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Application of pharmaceutical waste sludge compost alters the antibiotic resistome in soil under the Chinese cabbage system

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Highlights

- Cabbage associated with resistome was measured in amended soils.
- ARG profiles in amended soil and phyllosphere of cabbage were closely clustered.
- The amendment of compost might improve the accessibility of resistome in the soil especially for the Chinese cabbage system.

ABSTRACT

Antibiotic resistance is a global threat posing risks to public health. China, as the largest consumer and producer of antibiotics, is generating a large amount of pharmaceutical waste sludge from the antibiotic manufacturing industry, which has the potential to be released into the environments by anthropogenic activities. Land application of pharmaceutical waste sludge compost (PWSC) is a popular way of PWSC disposal, with large amount of antibiotics might hence be introduced into the soil environments and result in the development of antibiotic resistance genes (ARGs). ARGs in PWSC amended soil-plant systems, their transmission routes and potential ecological risks are still unknown. A high-throughput qPCR chip was used to profile ARGs in Chinese cabbage (Shanghaiqing, *Brassica chinensis* L.) and soils (including phyllosphere, root endosphere, rhizosphere soil, and bulk soil) with PWSC amendment, aiming to study the effect of PWSC application on ARGs soil-plant systems. A total of 249 ARGs and 12 mobile genetic elements (MGEs) were detected in all collected samples. The highest number of detected ARGs was in the soil samples (up to 181) compared to the above and below-ground components of Chinese cabbage. Our results demonstrated the PWSC amendment increased the diversity and normalized abundance of ARGs in the amended soil-Chinese cabbage system. Mantel test and Procrustes analysis revealed

the connection between ARG profiles and microbial communities (fungal and bacterial communities). Shared ARGs were identified among the Chinese cabbage phyllosphere, Chinese cabbage root, and rhizosphere soils, demonstrating a potential link between antibiotic resistome in Chinese cabbage and soils, with the amended soil as a key source of ARGs in the phyllosphere of Chinese cabbage. In summary, our findings provided novel evidence for a transmission route shared Zero-radius operational taxonomic units (ZOTUs) and ARGs were passed between the soil-Chinese cabbage system elements (i.e., amended soil, root, and phyllosphere) of ARGs from the PWSC amended soils to common green vegetables, highlighting a potential safety hazard of antibiotic resistome transfer from soils to the human food chain.

Keywords: Chinese cabbage; antibiotic resistome; pharmaceutical sludge; compost-amended soil; composting.

1. INTRODUCTION

Antibiotic resistance has become notorious in human pathogens (Allen et al., 2010). The spread of antibiotic-resistant bacteria (ARB) in the environment is contributing to the global crisis of clinically relevant antimicrobial resistance (AMR). Environmental pollution of ARB and antibiotic resistance genes (ARGs) can be attributed to commonly use of antibiotics in the animal industry (farm and agricultural industries), which are linked to anthropogenic activities such as intensive use of bio-organic fertilizers (e.g. animal manures and sewage sludge) in the agriculture (Chen et al., 2016; Chen et al., 2017; Cheng et al., 2013; Liu et al., 2019).

Appropriate usage of organic fertilizers on farmland can increase soil organic matter content,

67 but offering a pathway to recycle nutrients in the soil (Martínez Salgado et al., 2019). Studies have
68 proved that the unregulated use of untreated organic fertilizers can increase levels of antibiotic
69 resistome in farm settings (Chen et al., 2017; Su et al., 2015; Xie et al., 2016a). These antibiotics
70 and enriched ARGs in farm settings could further transfer from untreated organic wastes to soils
71 and gradually accumulate in vegetation (by absorption from the soil). For instance, supererogatory
72 ARGs were detected in tomato and Chinese cabbage after the use of swine excrement and struvite
73 as a soil amendment (An et al., 2018; Romain et al., 2013). Consumption of green products,
74 particularly raw greens, represents a typical pathway of direct human exposure to the soil
75 microbiome (Chen et al., 2019). ARGs can enter the human food chain or human pathogens via the
76 contaminated plants (e.g. fruits and vegetables) or via horizontal gene transfer (HGT), posing a risk
77 to human health (Amy et al., 2006; Gillings, 2017). China is one of the world's largest producers
78 and consumers of antibiotics (Qiao et al., 2018; Zhang et al., 2015; Zhu et al., 2013). Vast quantities
79 of pharmaceutical waste sludge (PWS) are produced from antibiotic manufacturing and wastewater
80 treatment processes. PWS harbors large amounts of mobile genetic elements (MGEs) and ARGs
81 (Tao et al., 2016), with a number of studies suggesting that PWS harbors more MGEs and ARGs
82 compared with the municipal waste sludge (Liu et al., 2014; Liu et al., 2012; Tong et al., 2017).
83 Waste sludge is often pre-treated before the land application (e.g. via aerobic composting,
84 anaerobic digestion, or sludge carbonization), with aerobic composting widely regarded as an
85 efficient way to minimize chemical and biological hazards from organic wastes (Bernal et al., 2009;
86 Liu et al., 2019; Su et al., 2015).
87 Previous studies have demonstrated that aerobic composting was promoted as an effective
88 means to reduce the concentration of antibiotics and ARGs in waste sludge (Selvam and Wong,

89 2017; Xie et al., 2016b; Zhang et al., 2019). Antibiotic resistome is a resistance reservoir of all the
90 ARGs and precursor genes in the pathogen and nonpathogen. It includes resistance elements carried
91 by both antibiotic-producing bacteria and pathogenic bacteria, and cryptic resistance genes (Wright,
92 2007). Whilst, ARGs can be effectively reduced through composting, the antibiotic resistome will
93 not be eliminated. Using Pharmaceutical Waste Sludge Compost (PWSC) as an organic fertilizer,
94 therefore, represents an underlying origin of human exposure to antibiotic resistome during
95 subsequent consumption of vegetables grown in compost-amended soils.

