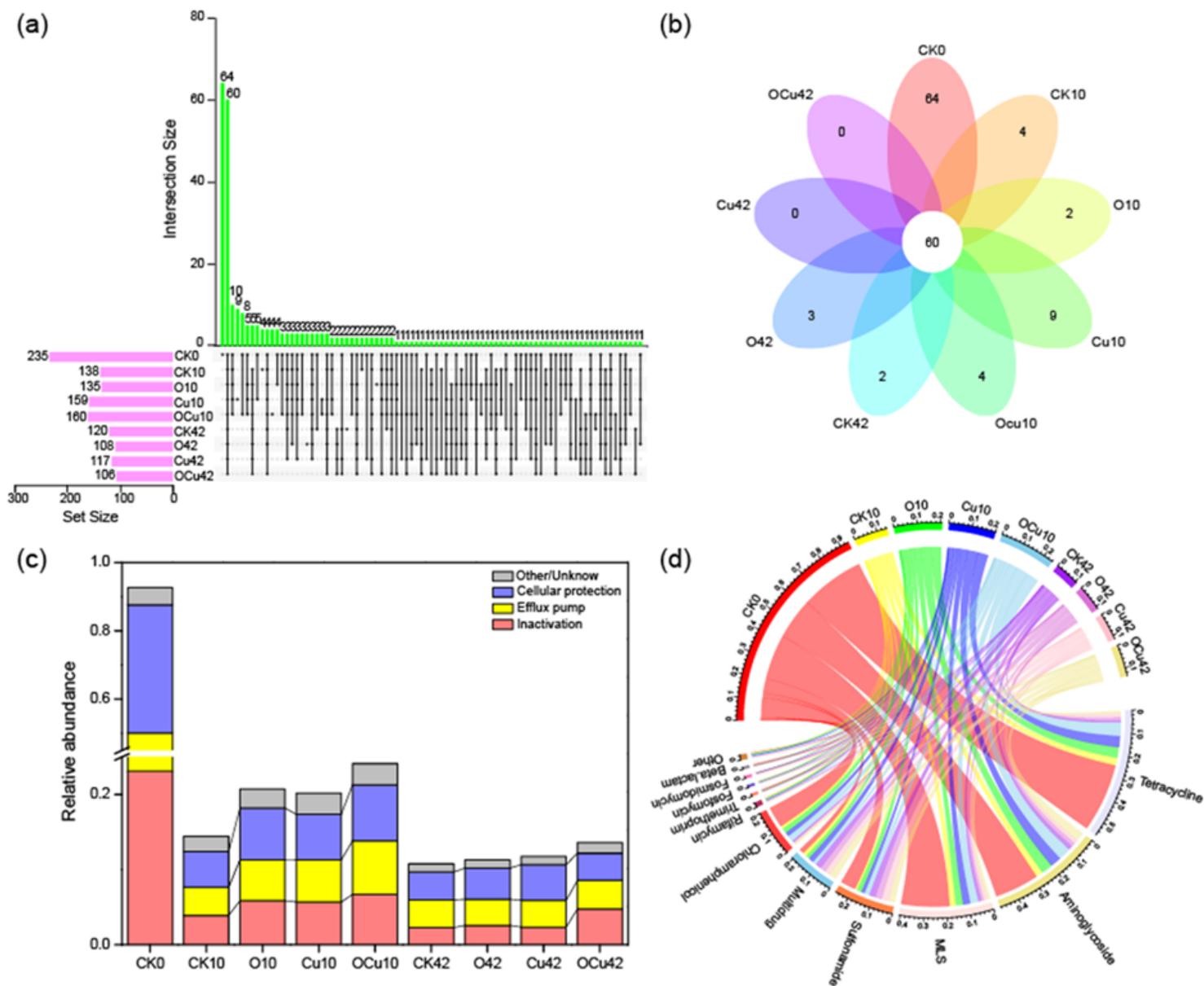


Figure 1

Setup venn and venn diagram showing the numbers of unique and shared ARGs during composting under different treatments (a and b). Changes in abundances of ARGs with different resistance mechanisms during composting (c). Changes in types and abundances of ARGs during composting (d). In the setup venn diagram, the horizontal histogram on the left represents the number of ARGs in each sample, a single point in the middle matrix represents a unique ARG present in the sample, the lines between points and points indicate ARGs that coexisted in different samples, and the vertical histogram represents the corresponding ARG number. MLS: macrolide–lincosamide–streptogramin; CK: with no added OTC or Cu; O: 10 mg/kg OTC; Cu: 500 mg/kg Cu; OCu: 10 mg/kg OTC + 500 mg/kg Cu.

