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# The Effect of Manure Application Rates on the Vertical Distribution of Antibiotic Resistance Genes in Farmland Soil

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**Abstract:** Manure application is the primary input route for antibiotic resistance genes (ARGs) in farmland soil. This study investigated the effects of varying the rates of five chicken manure applications on the accumulation and distribution of ARGs across different soil depths (0–20, 20–40, and 40–60 cm) using metagenomic sequencing. The results revealed that the distribution of ARGs in farmland soil was closely linked to soil depth and influenced to some extent by the fertilizer quantity after 30 days of fertilization. ARGs were predominantly concentrated in the surface soil and exhibited a significant decrease in type and abundance with an increased soil depth. Compared with soil treated with chemical fertilizers alone, chicken manure-treated surface soil presented a higher diversity and abundance of ARGs. However, the diversity and abundance of ARGs did not increase proportionally with the increasing ratios of chicken manure application (0, 25, 50, 75, and 100%). ARGs in soil primarily conferred resistance to host bacteria through antibiotic efflux pumps (~33%), antibiotic target alteration (~31%), antibiotic inactivation (~20%), and antibiotic target protection (~8%). Correlation analysis involving ARGs and soil microorganisms revealed widespread multidrug resistance among soil microorganisms. Furthermore, two genera of human pathogenic bacteria (*Pseudomonas* sp. and *Listeria* sp.) were identified as potential microbial hosts of ARGs in all treatments. Correlation analysis involving ARGs and environmental factors indicated that soil ARGs are predominantly influenced by heavy metals and microorganisms. This paper offers valuable insights for environmental risk assessments regarding the utilization of livestock manure resources. Additionally, it furnishes a scientific foundation for farmland application strategies pertaining to livestock manure.

**Keywords:** chicken manure; antibiotic resistance genes (ARGs); farmland soil; microorganisms; soil depths



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## 1. Introduction

Livestock manure is rich in nutrients (e.g., nitrogen, phosphorus, and potassium) that promote crop growth [1–5]. The application of manure to farmlands has been proven to be an effective strategy for mitigating soil constraints, augmenting soil organic matter, enhancing soil health, and boosting crop yields [6–9]. Prolonged manure application not only improves soil microbial characteristics but also enhances soil chemical properties, and thus, it plays a pivotal role in sustaining agricultural productivity and ecosystem services [10]. Concurrently, pollutants commonly found in manure, including heavy metals, antibiotics, antibiotic resistance genes (ARGs), and pathogens, may accumulate in soil with manure application, leading to soil contamination and posing risks to human health.

Although the careful administration of veterinary antibiotics can prevent animal illnesses and promote growth, significant portions of these antibiotics are not fully absorbed and utilized within the animal gut. With the rapid development of the livestock and

poultry industries, the consumption of veterinary antibiotics has gradually increased. The emissions of 80 veterinary antibiotics ranged from 23,110 tonnes/year to 40,850 tonnes/year in 2100–2020 [11]. Tetracyclines, sulfonamides, chloramphenicols, and quinolones account for 94% [11]. Approximately 75% to 95% of antibiotics, along with ARGs induced by the prolonged selective pressure of antibiotics, are excreted in feces and urine [12–14], and most of these excreted compounds retain their potency in soil [15]. Numerous studies have shown that the application of manure can elevate antibiotic concentrations in soil (from μg/kg to mg/kg levels) [16–18], increasing the number of drug-resistant bacteria and the abundance of ARGs [19,20].

The application of manure significantly enriches soil with carbon-containing substrates and nutrients, stimulating the growth and activity of soil microbial populations [21,22]. This enrichment increases soil microbial biomass and diversity [23,24], subsequently influencing the maintenance and distribution of ARGs. For example, numerous nutrient-rich bacterial communities have emerged in carbon-rich fertilizer soils [25]. Soil fertilized with manure exhibits a higher microbial biomass compared with soil that uses only chemical fertilizers [26]. Peng et al. [27] demonstrated that 3 years of continuous manure application could alter the structures of microbial communities and antibiotic resistance in soil. Moreover, Li et al. [20] revealed that manure application significantly enhanced the abundance and α diversity of ARGs in soil. The effect of manure application on soil ARGs is attributable not only to nutrient increase but also to antibiotics, heavy metals, and other factors. Gabini et al. [28] observed that 20 mg/kg of sulfamethoxazole caused only preliminary and short-term changes in soil bacterial community composition while no significant effect was noted on fungal communities. However, higher concentrations (100–300 mg/kg) of sulfamethoxazole induced substantial and persistent changes in the β diversity of both bacteria and fungi [29]. Additionally, the presence of heavy metals in livestock manure leads to the compound pollution of antibiotics in soil [30,31]. These heavy metals can act as selective agents for the proliferation of ARGs and promote their persistence in the environment [32]. For example, Kuppusamy et al. [33] discovered a positive correlation between soil ARGs and residues of tetracyclines, sulfonamides, quinolones, copper, and cadmium. However, most studies have, to date, been focused on the surface soil or on a few types of ARGs. Although these studies are very useful for understanding the environmental risks of ARG accumulation in farmland soils, there is little information on the vertical distribution of ARGs along the soil profile. In addition, insufficient attention has been paid to actual production conditions in studies. Soil depth leads to heterogeneity in the soil environment, and within farmland, the vertical variation of soil properties with depth is much greater than the horizontal spatial variability. Research on the vertical distribution of ARGs in farmland soils after manure application is helpful for improving the understanding of ARG environmental risks. The co-application of organic and chemical fertilizers is a common fertilization strategy in agricultural production. Understanding the effect of the ratio of manure to chemical fertilizer application on the accumulation of ARGs in farmland can help us formulate ARG environmental risk management strategies that better align with production needs. We believe that a reasonable application ratio of manure and chemical fertilizers can alleviate the accumulation of ARGs caused by the resource utilization of livestock manure in agricultural production to a certain extent and control the diffusion of ARGs into deep soil. This study aimed to investigate the influence of different manure application rates on the vertical distribution of ARGs in agricultural soils, assess the relationship between ARGs and microbial communities, and identify the principal environmental factors that influence ARG retention. The findings of this research can provide theoretical guidance for ecological risk assessment pertaining to the sustainable utilization of livestock manure.

## 2. Materials and Methods

### *2.1. Sample Collection*

The soil samples utilized in this study were collected from corn farmland in Changtu County (123°58′0.340″ E, 42°48′14.083″ N, Liaoning Province, China). Corn cultivation follows rotary tillage practices. The fertilizers consist of chemical fertilizers (self-made corn-specific fertilizers) and organic fertilizer (chicken manure after high-temperature composting). Various proportions of chemical and organic fertilizers were combined and applied to the farmland soil in three repeat regions. The chicken manure was labeled JF in figure and the application rates of organic fertilizers and sample numbers for different treatments are provided in Table 1. Sampling was performed at depths of 0–20 cm (Layer A), 20–40 cm (Layer B), and 40–60 cm (Layer C). Each sample comprised a blend of soils collected from five sampling points after fertilization for 30 days. A portion of the soil samples was stored at −80 °C in Whirl-Pak bags for the subsequent analysis of microorganisms, ARGs, and antibiotics. The remaining portion of samples was cleared of plant roots, large gravel particles, leaves, and other debris. After indoor drying, it was sifted through a 2 mm standard sieve and homogenized for the assessment of soil physicochemical properties and heavy metal content.