96 Our understanding of the fate of antibiotic resistome by following the application of composted
97 waste, has seldom been evaluated. In particular, the effect on indigenous soil, root, and phyllosphere
98 antibiotic resistome is a key knowledge gap. It is therefore imperative to elucidate the factors
99 influencing the fate of antibiotic resistome, which follows PWSC application to agricultural land in
100 order to develop effective tactics and technologies to alleviate the rapid spread of antibiotic
101 resistome.

102 High-throughput quantitative PCR (HT-qPCR) with 296 recognized primer sets covering
103 common 12 MGEs and 283 ARGs marker genes in the existing research (Zhu et al., 2013), was
104 employed together with Illumina sequencing of fungi ITS genes and bacterial 16S rRNA genes.
105 Analysis was targeted to: (1) assess the fate of four types of waste compost on the molecular
106 structure of ARGs, bacterial and fungal communities in the soil-Chinese cabbage system; (2)
107 explore the potential migration route of ARGs from the amended soil to Chinese cabbage, and (3)
108 identify the shared MGEs and ARGs between the Chinese cabbage and amended soil by bipartite
109 network analysis. The pot experiment aimed to assess the potential ecological risk of the use of
110 pharmaceutical waste sludge compost. We explored the impacting factors of ARGs in the crops and

proposed suggestions for agricultural utilization of pharmaceutical waste sludge compost.

2. MATERIALS AND METHODS

2.1 Properties and Materials

Four types of compost (refer to T1-T3 and S) with a range of characteristics by following production under different composting treatments were used in this pot experiment. Three types of PWSCs (coded T1-T3) were derived from carbon-rich amendments of pharmaceutical sludge compost. Sewage sludge compost (S) was obtained from an online sales platform (Ningbo, China) (Table 1). The PWSCs were air-dried, prior to being used in the experiments. Soils were collected from a vegetable plot (Ningbo, China), which had not received sewage sludge or been amended with other organic manure in the past five years. Prior to use, soils were sieved (mesh size: 2mm) and air-dried. Chemical and physical properties are described in Table 1, including the contents of heavy metals, antibiotics, total carbon, and total nitrogen in the initial samples. Total nitrogen and total carbon were evaluated via dry combustion in a CNS element analyzer (*Vario MAX, Elementar*) (Chen et al., 2017). Concentrations of antibiotics were analyzed by liquid chromatography-mass spectrometry (*LC-MS/MS. XevoG2-SQTOF, Waters*) (details are provided in Supplementary file). The content of heavy metals was determined with inductively coupled plasma- mass spectrometer (*ICP-MS, iCAP Qnova, Thermo Scientific*) after oxidative digestion of samples in sealed microwave digestion tubes (Zhu et al., 2013). The sample pH was determined by preparing a 1:5 solid sample/water (w/v) suspension (*Basic pH Meter, PB-10, Sartorius*). Determination of nitrate and ammonium in the soil was performed using an AA3 Continuous Flow Analytical System (*AA3, SEAL Analytical GmbH*) (Wang et al., 2019).

133 *2.2 Experiment Design*

134 Figure S1A shows a schematic of the experimental design. Each pot consisted of 1kg of soil
135 amended with or without 5% PWSC (w/w, dry weight) (Luo et al., 2016). Five treatments were
136 arranged: untreated soil without fertilizer application (hereinafter called CK), PWSC-T1 amended-
137 soil (hereinafter called T1), PWSC-T2 amended-soil (hereinafter called T2), PWSC-T3 amended-
138 soil (hereinafter called T3), and S amended-soil (hereinafter called S).

139 Each treatment was replicated for three times, giving 15 pots in total (Fig S1A). Soil samples
140 were then planted with Chinese cabbage (Shanghaiqing, *Brassica chinensis* L.) in rhizo-bags to
141 separate the bulk soil from rhizosphere soil. Each rhizo-bag contained 160g soil (30 μm nylon mesh,
142 4cm diameters, 6cm height), which only allowed the penetration of small molecular weight
143 compounds and prohibited the penetration of plant roots.

144 In this experiment, 14-day-old Chinese cabbage seedlings were potted in the soil and treated
145 with different fertilizer regimes in a greenhouse experiment located at the Institute of Urban
146 Environment, Chinese Academy of Sciences. Each pot was transplanted with 3 plants. Only one
147 plant was kept after survival. The experiment was conducted at room temperature (25-30 ℃) with
148 the relative humidity maintained at 70%. On a daily basis, deionized water was used for each pot to
149 maintain the soil water content throughout the experiment. All samples, including bulk soil,
150 rhizosphere soil, root, and Chinese cabbage leaves were collected after 60 days. The plants were
151 harvested at full maturity.