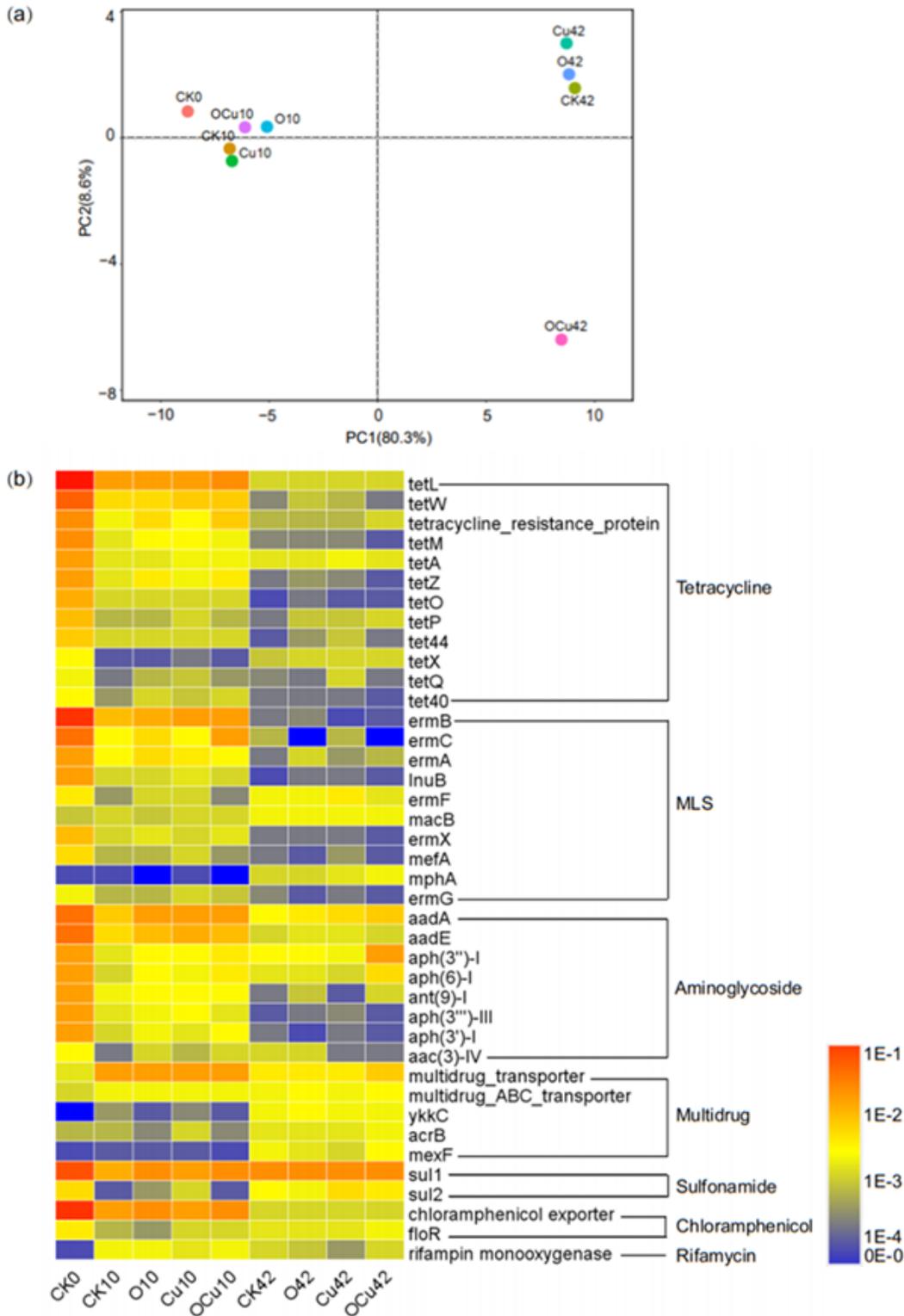


Figure 2

Variations in ARG subtypes during composting under different treatments. Principal component analysis of ARG subtypes (a). Heatmap showing the variations in ARG subtypes (average abundance > 1 × 10⁻³ copies/16S rRNA) during composting (b). MLS: macrolide–lincosamide–streptogramin; CK: with no added OTC or Cu; O: 10 mg/kg OTC; Cu: 500 mg/kg Cu; OCu: 10 mg/kg OTC + 500 mg/kg Cu.