**Table 1.** Application rate of organic fertilizer (chick manure) under different treatments.

| Soil Layers | Control Group (100% Chemical Fertilizer) | Treatment 1 (25% Organic Fertilizer Replaced Chemical Fertilizer) | Treatment 2 (50% Organic Fertilizer Replaced Chemical Fertilizer) | Treatment 3 (75% Organic Fertilizer Replaced Chemical Fertilizer) | Treatment 4 (100% Organic Fertilizer) |
|---|---|---|---|---|---|
| A (0–20 cm) | A0 | A1 | A2 | A3 | A4 |
| B (20–40 cm) | B0 | B1 | B2 | B3 | B4 |
| C (40–60 cm) | C0 | C1 | C2 | C3 | C4 |

### *2.2. Antibiotic and Heavy Metal Determination*

Eight antibiotics that were commonly used in livestock and occurring in poultry manure (oxytetracycline, tetracycline, doxycycline, tilmicosin, tylosin, sulfamonomethoxine, sulfamethazine, and sulfadiazine) were determined in this study [34–36]. The recoveries of the eight antibiotics measured via the external standard method [37] in the manure samples ranged from 70% to 120%. In particular, the antibiotics were extracted from 5.0 g of the manure samples by following this procedure: 20 mL of mixed extractant (0.4 g $Na_2EDTA$ + acetonitrile mixed with phosphate buffer at the ratio of 1:1, *v*/*v*) was added to the samples and shaken vigorously for 3 min and then centrifuged at 21,500× *g* for 5 min. The supernatant was transferred into a conical flask, adding 10 mL of mixed extractant. Then the extraction was repeated again. Finally, the pH of the extracting solution was adjusted to 2–2.5 and extraction was performed using HLB solid-phase extraction columns. The HLB columns was pre-activated with 6 mL methanol and 6 mL water. After extraction, each sample was vacuumed for 5 min and then 6 mL of methanol was used to elute the HLB columns. The eluate was collected and blown to near-dryness at 40 °C under nitrogen atmosphere, dissolved in 2 mL of 1:1 methanol: water *v*/*v*) with 0.2% formic acid, and centrifuged at 32,250× *g* for 10 min. Liquid chromatographic conditions for antibiotics analysis were set as follows: Poroshell 120 EC-C18 column (2.1 × 50 mm, 1.9 μm), flow rate of 0.3 mL/min, column temperature of 40 °C, injection volume of 2 μL, mobile phase A with 0.1% formic acid in methanol, and mobile phase B with 2.5 mmol/ammonium acetate (containing 0.1% formic acid) in water.

The concentrations of Cu, Zn, Cd, As, Pb, and Hg in the digested soil and manure were determined using inductively coupled plasma mass spectrometry (ICP-MS) (NexION 350, Perkin Elmer, Waltham, MA, USA). About 0.1 g soil/manure sample was placed in a digestion flask, and 5 mL HCl, 10 mL $HNO_3$, 2 mL HF, and 1 mL HClO were added, then the samples were digested using a graphite furnace digestion apparatus. The accuracy of

the experimental data was verified using reagent blank samples and duplicate soil samples. The recovery rate of the standard samples was 90–110% and the relative standard deviation (RSD) of the data was less than 5%.

### 2.3. Soil Physical and Chemical Indicators

All soil samples were air-dried at 25–30 °C in the laboratory, ground and sieved through a 0.25 mm mesh, and then analyzed for physicochemical properties. The pH was measured using a pH analyzer (Sartorius PB-10, Shanghai, China). The cation exchange capacity (CEC) was determined using atomic absorption spectrometry (AA700, Perkin Elmer, Waltham, MA, USA) [38]. The contents of total carbon (TC), total nitrogen (TN), and total phosphorus (TP) were measured with an elemental analyzer (Vario MICRO cube, Hanau, Germany). The concentration of potassium (K) was determined using an inductively coupled plasma mass spectrometer (ICP-MS) (NexION 350, Perkin Elmer, Waltham, MA, USA).

### 2.4. DNA Extraction and Metagenomic Sequencing

Manure/soil DNA was extracted using HiPure Soil DNA Kit B. Integrity and purity of extracted DNA were assessed using 1% agarose gel electrophoresis and its concentration and purity were determined using a NanoDrop 2000 ultra-micro spectrophotometer (Thermo Fisher, Wilmington, DE, USA) and a QuantiFluor fluorometer (Promega, Madison, WI, USA). PE150 bipartite sequencing was performed using the Illumina HiSeq/Illumina Novaseq/MGI2000 platform. Sequencing raw data (pass filter data) were removed from splices and low-quality sequences using the second-generation sequencing data quality statistics software, Cutadapt (v1.9.1), with primer and splicer sequences removed. The clean data for subsequent information analysis were obtained by removing bases with quality values lower than 20 at both ends, excluding sequences with N-base content greater than 10%, and retaining the minimum read length of 75 bp. Clean reads were assembled into overlapping clusters for each sample using MEGAHIT [39] and coding genes were predicted using the Prodigal software (v3.02) [40]. Then, the gene sequences of all the samples were integrated and further de-redundated by sequence clustering software MMseq2 (v11-e1a1c), which defaulted to 95% identity and 95% coverage for clustering to obtain a non-redundant gene set of unigene sequences. The representative sequences in the redundant gene set were compared with those in the NCBI NR database to obtain bacterial annotation and taxonomic information. Identification and analysis of ARGs were conducted based on the Comprehensive Antibiotic Research Database (CARD), employing a threshold set above 90.

### 2.5. Statistical Analysis

Figures were plotted using TB tools (heat map) and Origin 2021 (Origin Lab, Northampton, MA, USA). The Duncan test was performed using SPSS (27 IBM, Armonk, NY, USA) to compare the differences among treatments at a probability level of less than 0.05.