152 All harvested samples were placed into aseptic bags and delivered on ice to the lab promptly
153 after collection. A portion of the soil was sieved (mesh size: 2mm) to determine the selected soil
154 properties and the rest was stored at −80 °C for DNA extraction. Plant samples were brought back

155 to the laboratory immediately for processing (Fig S1B).

156 *2.3 Extraction and Purification of DNA Samples*

157 DNA was extracted from soil (bulk and rhizosphere soil) according to the operation guide
158 (*FastDNA Spin Kit for Soil (MP Biomedical, CA)*). Phyllosphere DNA was extracted according to
159 previously published methods with slight modifications (Chen et al., 2017; Duran et al., 2018;
160 Marasco et al., 2018).

161 Within 8 hours after harvesting, epiphytic microbes were isolated from the phyllosphere using
162 extensive shaking in sterile water and phosphate buffer (8.5 g L^{-1} Na_2HPO_4 anhydrous, 6.33 g L^{-1}
163 NaH_2PO_4, and pH = 6.5) added with 0.1% Triton X-100. Flushing liquid was stored as the epiphytic
164 fraction after filtering through 0.22 μm pore size membranes. The extraction for DNA in the filtrate
165 was processed using the *FastDNA Spin Kit for Soil*.

166 Root samples were rinsed with 80% ethanol and 0.25% NaClO to wash living microorganisms
167 of the root surfaces and followed by a sterile water-rinse step three times (at 1 min each). The
168 efficiency of the sterilization was evaluated through culturing on agar medium plates (15 g L^{-1} agar,
169 3 g L^{-1} $MgSO_4$, 10 mL L^{-1} glycerol, 1.5 g L^{-1} K_2HPO_4, 10 g L^{-1} hydrolyzed casein, and 10 g L^{-1}
170 protease peptone for solid medium). The root was treated and homogenized by liquid nitrogen
171 grinding plus. Then DNA was extracted with a *FastDNA Spin Kit for Soil* (Figure S1B).

172 *2.4 Microbial Community Profiling*

173 The DNA concentration of samples was determined through fluorescence quantitative analysis.
174 Samples were diluted to 20 ng μL^{-1} for downstream a two-step PCR amplification (Chen et al.,
175 2017). In the first step, the V4-V5 variable region of bacterial *16S rRNA (515F-907R)* (Jing et al.,
176 2015) and *fungal primers (gITS7 - ITS4)* (Ihrmark et al., 2012) were amplified (Table S1).

177 PCR amplification was performed in sterile conditions with technical triplicates. Details of the
178 PCR amplification program and reaction system were presented in Table S2. PCR products were
179 checked on a 1% agarose gel with negative control. Purification was followed for replicated
180 reactions: 1) the bacterial amplicons recovery of purified products was carried out following
181 previous methods (Chen et al., 2019; Zhu et al., 2018); and 2) fungal amplicons were purified using
182 the *Universal DNA Purification kit DP214*.

183 The DNA concentration was measured again with fluorescence quantitation analysis, and
184 pooling 300 ng DNA of each barcoded amplicon in one library for the relevant microbial group.
185 Barcoded amplicons sequencing was implemented with the *Illumina Hiseq2500* platform
186 (*MICROANALY, Hefei, China*).

187 *2.5 16S rRNA Gene and ITS Read Processing*

188 Raw data were pre-processed in *VSEARCH v2.12.0* (Rognes et al., 2016) and *USEARCH v10.0*
189 (Edgar, 2013), Zero-radius operational taxonomic units (ZOTUs) were denoised using the
190 *UNOISE3* (Edgar, 2016) algorithm with a 100% sequence similarity. Double-end amplicon
191 sequences were merged (minimum 50 bp overlap). After removing primers and barcode, chimeras,
192 and quality filtered (largest prospective error threshold of 1.0) were removed with *USEARCH v10.0*,
193 the rest of the high-quality amplicon reads were devoted to downstream analysis. Taxonomical
194 classification based on ZOTUs was performed using *VSEARCH v2.12.0* and the *Silva/UNITE* (*Silva*
195 *123/UNITE 8.0*) database (Quast et al., 2013).

196 ZOTUs of samples that were in low mass number (< 10 sequence compositions) generally
197 represent a PCR or sequencing error and were removed from the samples. All raw sequences were
198 stored in the public databases (*National Center for Biotechnology Information Sequence Read*

199 *Archive*). That session specification is numbered SRP227163. Alpha-diversity for each sample was
200 profiled through a measurement of Chao1 index, Buzas_gibson index and Shannon_e index, and
201 box plots were described to compare the diversity level of fungal and bacterial ZOTU.

202 *2.6 HT-qPCR and Data Analysis*

203 SmartChip Real-time PCR system (*Wafergen Inc.*, USA) was employed with HT-qPCR, which
204 is specifically used for large gene expression studies (Wang et al., 2014). Compared with traditional
205 qPCR, this system is a new dedicated high-throughput processing chip, which could greatly
206 accelerate the performance of all the levels. Compared with shotgun metagenomic sequencing, the
207 proposed system is more advantageous than shotgun metagenomic sequencing in operation,
208 analytical method, and absolute quantification analysis, although it might not have a big database
209 (Xiang et al., 2020). Primer sets targeting 283 ARGs, 12 marker genes for mobile genetic elements
210 (MGEs) (Ouyang et al., 2015) including of four universal integron-integrase genes (*intI-1(clinic)*,
211 *intI-1LC*, *intI2,* and *intI3*) and eight transposase genes, and one 16S rRNA gene (Table S5) were
212 included in recent research work (Zheng et al., 2019).

