Antibiotic resistance genes in layer farms and their correlation with environmental samples

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ABSTRACT Livestock farms are generally considered to be the important source of antibiotic resistance genes (ARGs). It is important to explore the spread of ARGs to reduce their harm. This study analyzed 13 resistance genes belonging to 7 types in 68 samples of layer manure including different stages of layer breeding, layer manure fertilizer, and soil from 9 laying hen farms in Guangdong Province. The detection rate of antibiotic resistance genes was extremely high at the layer farm in manure (100%), layer manure fertilizer (100%), and soil (> 95%). The log counts of antibiotic resistance genes in layer manure (3.34−11.83 log copies/g) were significantly higher than those in layer manure fertilizer (3.45−9.80 log copies/g) and soil (0−7.69 log copies/g). In layer manure, ermB was the most abundant antibiotic resistance gene, with a concentration of 3.19 × 10^9−6.82 × 10^11 copies/g. The average abundances of 5 antibiotic resistance genes were above 10^10 copies/g in the descending order ermB, sul2, tetA, sul1, and strB. The relative abundances of ARGs in layer manure samples from different breeding stages ranked as follows: brooding period (BP), late laying period (LL), growing period (GP), early laying period (EL), and peak laying period (PL). There was no significant correlation between the farm scale and the abundance of antibiotic resistance genes. Moreover, the farther away from the layer farm, the lower the abundance of antibiotic resistance genes in the soil. We also found that compost increases the correlation between antibiotic resistance genes, and the antibiotic resistance genes in soil may be directly derived from layer manure fertilizer instead of manure. Therefore, when applying layer manure fertilizer to cultivated land, the risk of antibiotic resistance genes pollution should be acknowledged, and in-depth research should be conducted on how to remove antibiotic resistance genes from layer manure fertilizer to control the spread of antibiotic resistance genes.

Key words: layer manure, soil, compost, antibiotic resistance gene

INTRODUCTION Intensive breeding of laying hens in confined spaces has led to colonization of poultry by resistant bacteria (Skowron et al., 2016). The use of antibiotics in livestock production may lead to the emergence of antibiotic resistant bacteria and reduce the effectiveness of antibiotics in treating and preventing human bacterial infections (Li et al., 2015; Tiedje et al., 2019). Antibiotics have caused great concern because of their extensive application in agriculture, aquaculture and the medical industry to prevent or treat bacterial infections in humans and animals, which has also caused uncertain threats to global public health. Animal excrement is the main source of residual antibiotics and has become a reservoir of resistant bacteria. There is increasing evidence that
antibiotic resistance genes (ARGs) can enter the environment through the discharge of animal manure, causing soil, water, and crop pollution (He et al., 2016). Unlike broiler farms in China, the use of antibiotics is strictly controlled during layer breeding, especially during the laying period. Therefore, there may be differences in antibiotic residues in the feces of laying hens and broilers. For example, the content of chlortetracycline (323 µg/kg), oxytetracycline (147 µg/kg), and doxycycline (303 µg/kg) in layer manure is significantly lower than that in broiler manure (Qiu et al., 2021). The antibiotic concentration is the important factor affecting ARGs (Tong et al., 2020). Previous investigations studied the ARG contents in broiler manure before and after antibiotic use. The results showed that the abundance of certain ARGs in the broiler coop increased even after composting after antibiotics were added to the broiler diet (Subirats et al., 2020). Therefore, antibiotics may lead to some differences in ARGs between laying hens and broilers. In addition, because the use of antibiotics during the incubation and growth periods is allowed for laying hens, most antibiotics are prohibited during the laying period. With growth and development, the abundance of ARGs should be significantly different.

Mainstream farming practices currently include the combined production of plants and animals. Before application of manure to land, composting, as an effective method to treat fecal waste and produce layer manure fertilizer, has become an important link in controlling the development of drug resistance (Cui et al., 2016). Composting is a biological oxidation process that is a harmless way to kill pathogens, and compost products can provide nutrients for crops (Liang et al., 2020). Composting can effectively reduce the concentrations of antibiotics. The antibiotic concentration decreased by more than 4,000-fold along the environmental transmission chain from manure samples from swine farms to aerobic compost, compost-amended agricultural soils, and neighboring agricultural soils (Gao et al., 2020). However, several studies have investigated ARGs have divergent fates during composting. Although compost significantly reduces the abundance of certain resistance genes, it cannot completely eliminate resistance (Cui et al., 2016; Xie et al., 2016; Qian et al., 2017; Zhao et al., 2017). In addition, the abundance of some resistance genes increased after composting (Xia et al., 2019).