## 3. Results and Discussion

### 3.1. Types and Abundance of ARGs in Manure

The identification and quantification of ARGs in manure are essential for evaluating the environmental ramifications of manure utilization in agriculture and formulating efficient fertilization methodologies [41–44]. A total of 462 ARG subtypes were identified in chicken manure. Among them, lincosamide (eighteen subtypes), aminoglycoside (fourteen subtypes), macrolide (eleven subtypes), chloramphenicol (nine subtypes), and glycopeptide (nine subtypes) were the primary types of ARGs (Figure 1A). ARGs that belonged to the lincomycin, macrolide, and aminoglycoside classes exhibited higher abundance, with 3187, 2651, and 1614 counts, respectively (Figure 1B). The major ARG subtypes in chicken manure were *tetA(58)*, *saur_walk_dap*, *msbA*, *bcrA*, *Ecol_fabG_TRC*, *macB*, and *novA*. Among these, *tetA (58)* and *bcrA* showed higher abundance (Figure 2). *tetA(58)* is a common gene that

confers resistance to tetracyclines while *bcrA* confers resistance to peptides. Both genes are widely distributed in chicken manure and soil [45–47]. Multidrug resistance genes are defined as ARGs that confer resistance to three or more antibiotics [48], facilitating the spread of ARGs among different bacteria [49]. The classification and analysis of ARGs in chicken manure revealed that subtypes of ARGs that are resistant to one-class drugs (40) >two-class drugs (23) > multidrug-resistant ARGs (19) (Figure 3). In terms of abundance, ARGs resistant to one-class drugs were still the largest (4032), but the abundance of multidrug-resistant ARGs (2653) > that of two-class drugs (1172). The major resistance mechanisms of ARGs detected in chicken manure were antibiotic target alteration (33.33%), antibiotic efflux pump (27.49%), antibiotic inactivation (17.75%), and antibiotic target protection (10.17%) (Figure S1). Antibiotics affect fundamental bacterial functions, including protein synthesis, RNA polymerase transcription, chromosome segregation, and folate metabolism. Antibiotic target alteration directly affects associated proteins or indirectly triggers the activation or inhibition of regulatory proteins that oversee these modifications, diminishing drug affinity and bolstering bacterial resistance [50]. Antibiotic efflux pumps are transporter proteins that expel toxic substrates out of cells and into the external environment [51]. Characterized by the expulsion of intracellular antibiotics from the cell via transporters, they assume a pivotal role in bacterial multidrug resistance and the development of numerous drug-resistant phenotypes [52].