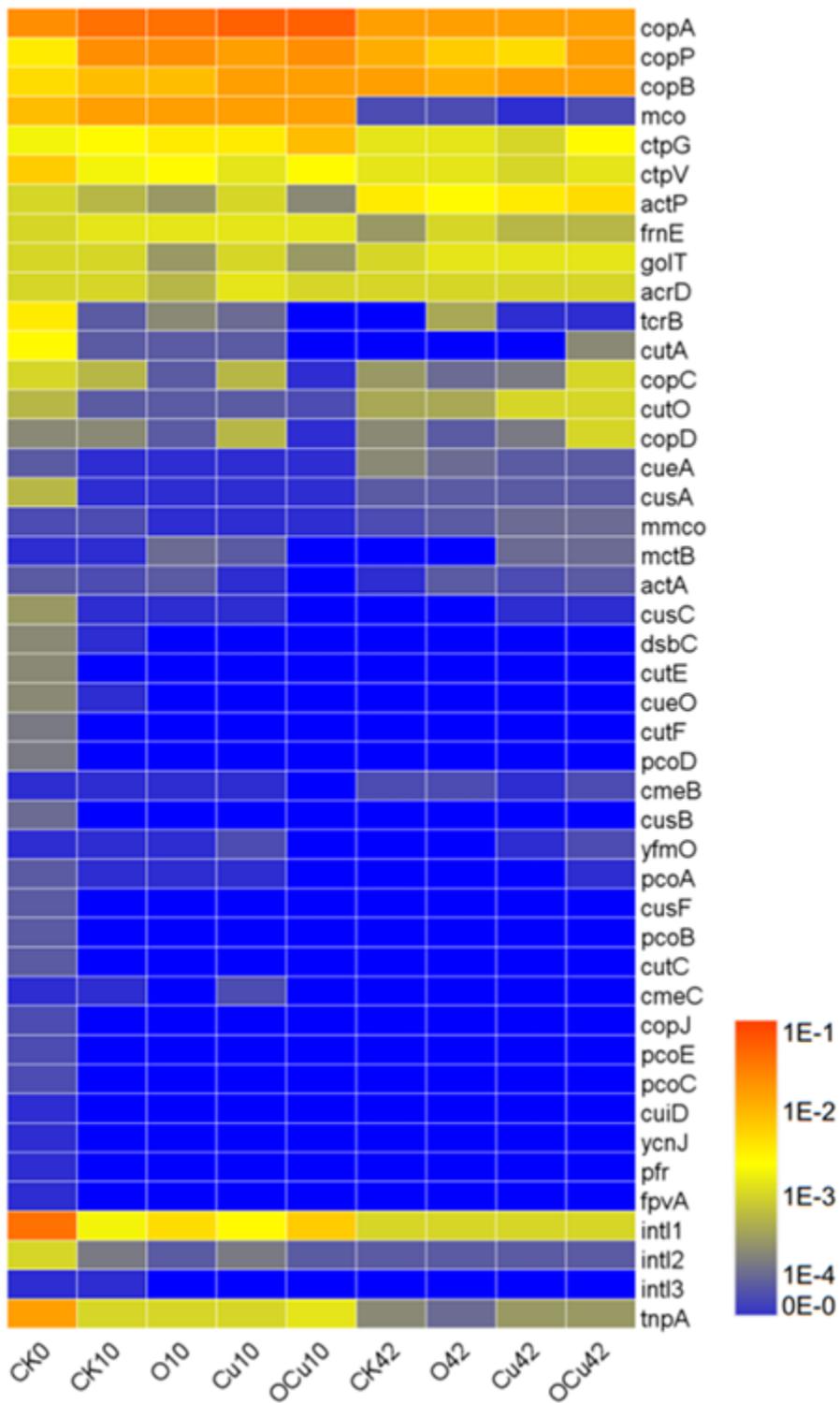


Figure 3

Changes in abundances of CRGs and MGEs during composting under different treatments. CK: with no added OTC or Cu; O: 10 mg/kg OTC; Cu: 500 mg/kg Cu; OCu: 10 mg/kg OTC + 500 mg/kg Cu.