213 The program and reaction system of HT-qPCR amplification was performed as outlined in
214 Table S2. The qPCR results were examined employing SmartChip qPCR Software. Wells with PCR
215 efficiencies are not in the normal range (1.8-2.2) or with multiple melting peaks were removed.
216 Then, according to eq 1, the amplification efficiency was converted to 2. Each experiment was
217 conducted and repeated three times and a detectable threshold cycle (CT) limit value was set to 31
218 (Zhu et al., 2013).

219 In the end, a valid value was determined only if all the repeated trials had amplification. The
220 normalized copy number of MGEs and ARGs was measured and transformed to absolute gene copy

numbers and normalized to 16S rRNA gene copy numbers, which were quantified respectively from the Wafergen platform (eq 2) (Chen et al., 2016). To minimize error as a result of differences in 16S rRNA gene abundance between samples, a normalized copy number of ARGs per bacterial cell were used and calculated as follows (eq 3). (Klappenbach et al., 2001; Stalder et al., 2014; Zhu et al., 2018)

$$C_T = C_T' \log_2 E \quad \text{(eq1)}$$

$$\textit{Relative Gene Copy Number} = 10^{(31-CT)/(10/3)} \quad \text{(eq2)}$$

$$\textit{Normalized copy number of ARG gene} = (\textit{Relative ARG gene copy number / Relative 16S rRNA gene copy number}) \times 4.1 \quad \text{(eq3)}$$

Where C_T means the threshold value, 4.1 represents the average number of 16S rRNA gene per bacterium, the related calculation is applied according to the Ribosomal RNA Operon Copy Number Database

2.7 Statistical Analysis

All data analyses in the plots that follow (e.g. box plots) were computed with the default settings of ggplot2 (Hadley Wickham et al., 2016) for the software R (R Team, 2010). Statistical correlation analysis (Spearman's correlation) was undertaken through SPSS software (PASW Statistics 18.0). A co-association network of ARGs was performed using R with the psych packages and igraph (Adair et al., 2018;Csardi and Nepusz, 2006). Visualized bipartite network analysis was based on Gephi.

Based on Bray-Curtis distance, the paper makes a PCoA analysis of the ARGs, bacterial, and fungi community profiles. Procrustes analysis and Mantel analysis of correlation between ARGs, bacterial, and fungal communities were used in R package, vegan (Oksanen et al., 2011).

3. Results and Discussion

3.1 Distribution of MGEs and ARGs in the Soil-Chinese Cabbage System

A total of 12 MGEs and 249 ARGs were detected among all samples, which was higher than previously reported for ARGs in soil samples (Chen et al., 2017; Zhang et al., 2019). The diversity of MGEs and ARGs ranged from 25 to 181 in all samples, with phyllosphere (CK) and rhizosphere soil (soil amended with PWSC T3/S) harboring the lowest (25) and the highest (181) detected number of ARGs, respectively. One possible explanation of this result is that the rhizosphere is usually considered as the most active area in plant microbiome and rhizospheric microorganism has the potential of increasing the spread of ARB and ARG around the rhizosphere.

A total of 249 ARGs were identified, representing nine types, of which the majority belonged to vancomycin resistance genes, beta-lactams, tetracycline, and MLSB resistance genes (Fig 1). The detection frequency of the initial soil was similar to the CK at the end of the experiment, which was statistically lower than in the soil samples amended with compost (Adonis, $P < 0.05$). After compost incorporated into the soil, diversity of MGEs and ARGs was raised in each sample, including soil, root endophytes, and phyllospheric samples.

ARGs were detected by HT-qPCR in all samples. This proved that ARGs generally exist in the environment (D'Costa et al., 2006). The number of ARGs detected in phyllospheric samples was lower than those in the soil and root endophyte samples following amendment with different fertilizer treatments (Fig 1). This could be a result of the fact that the soil bacterial community was in direct contact with the composted material, which contains high levels of antibiotics and ARGs (Zhu et al., 2017). The number of MGEs and ARGs detected in the initial soil and compost samples combined was less than the detected number of MGEs and ARGs in the soil amended with compost.

This phenomenon can be well explained by the following two reasons: (1) the application of organic fertilizer effectively facilitated microorganism activity in the soil and then promoted the spread of antibiotic resistance (Zhu et al., 2013), and (2) the antibiotic residues in PWSCs acted as selective agents and enriched ARGs in the soil (Baym et al., 2016; Nolivos et al., 2019).

The change of normalized abundance and the absolute abundance of MGEs and ARGs were different in soils amended with different composts. Figure S2 showed the absolute abundance of ARGs in all soil and phyllospheric samples ranged from 6.16×10^8 to 1.68×10^{10} and from 3.06×10^7 to 1.38×10^8 copies g^{-1} solid (dry weight), respectively. It is clear that following compost (T1, T2, T3, and S) amendment, the absolute abundance of ARGs in soil, rhizosphere, and phyllosphere were raised (Fig S2). Compared with CK, the T1 amendment significantly increased ($p < 0.05$) the absolute abundance of ARGs in rhizosphere soil samples, while S amendment significantly raised ($p < 0.05$) ARGs in soil and phyllospheric samples, suggesting that S compost may also have a strong effect on the Chinese cabbage antibiotic resistome (Fig S2). Our results showed that the S-compost consistently resulted in the highest concentration of antibiotic residues as well as the largest number of ARGs (Fig 1 and Table S1), which infers that the antibiotic residues might be responsible for the elevated number of ARGs.