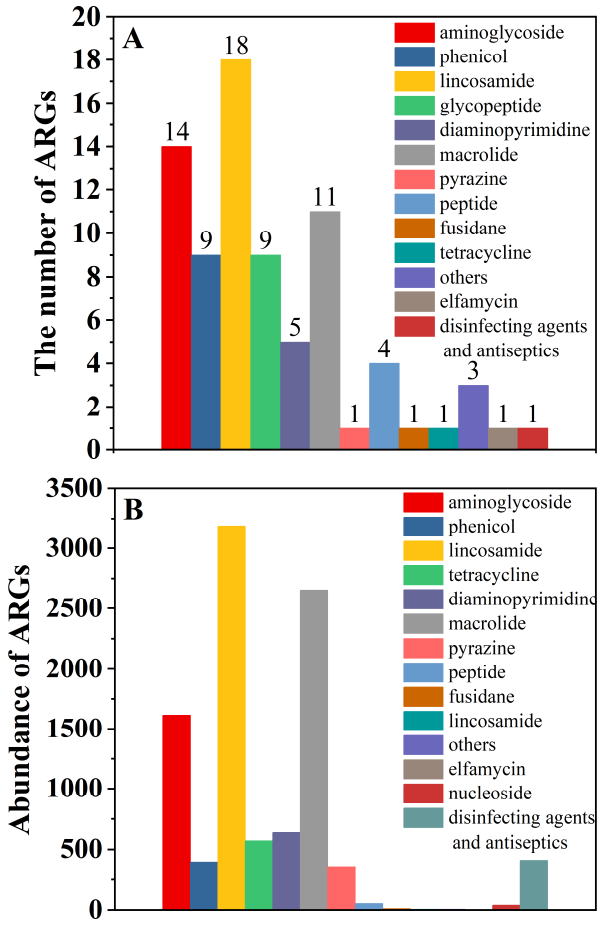


**Figure 1.** Quantities (**A**) and abundance (**B**) of ARGs in chicken manure.

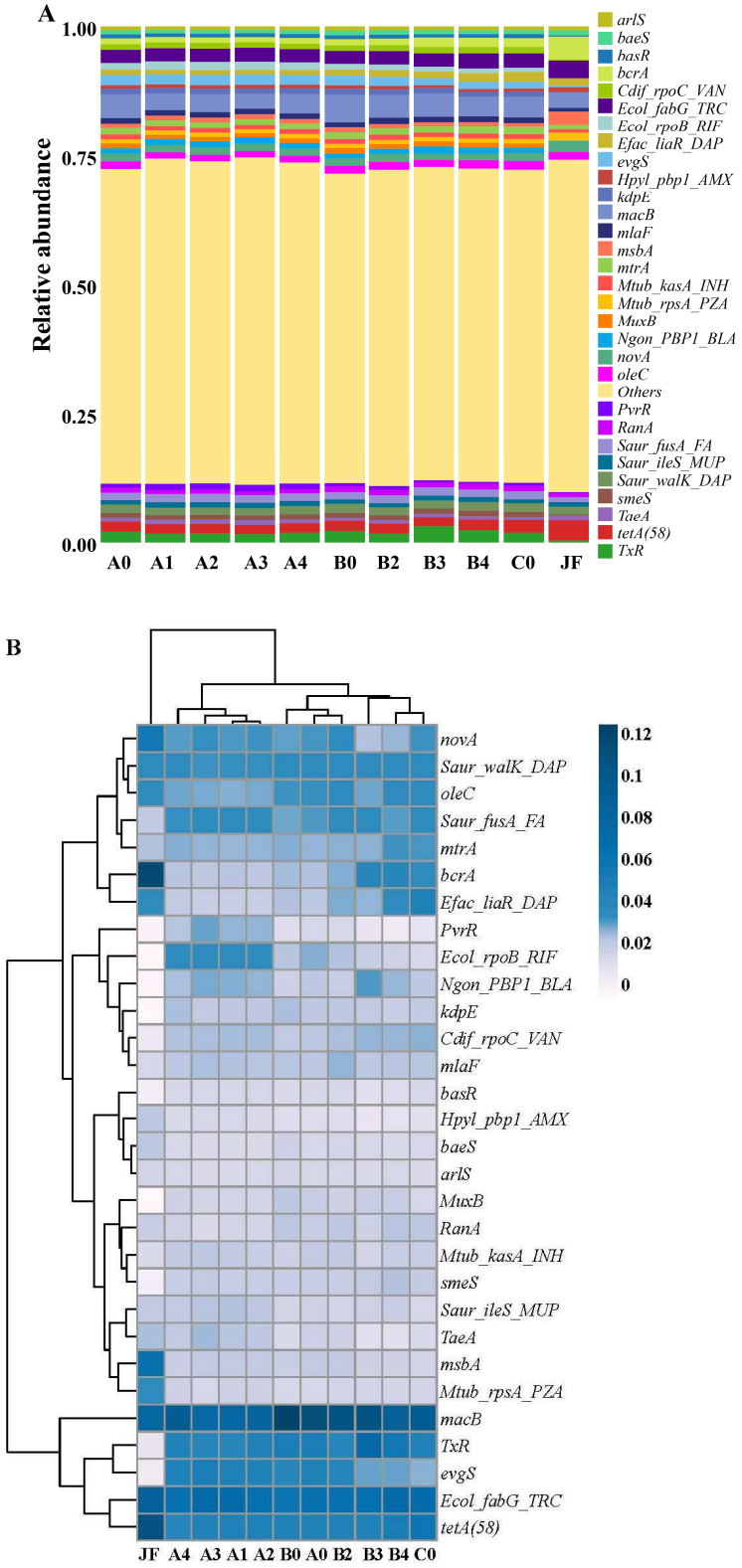


**Figure 2.** Changes in the relative abundance of major ARGs in soil and heatmap: subtypes and abundance of ARGs under different treatments (**A**) and the heatmap of ARGs under different treatments (**B**). A, B, and C represent the 0–20 cm, 20–40 cm, and 40–60 cm soil layers, respectively. The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively. JF: organic fertilizer (chicken manure after high-temperature composting).

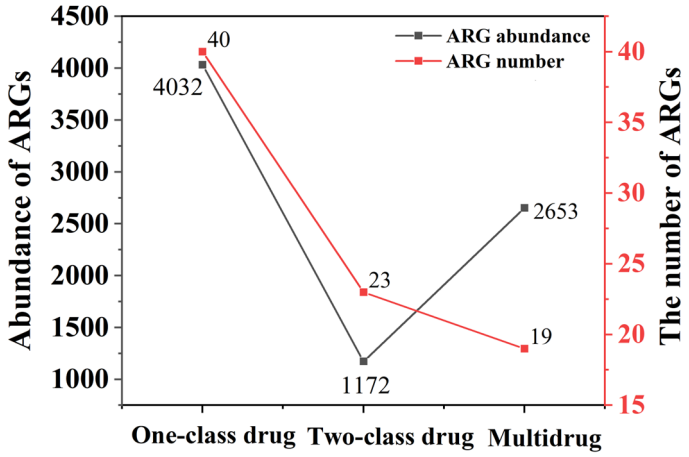


**Figure 3.** Abundance and quantities of ARGs in chicken manure in terms of resistance to drug classes.

### *3.2. Diversity and Abundance of ARGs in Fertilized Farmland Soil*

After the application of chicken manure to soil, various factors such as the diffusion of ARGs from manure, the alteration of environmental conditions, nutrient dynamics, and antibiotic stress can influence the relative abundance of ARGs in soil [53–55]. Although no significant positive correlation was observed between the quantity of applied chicken manure and the diversity of ARGs in soil, the soil treated with 100% chicken manure exhibited the highest diversity of ARG types (A4). Notably, the application of chicken manure contributed to an increase in the diversity of ARGs in agricultural soil. ARGs were still detected in soils treated only with chemical fertilizers, indicating that the application of organic fertilizers had led to the long-term presence of ARGs in farmland soils. A total of 652 ARG subtypes were identified in soil, with peptide, glycopeptide, fluoroquinolone, and elfamycin as the primary types of ARGs. The dominant ARGs detected in soil from each treatment included *macB*, *TRC*, *tetA (58)*, *Ecol_fabG_TxR*, *evgS*, *Saur_walK_DAP*, and *Saur_fusA_FA* (Figure 2A). A comparison between ARGs detected in chicken manure and those in soil post-application revealed similarities in major ARG subtypes, albeit with significant variations in their abundance (Figure 4). Variations in the abundance of resistance genes are intricately linked to microbial communities within the environment [56]. In particular, the relative abundance of *bcrA* in soil ranged from 58.66% to 77.83%, lower than that in chicken manure, while the relative abundance of *TxR* ranged from 73.94% to 86.56%, higher than that in chicken manure. The dominant ARGs observed in Layer A (0–20 cm) and Layer B (20–40 cm) were similar, with *macB* as the most prevalent ARG in both layers (Figures S2 and S3). *macB* confers resistance to macrolides in bacteria through the mechanism of antibiotic efflux pumps [57]. ARGs detected in soil predominantly exhibit four mechanisms of antibiotic resistance (Figure S1), including antibiotic target protection (~8%), antibiotic target alteration (~31%), antibiotic inactivation (~20%), and antibiotic efflux pumps (~33%) [58,59].

The distribution of ARGs in farmland soil was closely associated with soil depth and influenced by the application rate of manure. As shown in Figure 4A, the surface layer (Layer A, 0–20 cm) presented a rich diversity of ARGs. As the soil depth increased, the diversity of ARGs in each soil treatment decreased to varying extents (e.g., 41 subtypes of ARGs in A4, 16 subtypes of ARGs in B4, and none in C4). Notably, in the deepest soil layer (Layer C, 40–60 cm), no ARGs were detected in soils treated with chicken manure. The surface soil, as the primary recipient of manure, retained a more diverse array of ARGs. In terms of gene abundance, the ARG abundance in surface soil (Layer A) was significantly higher than that in deeper soils (Figure 5A). For instance, in soils treated with 100% chicken manure, the highest ARG abundance in Layer A was 347 (A4), which decreased to 109 in B4 and was absent in C4. However, the distribution of individual ARG types varied across soil layers. The abundance of fluoroquinolone ARGs, which was dominant in A4, decreased

by 85.45% in B4 and was entirely absent in C4. The abundance of peptide ARGs in B2 was 59, which was 47.9% higher than that in A2. The variation in individual ARG types distribution across soil layers is linked to microbial communities, antibiotics, and mobile genetic elements [20]. Conversely, the abundance of ARGs in the surface soil did not exhibit a significant increase with the rise in chicken manure application (e.g., the abundance values of A1, A2, A3, and A4 were 207, 173, 223, and 347, respectively). This result is attributed to the application of chemical fertilizers also influencing the maintenance of ARGs in soil [60]. The application of manure increased the diversity and abundance of ARGs in surface soil, with the most notable effect observed in soil treated only with manure. The accumulation of ARGs can foster the emergence of multidrug-resistant bacteria in the environment [61].