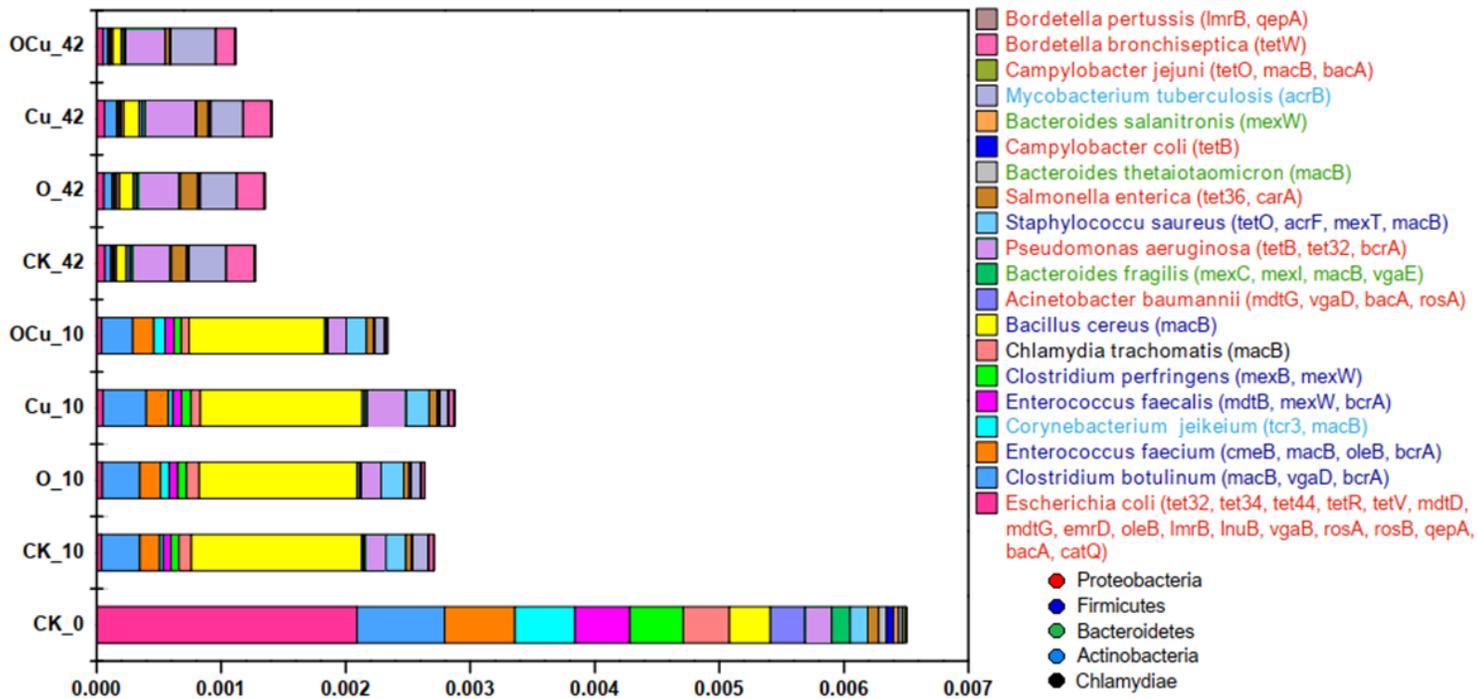


Figure 4

Dynamic changes in pathogenic hosts of ARGs during composting. ARGs in brackets indicate that they were carried by HPB. CK: with no added OTC or Cu; O: 10 mg/kg OTC; Cu: 500 mg/kg Cu; OCu: 10 mg/kg OTC + 500 mg/kg Cu.