Most studies assert that livestock manure is the important source of resistance genes in soil and water bodies and is an ARG reservoir (Zhu et al., 2013). However, manure is not directly applied to farmland but first undergoes harmless treatment processes. Composting is one of the important harmless treatment methods for manure. Therefore, the dynamics of the migration of ARGs from livestock manure to compost products to the surrounding soil provide a reference for better preventing the transfer of resistance genes and bacteria.

The objective of this study was to investigate the distribution characteristics of ARGs from layer manure to receiving environments. We analyzed the distribution of main ARGs in layer manure at different breeding stages, and provided a reference for the application of layer manure. We also investigated ARGs in layer manure from different growth periods and the dynamics of soil resistance genes at different distances from the farm. The study will provide a reference for scientifically and rationally reducing the diffusion of ARGs from layer manure to the ecosystem and evaluating the ecological risks that layer manure fertilizer may cause.

**MATERIALS AND METHODS**

**Laying Hen Farms and Sample Collection**

Nine representative laying hen farms with different waste management systems in Guangdong Province were selected for this study (Figure 1A). Manure, layer manure fertilizer and soil samples from laying hen farms at different farm scales were collected. The farm scales included small- and medium-; large-; and collectivization-scales. Detailed information on the laying hen farms is shown in Table S1. Manure samples were collected depending on the breeding periods and manure processing modes of the studied laying hen farms. Manure samples were taken during the brooding period (BP), growing period (GP), early laying period (EL), peak laying period (PL), and late laying period (LL), and different manure treatments included stacked manure (SM) and layer manure fertilizer (OM). Soil samples around the farm included soil from the farm (S1) and soil from farms 100 meters ($S_{100}$), 300 meters ($S_{300}$), and 500 meters ($S_{500}$) away. In total, 37 manure samples and 31 soil samples were collected. These samples were collected from 9 laying hen farms, which were named A to I (S1). Among the 9 laying hen farms, D, G, H, and I employed composting to treat the layer manure. All samples were stored in an ice box, immediately shipped to the laboratory and stored at −80°C.

**DNA Extraction**

DNA of the total microorganisms was extracted from manure and soil samples using the E.Z.N.A. Soil DNA Kit (OMEGA, Norcross, Georgia). The DNA concentration and purity were determined using a NanoDrop 2000 instrument (Thermo Scientific, Waltham, Massachusetts). The extracted DNA was stored in a freezer at −20°C until use.

**Quantification of Antibiotic Resistance Genes**

Previous studies have shown that tetracycline, sulfonamide, β-lactam, fluoroquinolone, macrolide, chloramphenicol, and streptomycin resistance genes are the most common ARGs in organic waste (Su et al., 2015). Therefore, 13 ARGs were determined, including tetracycline resistance genes ($tetA$, $tetM$, $tetQ$, and $tetX$), 2 sulfonamide resistance genes ($sul1$ and $sul2$), 1 β-lactam
resistance gene (\textit{bla}TEM), 2 fluoroquinolone resistance genes (\textit{oqxB} and \textit{qnrS}), 2 macrolide resistance genes (\textit{ermB} and \textit{ermC}), 1 chloramphenicol resistance gene (\textit{drfA7}), 1 multiple resistance gene (\textit{cfr}) and one streptomycin resistance gene (\textit{strB}). Mobile genetic elements (\textit{MGEs}) can spread resistance genes horizontally in bacteria (Forsberg et al., 2014). \textit{int1} is the key MGE for the horizontal transfer of ARGs. All of the ARGs