Based on the comparison of normalized results of MGEs and ARGs in bacterial cell numbers, we evaluated normalized abundance levels of MGEs and ARGs in the total microbial community (Fig S3). In the bulk soil, compost (T1, T2, T3, and S) applications consistently led to increases in the normalized abundance of MGEs and ARGs (Fig S3). In the rhizosphere soil, root endophytes, and phyllospheric samples, whilst there were differences in the normalized abundance of MGEs and ARGs, the increase in abundance did not always coincide compared with CK (Fig S3). In all samples,

287 the bulk soil harbored a greater resistome than the rhizosphere soil and the absolute abundance of
288 MGEs and ARGs in all soil samples were greater than that in the phyllosphere (Fig 2).

289 Our results were consistent with a previously published research confirming that the
290 rhizosphere was a key area for propagation and spread of antibiotic resistome and horizontal transfer
291 of ARGs (Zhang et al., 2019). Previous studies have postulated that the rhizosphere microbes
292 provide a range of ecosystem services for plants, such as nutrient acquisition, and abiotic stress
293 tolerance (Meena et al., 2017; Mendes et al., 2014).

294 Hence, this study further highlights the importance of studying ARGs and ARB in the
295 rhizosphere. Diverse and abundant ARGs were detected in different environments using different
296 new molecular tools. The metagenomic approach is considered as a feasible method for studying
297 the environmental dimension of antibiotic resistance. For example, Li et al. (2015) identified 260
298 ARG from 6 different typical environment samples including soil, water, sediment, excrement,
299 wastewater biofilm, and sludge. Moreover, diverse ARGs (381 ARG subtypes) were also detected
300 in urban sewage samples from 32 municipal wastewater treatment plants by Su et al. (2017). High-
301 throughput qPCR based ARG chip as another popular approach for studying ARGs in environment,
302 has the advantage of measuring the absolute abundance of ARGs, which provide a more intuitive
303 way to describe the potential risk. We propose combining various alternative methods might have
304 great potentials in providing more comprehensive understanding of ARGs in environments and their
305 dissemination.

306 In addition to the changes in the diversity and abundance of MGEs and ARGs, even ARG
307 subtypes and MGEs turned out to have more variants (interactions) in network analysis (correlation
308 coefficient > |0.6| and P-value < 0.05), mostly in large proportions (Fig S4). Co-occurrence styles

among MGEs and ARG subtypes in the soil-Chinese cabbage system were evaluated using network analysis. Network analysis results suggested that the use of composts (T1, T2, T3, and S) might promote the propagation and spread of ARGs by way of HGT (Fig S4, Table S1). Of all networks, the node was considered the "hub", chiefly meaning the most continually connected or the most abundant node in each network. For example, *vanC2/vanC3* was the hub gene for the network in the control soil-Chinese cabbage system (CK) (Fig S4A), *ermX* was a "hub" gene for the network in T1 amended-soil (Fig S4B), $bla_{\text{CTX-M-01}}$ was a "hub" gene for the network in T2 amended-soil (Fig S4C), *tetG-01* was a "hub" gene for the network in T3 amended-soil (Fig S4D) and $bla_{\text{IMP-02}}$ was a "hub" gene for the network in S amended-soil (Fig S4E).

Our network analyses also indicated that MGEs co-occurred with diverse ARG subtypes in cabbage-soil systems. Among the detected MGEs, the transposase genes (e.g., *tnpA*-01, *tnpA*-02, *tnpA*-04 and *tnpA*-05) and the integrase genes (e.g., *intI-1LC* and *intI-1(clinic)*) showed significant co-occurrence with related ARGs in the S amended soil-Chinese cabbage system, with matching degrees of 30, 33, 32, 32, 30 and 30 (Fig S4E). Bray-Curtis distance and PCoA analysis (Fig S5) indicated that the structure and distribution of ARGs in root endophytes and phyllospheric samples were significantly different from soil samples (Adonis, $P < 0.01$).

Network analysis is deemed to be one of the most effective methods to uncover the co-occurrence patterns of MGEs and ARGs (Forsberg et al., 2014; Li et al., 2015). Network analysis carried out in this study, supported previous observations regarding the relationship between ARGs and MGEs (An et al., 2018; Li et al., 2015; Quintela-Baluja et al., 2019). Network analysis also demonstrated that the relationship between ARGs and MGEs became more intertwined and complex following the introduction of compost into the soil eco-system. The ARGs exhibited a complex

interaction with the MGEs, resulting in a more uniform deformation compared with the previous study (Liu et al., 2019). Co-occurrence networks represented that there was an "easy correlation" between two genes, but without unequivocal demonstrable evidence of direct association and interaction. It still needs further identifying specific ARGs-MGEs interactions and associates them with microbial community structures and ecological functions.

3.2 Microbiota in Soil and Chinese Cabbage Samples

A total of 3,924-69,847 and 2,525-93,570 effective tags were acquired for the bacterial and fungal communities, respectively, through 16S rRNA and ITS amplicon sequencing of all samples.