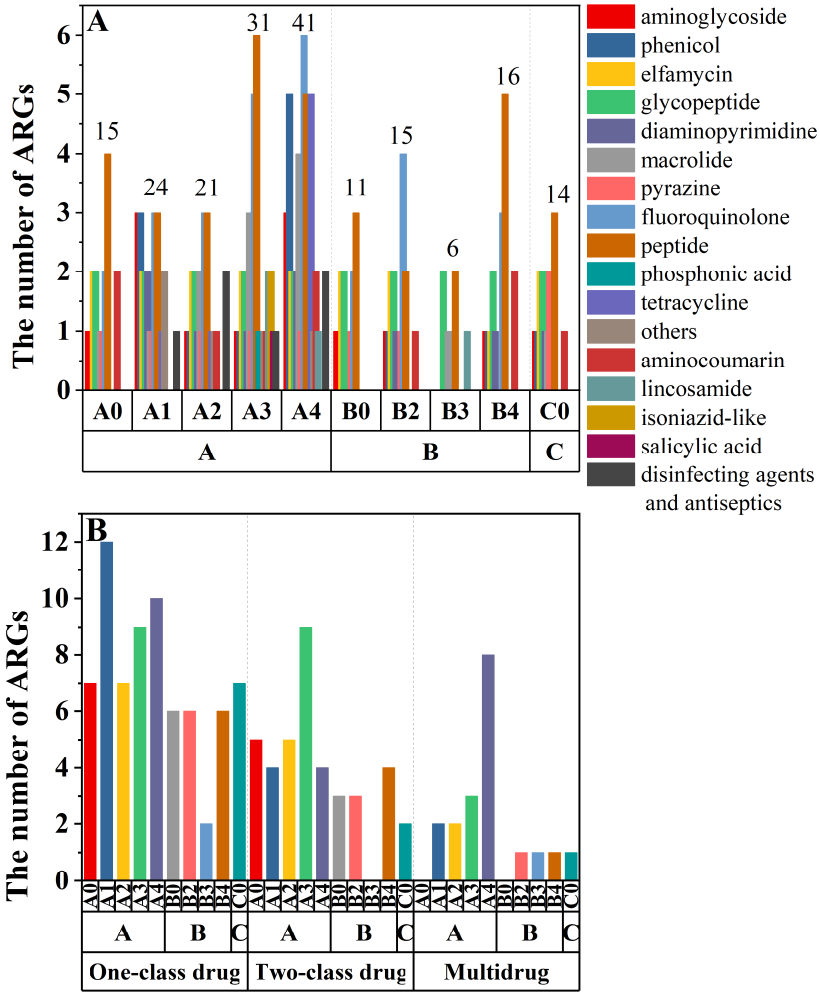


**Figure 4.** Quantities of ARGs (**A**) and drug resistance classes (**B**) in different soil layers (A: 0–20 cm, B: 20–40 cm, C: 40–60 cm). The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively.

According to the analysis of ARG resistance classes in soil (Figure 4B), ARGs that were resistant to only one-class drugs comprised the highest number (648) while the abundance of multidrug-resistant ARGs was the lowest (137) (Figure 5B). This distribution was aligned with the proportion of ARG resistance types observed in chicken manure. All types of ARG resistance were predominantly concentrated in the surface soil (Layer A), with abundances decreasing significantly with an increased soil depth. For example, in samples treated with 100% chicken manure, the numbers of ARGs that were resistant to one class of drugs were ten (A4), six (B4), and zero (C4), while the numbers of ARGs that were resistant to

two classes of drugs were four (A4), four (B4), and zero (C4). No significant correlation was observed between ARG resistance types in soil and the application amount of chicken manure, which was also true for abundance (Figure 5A). The abundance of multidrug-resistant ARGs was the highest in topsoil treated with 100% organic fertilizer while the abundance of ARGs that were resistant to one and two classes of drugs was higher in surface soil treated with 75% organic fertilizer.

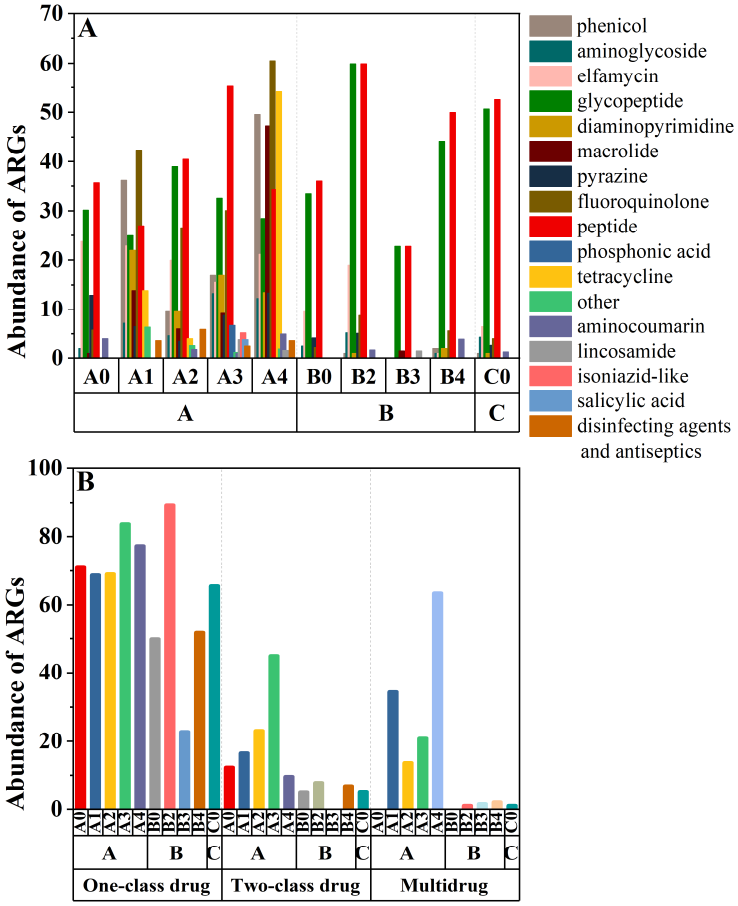


**Figure 5.** Abundance of ARGs (**A**) and drug resistance classes (**B**) in different soil layers (A: 0–20 cm, B: 20–40 cm, C: 40–60 cm). The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively.

### *3.3. Soil Microbial Community in Fertilized Farmland Soil*

Nutrients, microorganisms, ARGs, antibiotics, and heavy metals present in manure can directly or indirectly influence the accumulation and maintenance of ARGs in soil [62–65]. The contents of carbon, antibiotics, and heavy metals in soil treated with chicken manure increased to varying degrees (Figure S4). These components exhibit the potential to affect the structure of the microbial community in soil, thereby influencing the maintenance of ARGs. The predominant bacterial phyla identified in chicken manure were Actinobacteria, Firmicutes, and unclassified with relative abundance values of 46.95%, 40.95%, and 10.82%, respectively (Figure 6). Actinobacteria and Firmicutes were recognized as major bacterial hosts of ARGs [66–69]. Changes in the relative abundance of species at the phylum level in soil from different treatments are depicted in Figure 6A, revealing that Chloroflexi (1.03–7.67%), Verrucomicrobiota (0.84–10.61%), Firmicutes (0.49–1.17%), Gemmatimonadetes (2.96–8.47%), unclassified (11.42–14.29%), Actinobacteria (7.06–20.46%), Acidobacteria (12.2–30.02%), and Proteobacteria (14.86–46.93%) were the predominant bacterial phyla in all treated samples.