Figure 1. Sample collection and copy numbers of the main ARGs in the sample. (A) Distribution map of nine layer farms (A–I); (B) broad-spectrum quantitative profiles of 13 ARGs and \textit{int1} in layer manure, layer manure fertilizer, and soil. The first letter of the sample name represents the farm; the second represents the sample type, manure (M), layer manure fertilizer (LM) or soil (S); the third represents one of the following, brooding period (BP), growing period (GP), early laying period (EL), peak laying period (PL), late laying period (LL), stacked manure (SM), soil at the farm (SI), soil within 100 meters of the farm (S100), soil within 300 meters of the farm (S300), soil within 500 meters of the farm (S500). The absolute ARG concentrations were used for plotting. Black cells indicate the absence of corresponding ARGs in a certain sample. Abbreviation: ARGs, antibiotic resistance genes.
and MGEs were analyzed by PCR and agarose electrophoresis. The detected ARGs and MGEs were analyzed further by qPCR using an iCycler IQ5 thermocycler (Bio-Rad, Hercules, California).

After PCR, the products of all genes were subjected to gel electrophoresis and recovered using a Gel Extraction Kit (OMEGA). The recovered product was ligated into the pMD18-T vector. Standard curves were constructed in which each plasmid was diluted in a 10-fold gradient, and the diluted plasmids were used as templates for qPCR. The qPCR mixture in a volume of 20 μL contained 1 μL of DNA template, 0.5 μL of each primer (ShengGong, China), 10 μL of SYBR qPCR mix (TaKaRa, Japan), and 8 μL of ddH2O. The thermal cycling steps for qPCR amplification were as follows: 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, annealing temperatures (shown in Table S2) for 30 s, 72°C for 30 s; 72°C for 10 min; and a 4°C hold (Yin et al., 2017). Each dilution gradient was repeated 3 times, and 3 negative controls were made at the same time. The lower limit of quantification (LOQ) for each qPCR assay was higher than the result of the negative controls (Table S3). A melting curve of qPCR products was constructed. The squared correlation coefficients were calculated. The amplified products were cloned and sequenced. The sequencing results were submitted into GeneBank with the accession number of KC507994-KC507999. The detection of ARGs and MGEs was analyzed by PCR and agarose electrophoresis. The detected ARGs and MGEs were analyzed further by qPCR using an iCycler IQ5 thermocycler (Bio-Rad, Hercules, California).

RESULTS AND DISCUSSION

Distribution Characteristics of ARGs in Layer Manure, Layer Manure Fertilizer, and Soil

In this study, seven classes of ARGs, namely, tetracycline, sulfonamide, β-lactam, quinolone, macrolide, chloramphenicol, and streptomycin resistance genes, were examined because of their widespread contamination of the farm environment. The frequencies of ARG detection in the 3 environmental samples are shown in Table 1. Notably, the detection frequency of the target genes was 100% in layer manure (n = 3 3) and layer manure fertilizer (n = 4). The detection frequency of the target genes was above 90% (n = 31) in soil. Among all samples (n = 68), the highest detection frequency was 100% (tetA, tetQ, and blaTEM), the second was 98.53% (tetX, sulI, strB, and intI), the third was 97.06% (tetM, ermB, sul2, qnrS), and the lowest was 95.59% (ermC, qoxB, and cfr).

Although all 13 ARGs were detected in layer manure, layer manure fertilizer, and soil, their abundances were different among the sample types. As shown in Figure 1B, the abundance of detected genes in layer manure was significantly higher than that in layer manure fertilizer and soil (P < 0.05). Lee et al., 2017 and He et al. (2016) previously reported that layer manure could be a main source of ARG contamination in layer farm environments. Based on the measurements within the 3 types of samples, the macrolide resistance gene ermB showed the highest abundances in layer farm

Table 1. Log count of ARGs and intI in layer manure, layer manure fertilizer, and soil (copies/g).