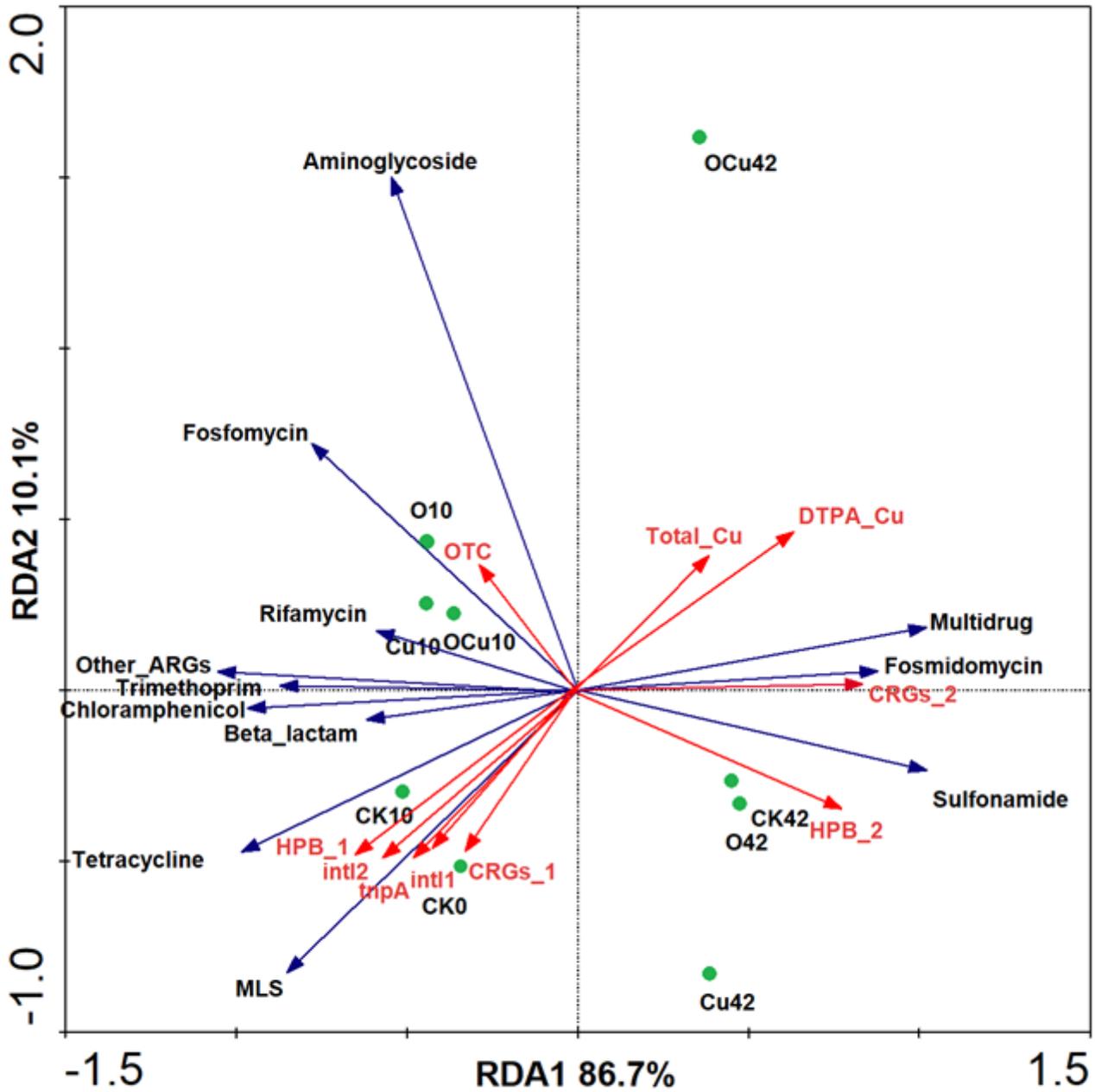


Figure 5

Redundancy analysis based on changes in HPB, MGEs, CRGs, and ARGs. ARGs were treated as species variable (dark blue lines) and others as environmental variables (red lines). HPB1-2 and CRG1-2 were extracted from the two principal axes obtained for HPB and MRGs by principal component analysis. MLS: macrolide–lincosamide–streptogramin; CK: with no added OTC or Cu; O: 10 mg/kg OTC; Cu: 500 mg/kg Cu; OCu: 10 mg/kg OTC + 500 mg/kg Cu.

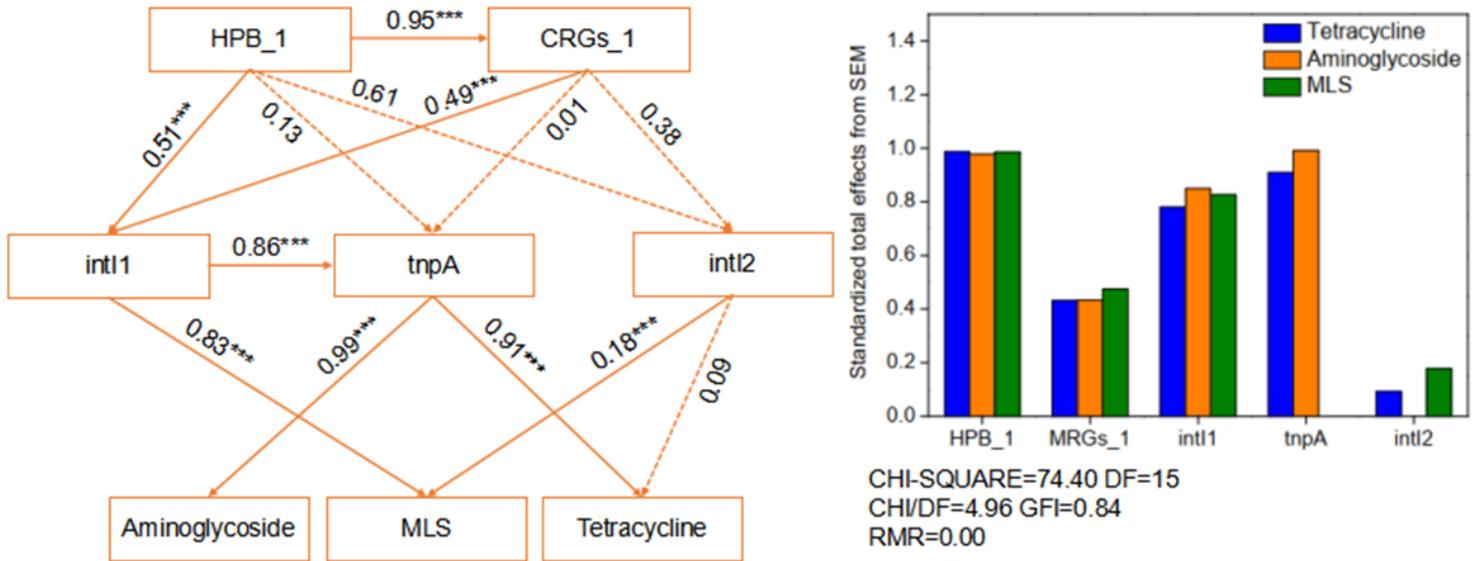


Figure 6

Structural equation models showing the direct and indirect effects of HPB_1, CRG_1, tnpA, int1, and int2 on ARG profiles during composting. Solid and dashed arrows indicate significant and nonsignificant relationships, respectively. Significance levels are indicated as: *P < 0.05, **P < 0.01, and ***P < 0.001. Standardized total effects (direct plus indirect effects) were calculated based on the structural equation models. MLS: macrolide–lincosamide–streptogramin.

Supplementary Files

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