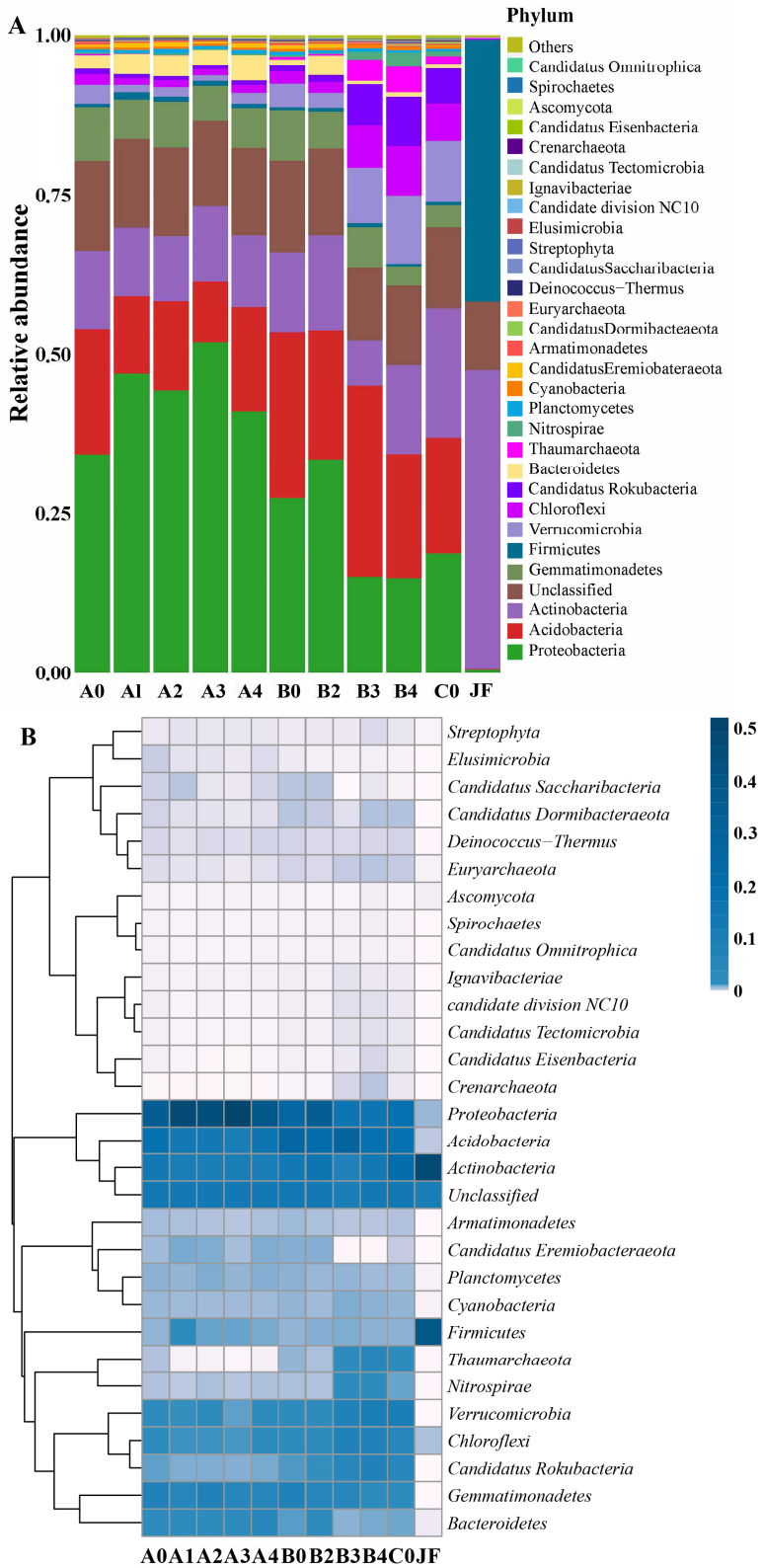


**Figure 6.** Changes in the relative abundance of microbial species in different soil layers: the relative abundance of species at the phylum level under different treatments (**A**) and heatmaps of species at the phylum level under different treatments (**B**) (others represent all phyla or genera with a relative abundance of less than 1%). A, B, and C represent the 0–20 cm, 20–40 cm, and 40–60 cm soil layers, respectively. The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively. JF: organic fertilizer (chicken manure after high-temperature composting).

There were significant differences in the structures of bacterial communities in soils at different depths (Figures S5 and S6). In Layer A soil, the dominant phyla were Proteobacteria, Acidobacteria, Actinobacteria, unclassified, and Gemmatimonadetes. In Layer B soil, the relative abundance of Proteobacteria and Gemmatimonadetes decreased by 48.06% and 14.19%, respectively, while the relative abundance of Acidobacteria and Actinobacteria increased by 66.48% and 7.69%, respectively. Nutrients in manure can promote the growth and reproduction of soil bacteria, enhancing bacterial diversity [70]. However, heavy metals exert a suppressive effect on microbial activities [71]. Therefore, changes in nutrient composition and heavy metal content in soil collectively influence the structure of the microbial community.

Compared with the control treatment that only used chemical fertilizers, the application of chicken manure exerted varied effects on the bacterial community structure in soil. At the phylum level, Proteobacteria exhibited an increase in Layer A soil (levels in A1, A2, A3, and A4 were 37.18%, 27.47%, 51.42%, and 19.41% higher than the control group A0) (Figure 6A). Proteobacteria, which consist of Gram-negative bacteria, prefer environments with a high nutrient content and are significantly affected by the type of fertilizer used [72,73]. Consequently, Proteobacteria decreased by 58.45% in Layer B soil compared to layer A soil. Acidobacteria also declined in layer A soil (it decreased by 38.32%, 29.22%, 51.57%, and 16.43% in A1, A2, A3, and A4 compared to control A0). Acidobacteria, which are oligotrophic bacteria, can thrive in complex environments and under oligotrophic conditions [74]. Hence, Acidobacteria exhibited higher relative abundance in deeper Layer B soil. Due to the complex conditions of field soil, changes in the bacterial community structure were only correlated with the application rate of chicken manure in surface soil.

Figure S7 shows the KEGG cluster and functional analyses of microbial community genomes in chicken manure and soil. The enzymes primarily involved in genes were glycosyltransferases and glycoside hydrolases. The major functional pathways were related to metabolism, genetic information processing, and environmental information processing.