3.2.1 Bacterial Community

Bacterial community data were further evaluated using the Buzas_gibson and Shannon_e diversity index to explore changes in bacterial diversity. Compared with those of the CK, the Shannon_e and Buzas_gibson indices increased in the soil amended with fertilizer (Fig S6). The Shannon_e and Buzas_gibson indices of the bacterial community were more sensitive to the T2 than that of the other groups (Fig S6). In bulk and rhizosphere soil but not showed the cabbage phyllosphere, alpha diversity of bacterial after application of compost was raised significantly with obvious features ($P < 0.05$) (Fig S6). Calculated alpha diversity revealed samples from the phyllosphere had the lowest bacterial alpha diversity, followed by bulk soil and then rhizosphere soil (Fig S6).

The bacterial community structure of the soil (bulk soil and rhizosphere soil) samples at the phylum level was shown in Fig S7A. Firmicutes, Actinobacteria, Acidobacteria, and Proteobacteria were the four dominant phyla in all soil (bulk soil and rhizosphere soil) samples, with their relative

353 abundance accounted for 68.2-79% totally (Fig S7A). At the class level, Bacilli (ranging from 7.88
354 to 18.5%), Acidobacteria (ranging from 8.28 to 16.2%), and Alphaproteobacteria (ranging from 7.4
355 to 15.2%) were the dominant classes of all soil (bulk soil and rhizosphere soil) samples (Fig S7B).
356 *3.2.2 Fungal Community*
357 Compared with the bulk soil, Chao1 and Richness indices increased in the rhizosphere soil.
358 Comparatively, when amended with T1, T2, T3, and S, the Chao1 index declined by 7.36%-30.60%
359 and 7.49%-15.90% in rhizosphere soil (except for T1) and bulk soil, respectively (Fig S9).
360 In the bulk soil, at the phylum level, the abundance of Ascomycota accounted for 73.93-92.13%
361 of the total composition of all libraries, followed by Chytridiomycota (0.11-20.78%),
362 Basidiomycota (1.00-7.00%), and Zygomycota (0.34-5.58%) (Except for CK). (Fig S10 A) In
363 rhizosphere soil, at the phylum level, the abundance of Ascomycota accounted for 88.9-98.1% of
364 the total composition of all libraries, followed by Zygomycota (0.32-6.57%), Basidiomycota (0.63-
365 4.57%), and Chytridiomycota (0.01-2.02%) (Fig S10 A).
366 In all soil samples, in comparison to the CK, the abundance of Ascomycota increased following
367 the addition of compost, the highest value reached at 36.03% (Fig S10 A). The abundance of
368 Ascomycota in samples from bulk soil had the lowest value, followed by rhizosphere soil (Fig S10
369 A). Meanwhile, those abundance of Basidiomycota and Zygomycota increased by 0.24-0.83%
370 (except for treatment S) and 0.04-6.25%, respectively.
371 In the phyllosphere, compared with the CK, the Chao1 index and Richness increased by 28.42%
372 and 30% respectively in treatments spiked with T3, with minimal changes after the addition of T1,
373 T2, and S (Fig S9 C and D). Ascomycota and Basidiomycota were the dominant fungal community
374 in the phyllosphere and accounted between 68.94- 90.61% of the total composition in all libraries.

375 In comparison to the control treatments, species of Ascomycota were more sensitive to the addition
376 of T1. Consequently, a decrease ranging between 34.51 and 95.38% was observed for
377 Basidiomycota (except for S), while increases between 4.28-41.49% were found for Ascomycota
378 (Fig S10 E).

379 Our findings support previous research, which observed that the addition of fertilizer can affect
380 the composition and structure of the plant rhizosphere fungal and bacterial communities. The
381 additional compost has been shown to positively influence bacterial richness and diversity in soil,
382 and that over time, the bacterial community structure becomes more stable. (Kavamura et al., 2018)

383 Soil microbial communities affect plant growth, resource use efficiency, and health, especially
384 the microbial communities that are picked up according to vegetation to formation the rhizosphere
385 microbiome (Berendsen et al., 2012; Rodrigo et al., 2013). The bacterial and fungal community
386 compositions in phyllospheric samples were similar to the rhizosphere soil samples, particularly at
387 the phylum level (Fig S7, S8, and S10). We can conclude that a certain amount of fungal and
388 bacterial communities, derived from amended soil, subsequently migrated to the vegetable tissue
389 and colonized the endophyte.

390 However, philosophic samples, principal fungal and bacterial families were different from the
391 soil samples (Fig S7, S8, and S10). It has been suggested that in comparison to endophyte samples,
392 phyllospheric samples, are more sensitive to the airborne fungal and bacterial community than the
393 soil fungal and bacterial communities, which would also support our findings (Yan et al., 2019). We
394 observed differences in phyllospheric samples and bulk soil samples for the major ARGs detected
395 (aminoglycoside, beta-lactams, and multidrug resistance genes) (Fig 1 and 2). This finding may be
396 ascribed to the unique microbial community composition in the phyllosphere, where particular

397 microbial ZOTUs are able to port only specific types of ARGs (Li et al., 2015).