| Genes | Manure | LM | Soil | Total |
|-------|--------|----|------|-------|
| tetA | 8.60   | 11.47 | 10.24 ± 0.77 | 100 | 7.27 | 9.53 | 8.36 ± 1.02 | 100 | 3.86 | 7.06 | 5.44 ± 0.79 | 100 |
| tetM | 7.77   | 10.35 | 9.4 ± 0.59 | 100 | 6.75 | 8.52 | 7.39 ± 0.67 | 100 | 6.36 | 4.46 | 1.39 | 93.55 | 97.06 |
| tetQ | 7.20   | 10.31 | 9.69 ± 0.98 | 100 | 6.60 | 8.91 | 7.32 ± 0.93 | 100 | 4.22 | 6.89 | 5.38 ± 0.56 | 80 |
| tetX | 7.56   | 11.38 | 9.48 ± 0.83 | 100 | 7.45 | 9.40 | 8.28 ± 0.71 | 100 | 7.27 | 8.62 | 7.12 | 96.77 | 98.53 |
| ermB | 9.50   | 11.83 | 10.53 ± 0.58 | 100 | 7.46 | 9.17 | 8.36 ± 0.66 | 100 | 7.26 | 5.87 | 1.26 | 93.55 | 97.06 |
| ermC | 7.27   | 10.95 | 8.51 ± 0.81 | 100 | 8.67 | 9.67 | 9.2 ± 0.35 | 100 | 6.39 | 3.67 | 1.50 | 90.32 | 95.59 |
| sulI | 8.16   | 11.45 | 10.04 ± 0.85 | 100 | 8.63 | 10.46 | 9.35 ± 0.68 | 100 | 7.45 | 4.72 | 1.35 | 96.77 | 98.53 |
| sul2 | 8.70   | 11.51 | 10.36 ± 0.74 | 100 | 7.71 | 10.45 | 9.88 ± 1.02 | 100 | 7.69 | 4.72 | 1.37 | 93.55 | 97.06 |
| blaTEM | 6.38 | 9.38 ± 1.22 | 100 | 5.88 | 9.10 | 6.77 ± 1.35 | 100 | 3.4 | 6.14 | 5.15 ± 0.64 | 100 | 90.32 | 95.59 |
| qoxB | 3.34   | 10.51 | 6.40 ± 1.73 | 100 | 3.92 | 7.07 | 4.83 ± 1.30 | 100 | 4.91 | 3.41 | 1.26 | 90.32 | 95.59 |
| qnrS | 5.28   | 11.03 | 8.02 ± 1.34 | 100 | 4.06 | 7.00 | 5.08 ± 1.15 | 100 | 5.30 | 3.66 | 0.93 | 93.55 | 97.06 |
| cfr  | 5.14   | 9.01 | 7.20 ± 1.08 | 100 | 3.45 | 8.06 | 5.66 ± 1.67 | 100 | 4.53 | 3.1 | 1.03 | 90.32 | 95.59 |
| strB | 7.78   | 11.16 | 10.00 ± 0.82 | 100 | 7.44 | 9.58 | 8.52 ± 0.79 | 100 | 6.39 | 4.82 | 1.14 | 96.77 | 98.53 |
| intI | 8.25   | 11.39 | 9.87 ± 0.81 | 100 | 7.84 | 9.80 | 8.86 ± 0.84 | 100 | 7.20 | 5.36 | 1.24 | 96.77 | 98.53 |

Abbreviation: ARGs, antibiotic resistance genes.
environments, in the range of $7.48 \times 10^5$–$3.40 \times 10^{10}$ copies/g. Studies in broiler farms also found that the relative abundance of the \textit{erm}B gene was the highest (Yang et al., 2020), which should create awareness regarding the spread of this resistance gene. The abundance of macrolide resistance genes may be related to the greater use of macrolides during feeding (Zhang et al., 2017). In addition, the absolute abundance of \textit{tet}A, \textit{tet}M, \textit{tet}Q, and \textit{tet}X was $2.88 \times 10^5$–$2.95 \times 10^{11}$ copies/g. Tetracycline resistance genes were the most common types of ARGs. Previous studies reported that tetracycline resistance genes were the predominant ARGs in livestock and poultry manure (Wen et al., 2019). Adding 2 g/L of chlortetracycline to drinking water can increase the abundance of tetracycline resistance genes in chicken feces (Xiong et al., 2018). And studies have shown that 10 ug/L tetracycline can promote the horizontal transfer of tetracycline resistance genes, which shows that even low dose antibiotics can promote the increase of ARGs abundance (Jutkina et al., 2016). The abundance of sulfa resistance genes (\textit{sul}2) was $5.25 \times 10^4$–$3.74 \times 10^{11}$ copies/g, second only to \textit{erm}B. From layer manure to organic fertilizer, the levels of the \textit{sul}2 decreased from $2.29 \times 10^{10}$ copies/g to $9.55 \times 10^8$ copies/g, respectively. Birgit et al. (2016) had similar results in a study in which the levels of the \textit{sul}2 genes dramatically decreased during composting. The changes in the number of host bacteria may be an explanation for the increase or decrease of ARGs during the composting (Ben et al., 2017). In addition, fluoroquinolone resistance genes \textit{qox}B ($2.51 \times 10^6$ copies/g) and multiple resistance genes \textit{cfr} ($1.58 \times 10^7$ copies/g) were the least abundant in layer manure which indicated a smaller risk of transmission of these genes.