The differences in microbial function and classification composition among different layers of soil were analyzed (Figure 7). According to the analysis of similarities (ANOSIM), the differences in microbial function (Figure 7A) and classification (Figure 7B) between groups in Layers A and B were greater than those within groups ($R > 0$, $p < 0.05$). The principal component analysis (PCA) based on the Bray–Curtis distance matrix (Figure 7) indicated that the first two principal components (PCs) explained 62.04% of the variance in microbial communities. PC1 was a principal component associated with soil layers, accounting for 40.91% of the variance, while PC2 was a principal component associated with treatments within groups, accounting for 21.13% of the variance (Figure 7C). The correlation between the soil layers and microbial communities was greater than that between the treatments within the groups and microbial communities. Soil properties, such as chemical composition and physical structure, change from surface to deeper layers; hence, significant changes also occur in community composition and functional characteristics with depth in the soil microbiome [75]. The compositions of microbial communities in soil samples with different fertilization treatments exhibited differences on the PC axis (Figure 7D). On the PC1 axis, each treatment group was relatively scattered, with chicken manure (JF) distributed in the positive direction, while the other treatment groups were mostly distributed on the negative axis of PC1. On the PC2 axis, JF and C5 were distributed on the positive axis while Groups A and B were distributed on both the positive and negative axes, with group A mainly in the negative direction. The results indicated that PC1 was a principal component related to the characteristics of chicken manure, and it could explain 50.42% of the variance. PC2 was a principal component related to soil depth, accounting for 18.46% of the variance. The microbial community structures in A1, A2, A3, and A4 were similar while those in JF and soil samples with different fertilization rates showed significant differences.

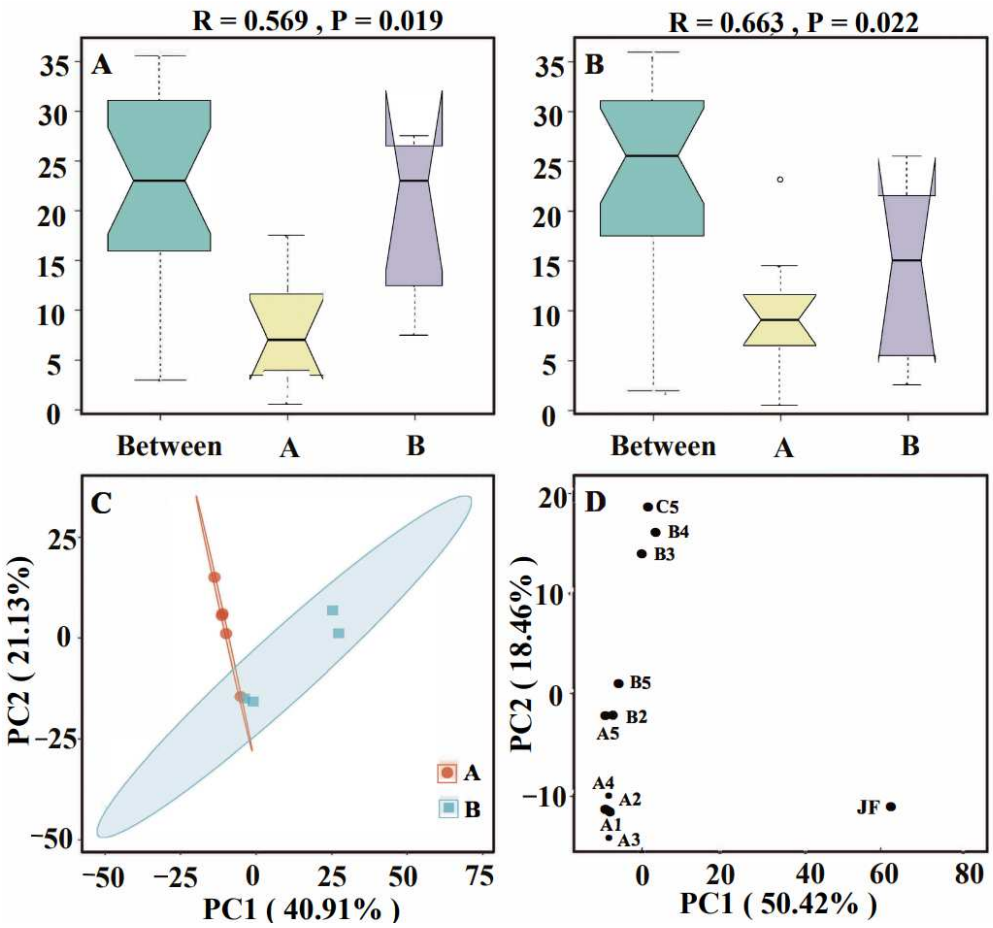


**Figure 7.** Differences in microbial function and classification composition at different soil layers: differences in microbial function (**A**) and classification (**B**) among ANOSIM groups; PCA of microbial community distribution characteristics in different soil layers (**C**) and fertilization rates (**D**) based on the Bray–Curtis distance matrix.

### *3.4. Correlation Analysis of ARGs with Microorganisms in Soil*

The correlation between the major ARGs and microorganisms in soil and their antibiotic resistance types were analyzed. Figure 8 shows that multidrug-resistant ARGs are widely found in soil microorganisms, and microorganisms can harbor multiple ARGs. The presence of multiple ARGs in the same potential microbial host increases the risk of ARGs spreading among pathogens [76–78]. Upon comparing the potential microbial hosts of ARGs in soil with pathogenic bacteria in the NCBI database (Table S1), the potential microbial hosts of ARGs in all the samples encompassed three species of human pathogenic bacteria: *Pseudomonas* sp. (*Pseudomonas aeruginosa LESB58* and *P. aeruginosa PAO1*) and *Listeria* sp. (*Listeria monocytogenes EGD-e*). The three pathogenic genera simultaneously contain multiple ARGs and nearly every pathogen has developed resistance to at least one antibiotic. The antibiotic resistance mechanism of *Pseudomonas* involved antibiotic efflux and the alteration of antibiotic targets while that of *Listeria* was antibiotic inactivation. *Listeria*, which consists of a series of Gram-positive bacteria, can obtain resistance genes from plasmids and associated transposons possessing various resistance mechanisms [79]. The dissemination of ARGs resulting from manure application and their accumulation in pathogenic bacteria significantly increase risks to human health.