398 *3.3 Pathway of ARGs from PWSC to Phyllosphere*

399 Diverse shared ARGs were found among rhizosphere soil (compost amended-soil), root, and
400 Chinese cabbage phyllosphere, and those only detected in individual samples were represented
401 utilizing a bipartite network analysis (Fig 2). Seven categories of ARGs (I-VII) were identified based
402 on the detected sample types, with two categories identified, where the antibiotic resistome was
403 shared between the Chinese cabbage phyllosphere and environment following compost treatment
404 (namely, IV and VI).

405 Cluster IV contained 20 (Fig 2a), 12 (Fig 2b), 1 (Fig 2c), and 18 (Fig 2d) ARGs, respectively,
406 which were detected simultaneously in the Chinese cabbage phyllosphere and root samples. Cluster
407 VI contained 19 (Fig 2a), 15 (Fig 2b), 12 (Fig 2c), and 21 (Fig 2d) ARGs, respectively, which were
408 detected simultaneously in the Chinese cabbage phyllosphere and the amended rhizosphere soil.
409 The genes in these two categories conferred resistance to multidrug, beta-lactamase, aminoglycoside,
410 MLSB, vancomycin, and tetracycline, and were possible candidates for moving resistance from the
411 soil, to the endophyte and phyllosphere. The ARGs in Cluster VII were shared in the middle of three
412 compartments (Chinese cabbage phyllosphere, rhizosphere soil, and root). In PWSC-T1 amended
413 soils, these genes included 69 ARGs and 5 MGEs (*tnpA*-02, *tnpA*-04, *tnpA*-05, *intI-1 (clinic)*, *intI-*
414 *1LC*), most of which conferred multidrug resistance as well as resistance to beta-lactamase,
415 aminoglycoside, MLSB, and tetracycline (Fig 2a).

416 In PWSC-T2 amended soils, these genes included 53 ARGs and 6 MGEs (*tnpA*-01, *intI3*, *tnpA*-
417 02, *intI-1LC*, *intI-1(clinic)*, *tnpA*-05), most of which conferred multidrug resistance and resistance
418 to beta-lactamase, sulfonamide, aminoglycoside, and MLSB (Fig 2b). In PWSC-T3 amended soil,

these genes included 60 ARGs and 3 MGEs (*intI-1LC*, *intI-1(clinic)*, *tnpA*-01), the majority of which conferred resistance to vancomycin, sulfonamide, aminoglycoside, multidrug, beta-lactamase, and MLSB (Fig 2c). In S amended-soil, these genes included 60 ARGs and 5 MGEs (*tnpA*-02, *tnpA*-04, *intI-1LC*, *intI*-1(clinic), *tnpA*-05), most of which delivered multidrug resistance as well to aminoglycoside, beta-lactamase and MLSB (Fig 2d). The bipartite network graph of PWSC treatment (T1-T3) revealed a similar layout to the S treatment. However, there were generally fewer shared ARGs clusters between the PWSC amended rhizosphere soil, Chinese cabbage phyllosphere, and root endophyte samples (Fig 2).

Our findings provide further evidence that compost amended soil-derived ARGs are a key origin of the antibiotic resistome found in plants (Chen et al., 2017; Zhang et al., 2019; Zhu et al., 2017). To understand further the migration pattern of the PWSC-derived antibiotic resistome to Chinese cabbage tissues and soil. We have illustrated the possible routes in the soil-plant system (Fig 2). The migration of ARGs from amended soil to plant through the plant tissues was accounted for as an inherent route.

All categories in the same soil-Chinese cabbage system shared and exchanged ARGs effortlessly across categories, especially the exchange of shared ARGs. For example, in the PWSC-T1 treatment, we found that 121 ARGs were shared between root endophyte and rhizosphere soil. Of most interest were the 74 genes shared solely by rhizosphere soil and Chinese cabbage phyllosphere, which provided clear evidence of ARGs migrating from amended soil to the Chinese cabbage phyllosphere. The specific concern is infections caused by multidrug-resistant gram-positive pathogens. Vancomycin, which is an important medical measure to assure the safe running of the hospital system, is the last line of defense to avoid the hospital infection from complete

441 collapse. Agent resistant genes to vancomycin, except for T1, were not detected in the phyllosphere
442 in this study. A study on biosafety is an important aspect among studies on applying fertilizer of
443 PWSC.
444
445 *3.4 Shared Microbiota and Antibiotic Resistome among Chinese Cabbage and Amended Soil*
446 *Samples*
447 Procrustes analysis and Mantel analysis were used to explore shared ZOTUs associated with
448 the antibiotic resistome composition from Chinese cabbage phyllosphere, root, and rhizosphere soil
449 samples. Our study indicated that ARG profiles were associated with the shared bacterial structures
450 and composition in both the control group and treatment groups, especially for T1 (Fig 3). Further
451 Procrustes analyzes of the ARG profiles and bacterial 16S rRNA gene clustered the samples
452 automatically according to the type of sample, and the foundation of Bray-Curtis dissimilarity
453 metrics, the results offered high accuracy. (Table S3, Fig 3)
454 We also described the fungal communities' roles in the dissemination of ARGs. Our findings,
455 in agreement with previous studies, show that ARG profiles were remarkably associated with the
456 shared fungal structures and composition in both the control group and treatment group. Procrustes
457 analyzes of ARG profiles and fungal ITS gene clustered the samples automatically according to the
458 type of sample, and the foundation of Bray-Curtis dissimilarity metrics fitting the results offered
459 high accuracy (Table S4, Fig 4).
460 Studies have suggested that shared ARGs were found in both the endophytes and phyllosphere
461 of plants cultured in soil amended with manure. For instance, Bai et al. (2015) observed that in terms
462 of taxonomic structure, leaf and root microbiota, were extensively overlapped whilst. Knief et al.