### Resistance Gene Abundance in Layer Farms of Different Scales

Few studies in the past have focused on the relationship between ARGs and the scale of farms. Therefore, we analyzed the correlation between layer farms of different scales and ARGs. Previous studies have found that ARGs were mainly subjected to impacts from environmental factors and microbial communities (Cao et al., 2020). The samples from manure and soil are concentrated on the left and right of the figure, respectively, while the samples from layer manure fertilizer are mainly concentrated in the middle (Figure 2). In addition, the samples of the same scale were not clustered together. Principal coordinate analysis (PCoA) demonstrated that the difference in the abundance of ARGs between samples mainly depended on whether they originated from manure, layer manure fertilizer or soil, and there was no significant correlation with the scale of the layer farm. The above results indicate that the farm scale did not affect the abundance of ARGs.

![Figure 2](image_url) **Figure 2.** Principal coordinate analysis (PCoA) showing the clustering of communities based on b-diversity. The nodes are shaped according to the types of substances, including layer manure (triangle), layer manure fertilizer (round), and soil (square). The color from light to dark indicates the scale of the farm from small to large, respectively.
Abundance of ARGs and int1 in Different Breeding Stages

Ma et al. (2016) detected 4 tetracycline ARGs in 20-day-old layer manure, and as many as 16 ARGs in 80-day-old layer manure. To study the dynamics of ARGs over the breeding cycle of laying hens, we collected layer manure from different periods and analyzed the relative abundance of the main ARGs (Figure 3A). The comparison of ARG profiles indicated that the abundance of ARGs in different breeding periods was quite different. The relative abundance of ARGs in the manure of layers at different breeding stages from high to low was as follows: the brooding period, the late laying period, the early laying period, the growing period, and the peak laying period. This may be related to the heavy use of antibiotics during the brooding period (Rivera-Gomis et al., 2021). The relative abundance during the brooding period was significantly higher than that during the other breeding stages ($P < 0.05$). The reason for this phenomenon is that the relative abundance of tetA (Figure 3B), blaTEM (Figure 3C), and oqxB (Figure 3D) in the brooding period was significantly higher than peak laying period ($P < 0.05$). The decrease in the abundance of tetA, blaTEM, and oqxB may be related to the prohibition of antibiotics during the laying period, which reduces the risk of residual resistance genes in eggs. The relative abundance of ermB varied from $1.22 \times 10^{-1}$ to $2.34 \times 10^{-1}$ during the integral breeding period. Moreover, a study also found the macrolide resistance gene (erm-ARG) ermB in the waste of intensive chicken farms with high abundance (Mu et al., 2015). The relative abundance of the sulfonamide resistance gene $su2$ ($9.13 \times 10^{-2}$ to $1.54 \times 10^{-1}$) was always high during the breeding process. Previous studies have shown that $sul1$ and $sul2$ are very abundant sulfonamide resistance genes in manure and soil to which manure is applied (Zhao et al., 2017). Therefore, ermB and $sul2$ consistently maintained a high abundance without a significant decrease, which indicates that they pose a high risk during the laying period and can exist more stably without selection pressure. However, this study is based solely on a snapshot of various samples collected at a single time in point, so the conclusion drawn is limited to one observation.

The Relative Abundance of ARGs and int1 in LM and SM

Composting is widely used to treat and reutilize animal manures for ARG and pathogen reduction (Guo et al., 2020; Peng et al., 2020). Stacked manure is manure that has not been composted, just piled manure.