(2012) observed that the dinitrogen reductase gene and dinitrogenase gene, were in both the rhizosphere and phyllosphere metagenome, indicating that a functional gene level, these genes were partially overlapping in the leaf and root microbiota.

Shared ZOTUs and shared ARGs imply that phyllosphere and root microbiota might interconnect fungal and bacterial communities including antibiotic-resistant microbiota which have the potential to move between the rhizosphere and phyllosphere (Beattie and Lindow, 1999; Ruiz-Pérez et al., 2016). A study has indicated that roots can effectively recruit soil bacteria or fungal communities to colonize the root, allow antibiotic resistome to migrate from the soil into the phyllosphere and roots (Bulgarelli et al., 2012). This might be a key migration path for the antibiotic resistome in organic fertilizer to move into plants, whereby in this process, the roots can act as a bridge. The results showed that shared ZOTUs support the origin of ARGs by a host in the bacterial or fungal communities. Ultimately, the phyllosphere and root endophyte in Chinese cabbage forms their own ARG compositions.

4. Conclusions

In summary, this research showed that the application of both PWSCs (T1-T3) and S increased the diversity and absolute abundance of the ARGs in the phyllosphere, root, rhizosphere soil, and bulk soil of Chinese cabbage. ARGs were examined in the Chinese cabbage microbiome and disproportionally distributed in distinguishing parts (e.g. Phyllosphere and root) of Chinese cabbage. Enrichment of Chinese cabbage antibiotic resistome was associated with fungal and bacterial taxa. Based on these findings, we suggested that rhizosphere soil, root, and phyllosphere microbiota might interconnect with microbial communities (fungal and bacterial communities), including

antibiotic-resistant microbiota, potentially moving between the rhizosphere and phyllosphere. In this process, we propose that the roots play the role of a bridge.

Nevertheless, shared antibiotic resistome in the amended soil-plant system indicates a feasible pathway of ARGs migration into human pathogens and the microbiome via the food chain (e.g. Vegetable and salad). These observations are critical for the assessment of public health risks on Chinese cabbage grown in the soil amendment dealt with PWSCs. Soil utilization of sewage sludge is an important universal method of treating sewage sludge. How to prevent the biological pollution is always a much-discussed problem, especially on the pharmaceutical waste sludge. This research has significant implications for the recycling of pharmaceutical waste sludge, to improve the sustainable agricultural practices and the human health protection.

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703 Table 1. Chemical properties of fertilizers and soil.

Items	Soil	PWSC T1	PWSC T2	PWSC T3	S
pH	7.52	7.43	8.06	6.94	8.73
TC	17.74	283.1	242.2	280.5	146.2
TN	2.71	9.45	12.95	11.27	9.59
Cr	78.18	47.73	226.58	36.43	13.35
Cu	34.81	65.53	164.41	229.65	95.38
Pb	38.45	5.87	14.35	1.91	4.08
Zn	132.37	148.85	256.64	320.68	290.66
Cd	0.37	0.48	0.51	0.45	0.45
Tetracycline	-	4.96	3.86	4.06	8.79
Oxytetracycline	-	2.33	1.12	1.97	4.68
Doxycycline	-	2.56	2..13	1.86	3.33
Sulfadiazine	-	0.81	0.31	0.54	0.34
Sulfamethazine	-	0.23	0.12	0.10	0.05
Ciprofloxacin	-	4.32	4.12	3.24	5.66
Enrofloxacin	-	0.32	0.29	0.31	0.51
Chlorotetracycline	-	0.54	0.59	0.48	0.46

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714 Figure 1. The detected MGEs and ARGs in soil samples (including initial soil, rhizosphere soil and
715 bulk soil), Chinese cabbage phyllosphere and compost (including T1, T2, T3 and S). ARGs were
716 classified according to the antibiotic they resisted. Control treatments (CK), PWSCs of T1
717 treatments (T1), PWSCs of T2 treatments (T2), PWSCs of T3 treatments (T3), sewage sludge
718 compost treatments (S) respectively. The mean values of three replicates, data as means ± SD.
719
720 Figure 2. Bipartite network illustrating the shared genes between Chinese cabbage phyllosphere,
721 root, and rhizosphere soil (compost amended soil) cultured in soil amended with compost (T1 (a),
722 T2 (b), T3 (c) and S (d)).
723
724 Figure 3. Procrustes analysis is unveiling the significant association between the absolute abundance
725 of ARGs and shared bacterial taxa composition (16S rRNA gene ZOTUs) relied on the Bray-Curtis
726 distance. a, without the application of manure, b, with application of TI composting, c, with
727 application of T2 composting, d, with application of T3 composting, e, with application of S
728 composting.
729
730 Figure 4. Procrustes analysis unveiling the significant association between the absolute abundance
731 of ARGs and shared fungal taxa composition (ITS read ZOTUs) relied on the Bray-Curtis distance.
732 a, without application of composting, b, with application of TI composting, c, with application of
733 T2 composting, d, with application of T3 composting, e, with application of S composting.