Figure 3. The relative abundance of ARGs and int1 in different breeding stages. (A) Changes in ARGs and int1 in different breeding stages. (B–D) Changes in tetA, sul1 and blaTEM in different breeding stages. Brooding period (BP), growing period (GP), early laying period (EL), peak laying period (PL), late laying period (LL). Different letters indicate significant differences between the means (Kruskal-Wallis test; $P < 0.05$) of treatments. Abbreviation: ARGs, antibiotic resistance genes.
together for several months. The distributions of detected ARGs in stacked manure and layer manure fertilizer are shown in Figure 4. It can be seen that there was no significant difference in ARGs between stacked manure and layer manure fertilizer ($P > 0.05$), but the value between the upper and lower quartiles in stacked manure was notably higher than that in layer manure fertilizer. OqxB and cfr had the lowest relative abundances in stacked manure and layer manure fertilizer. The relative abundances of tetM, tetQ, oqxB, and cfr were less than $1.4 \times 10^{-2}$. Composting resulted in better ARG reduction efficiency in layer manure fertilizer than did manure stacking. These results suggested that specific compost treatment components can effectively decrease the concentration of ARGs in layer farms. Current studies demonstrate the effective elimination of ARG residues, and the absolute abundance of TRGs, MLSBRGs, $\beta$-LRGs, and PRGs significantly declined after composting (Zhang et al., 2016; Cheng et al., 2019). In layer manure fertilizer, the relative abundances of sul1 and sul2 were higher than those of other ARGs. The results indicate that composting had lower removal efficiency for sulfonamide resistance genes than for other ARGs. Holman et al. (2016) found similar results and showed that sul1 was more persistent than most tetracycline and macrolide resistance genes during cattle manure composting. The removal efficiencies of tetX, ermC, and intI from compost were also limited. Many studies have reported that composting may not completely remove ARGs, so compost products are still considered an important reservoir for ARGs (Su et al., 2015; Qian et al., 2016). In general, composting is a vital link for controlling the dissemination of antibiotic resistance, however, close attention should still be given to those genes that are not easily removed.

### The Relative Abundance of ARGs and int1 in Soil

Poultry is currently regarded as a reservoir from which multidrug resistance can be readily transferred to the surrounding ecosystem (Dandachi et al., 2020). ARGs in animal manures enter the soil through manure application, which leads to significant increases in ARGs in soil (Chen et al., 2019a, b). In addition to being directly affected by layer manure fertilizer, the soil is also affected by groundwater and rain due to the proximity to the layer farm (Nnadozie and Odume, 2019; Zhu et al., 2021), so the distance to the layer farm may affect the abundance of ARGs in the soil. Therefore, we analyzed the distribution of ARGs in the soil at different distances from the farm, as shown in Figure 5A. The relative concentration of total ARGs in SI was $2.51 \times 10^{-2}$, which was higher than that in surrounding soil. However, the difference between the groups was not significant. We found that the abundance of tetA in the soil outside the laying hen farm was lower than that in the farm, especially the S100 and S300 were significantly lower than the SI. (Figure 5B). This indicated that the risk of tetA transmission outside the farm was lower than that on the farm. Moreover, the 2 ARGs, blaTEM and ermB, had high abundances both inside and outside the layer farms and did not decrease significantly with increasing distance. Therefore, these 2 resistance genes may be more likely to spread in the soil off-site, posing an ARG contamination risk.

The relative abundance of the total ARGs gradually decreased with distance from the farm, but the difference was not significant. This indicates that the relative abundance of ARGs in the soil is mainly affected by

![Figure 4](https://example.com/figure4.png)
layer manure fertilizer, but has a low correlation with the distance from layer farms.

Correlation Analysis of ARGs in Different Environments

Layer manure, which is processed into organic fertilizer or directly applied to the soil, contains a large number of ARGs. Therefore, it is fundamental to research the spread of ARGs in different environments. The soil samples collected in this study were all applied with layer manure fertilizer instead of directly applying layer manure. Layer manure fertilizer is a harmless product from layer manure after aerobic composting. In the present study, significant correlations were found among various ARGs in particular environments. Nevertheless, the correlation of ARGs in different samples varied greatly (Figure 6). In the 3 environmental samples, _ermB_ was significantly correlated with _tetM_ ($P < 0.05$). According to a study by Jia et al. (2017), _tetM_ and _ermB_ can be acquired by the same microorganism. Therefore, _tetM_ and _ermB_ may be carried by the same microorganism to contaminate the environment (Shen et al., 2008).

In layer manure, there was a weak correlation among ARGs, but after composting, the correlation among ARGs was enhanced in layer manure fertilizer. In organic matter, _StrB_ had a positive correlation with 9 other ARGs. _tetA, tetQ, tetM, ermB, oqpB, and blaTEM_ in layer manure fertilizer showed a significant positive correlation with each other ($P < 0.05$). The ARGs in layer manure showed a lower correlation than those in layer manure fertilizer and soil. It is worth noting that _tetA_ had a significant positive correlation between layer manure fertilizer and soil ($P < 0.01$), and _tetQ, tetM, ermB_ and _blaTEM_ showed similar results ($P < 0.05$). This result highlights the possibility of ARGs spreading from layer manure fertilizer to soil. The results from a recent study showed that compost application to land could lead to enhanced contamination levels of ARGs in compost-amended soils (Xu et al., 2015; Ben et al., 2017). Many studies have indicated that livestock manure is an important source of ARGs in the environment (Cerqueira et al., 2019; Liao et al., 2019). However, from our investigation, the high abundance of ARGs in manure does not mean a high risk of ARG transmission because composting has become an efficient method to decompose manure waste and produce layer manure fertilizer. Notably, compared with the ARGs in the layer manure, the ARGs in the layer manure fertilizer had a significant correlation with the ARGs in the soil. This result means that we need to pay more attention to the content of ARGs and new pollutants in compost products.

ARGs and MGEs have often been linked to or located in integrons (Cao et al., 2020). In this study, _int1_ was significantly correlated with _ermB, sul2_, and _tetM_ in layer manure fertilizer and soil ($P < 0.01$), whereas these factors were weakly correlated in layer manure. Studies found that _tetM_ exists in conjugative plasmids of various gram-positive and gram-negative bacteria (Ojo et al., 2006; Pachulec and Does, 2010; Shaskolskiy et al., 2018). During swine manure composting, _int1_ was significantly correlated with _tetM_ and _ermB_ (Qian et al., 2019). Previous studies have shown that integrons can integrate exogenous ARG cassettes, which promote multidrug resistance (Rapa and Labbate, 2013). In addition, ARG cassettes are usually located in plasmids or transposons, making them easy to disseminate in the environment (Partridge et al., 2018). These findings suggest that the promotion of horizontal gene transfer by _int1_ may be an important contributor to dissemination in different media. Huddleston (2018) suggested that _int1_ participates in Horizontal gene transfer (HGT) processes and induces bacteria to acquire ARGs (e.g., _sul1, dfrA1, tetA_, and _floR_) in the environment. Furthermore, compared with manure, ARGs in layer manure fertilizer are more related, which may be caused by the existence of a common host for multiple ARGs. The correlation of ARGs in soil was also significantly higher than that in

Figure 5. The abundance of ARGs and _int1_ in the soil at different distances from the farm. (A) The relative abundance of ARGs and _int1_ in soil. (B) The difference in the abundance of _tetA_ in the soil. Soil on the farm (SI), soil within 100 meters of the farm (S100), soil within 300 meters of the farm (S300), soil within 500 meters of the farm (S500). Different letters indicate significant differences between the means (Kruskal-Wallis test; $P < 0.05$) of treatments. Abbreviation: ARGs, antibiotic resistance genes.
manure. The ARGs in soil may mainly come from layer manure fertilizer, which also confirms our speculation. This result illustrates that composting may lead to the multidrug resistance of microorganisms and aggravate the harmful effects of resistant bacteria. Eventually, animal husbandry and even humans themselves may be in crisis, so we need to pay more attention.

CONCLUSIONS

This study investigated the distribution of ARG in layer manure, layer manure fertilizer, and soil. The number of ARGs in layer manure during the incubation period was significantly higher than that in other periods, and the scale of the layer farm had no significant effect on the abundance of ARGs. We found that the correlation of ARGs in layer manure fertilizer was significantly higher than that of ARGs in layer manure, which may be due to the emergence of multidrug resistant bacteria. Furthermore, the ARGs in the soil and layer manure fertilizer show a significant positive correlation, indicating that the ARGs in the soil may come from layer manure fertilizer instead of directly from layer manure.

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DISCLOSURES

We declare that no conflict of interest exits in this manuscript, and manuscript is approved by all authors for publication. This manuscript is original and has not been published in whole or in part previously.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101485.

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