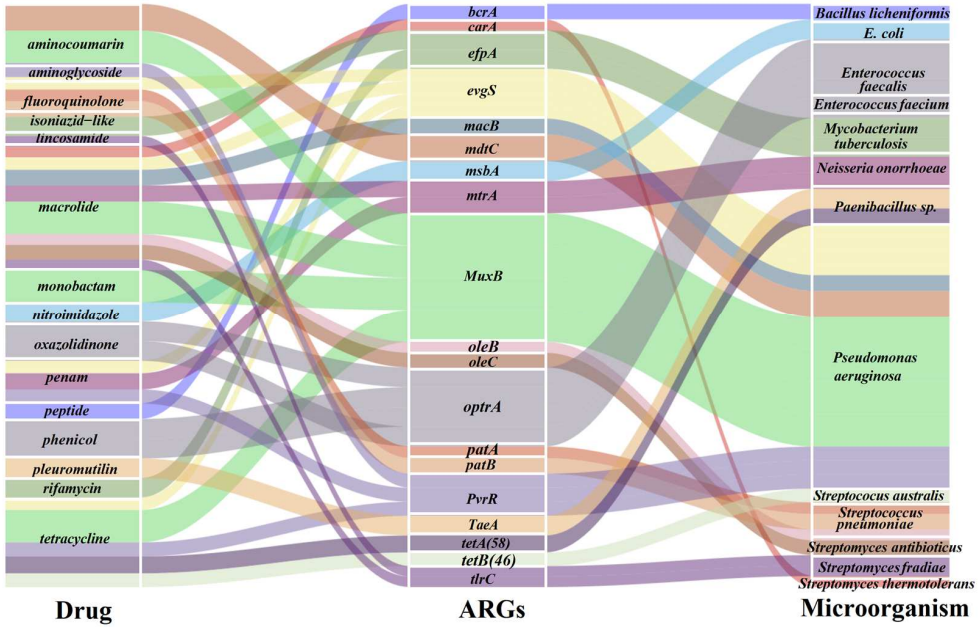


**Figure 8.** Mulberry plot of microbes, ARGs, and antibiotic resistance types in soil.

### *3.5. Correlation Analysis of ARGs in Soil with Environmental Factors*

The effects of environmental factors on ARGs in soil were assessed with an aggregation boosting tree (ABT). The analysis of Bray–Curtis distance indicated that factors that influenced the abundance of ARGs included heavy metals, microorganisms, antibiotics, and nutrients, with relative effects of 26.9%, 23.7%, 16.5%, and 17.4%, respectively (Figure 9A). The redundancy analysis (RDA) of ARG abundance and environmental factors further suggested that the selected microorganisms accounted for 71.3% of abundance changes in ARGs, with RDA1 and RDA2 accounting for 41.5% and 29.8%, respectively. Among them, *Enterococcus faecalis* exerted the most significant effect on ARGs. The abundance of *E. faecalis* was positively correlated with *macB*, *TxR*, *olec_walk_DAP*, *saur_walk_DAP*, and *evgS*, but negatively correlated with *tetA(58)*, *Ecol_fabG_TRC*, *Saur_fusA_FA*, and *novA*. Meanwhile, the abundance of *E. faecalis* was positively correlated with *P. aeruginosa*, *Streptomyces fradiae*, and *Neisseria gonorrhoeae* [80]. *E. faecalis* is an opportunistic pathogen of animals and humans. It not only acts as the primary host of multidrug-resistant bacteria but also possesses unique virulence factors that facilitate the transfer of ARGs and virulence genes. It is also an indicator of food and manure contamination [45,81].

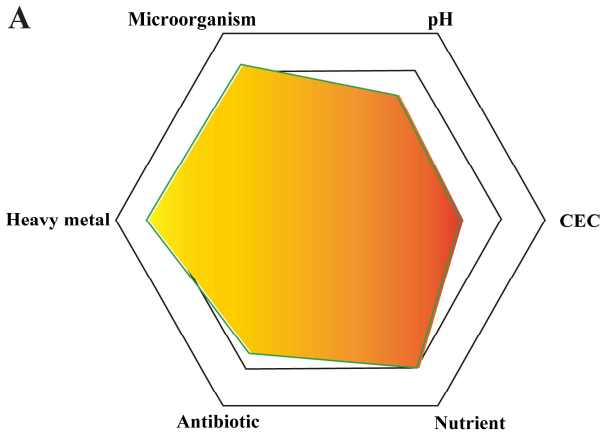


**Figure 9.** *Cont.*

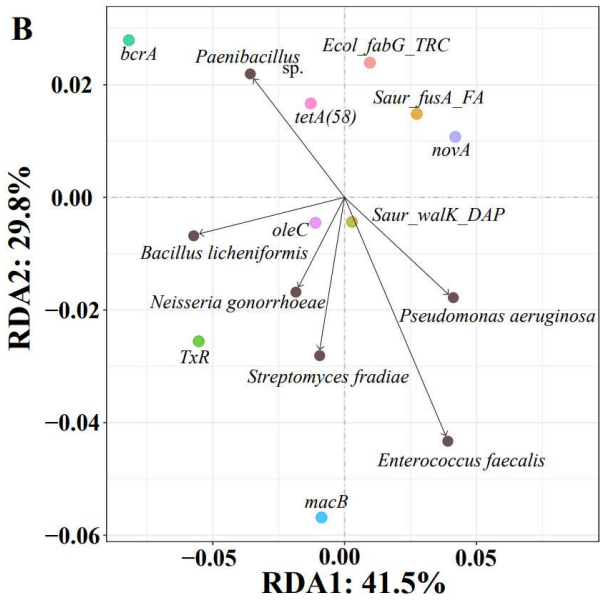


**Figure 9.** ABT assessment of the effect of environmental factors on ARGs (based on the Bray–Curtis distance) (**A**) and RDA based on ARGs and microbial abundance (**B**).

## 4. Conclusions

A total of 462 ARG subtypes, which are predominantly resistant to lincomycin, aminoglycoside, macrolides, chloramphenicol, and tetracyclines, were detected in chicken manure. After manure application, 652 ARG subtypes were detected in soil after 30 days. Among them, peptide, glycopeptide, fluoroquinolone, and elfamycin were the primary types of ARGs. Variations in environmental conditions resulted in disparities in the composition of ARGs. The distribution of ARGs in farmland soil was closely related to soil depth and influenced by the amount of manure applied. Although the types and abundance of ARGs were significantly higher in surface soil treated with chicken manure compared with the control samples treated only with chemical fertilizers, they decreased significantly with an increased soil depth. However, ARGs did not exhibit a gradual change with increasing fertilizer application. The subtype compositions of ARGs in chicken manure and soil were similar, but differences in abundance were observed. Resistance to one class of drugs was identified as the major type of drug resistance among ARGs in soil and chicken manure.

The analysis of soil microbial communities revealed that Chloroflexi, Verrucomicrobia, Firmicutes, Gemmatimonadetes, an unclassified phylum/phyla, Actinobacteria, Acidobacteria, and Proteobacteria were the dominant bacterial phyla in all the treated samples. The structure of soil bacterial communities was affected by the amount of chicken manure applied. ARGs in soil endowed host bacteria with resistance through antibiotic efflux pumps (~33%), antibiotic target protection (~8%), antibiotic target alteration (~31%), and antibiotic inactivation (~20%). Further analysis showed that multidrug-resistant ARGs were widespread among soil microorganisms, with the potential microbial hosts of ARGs including two human pathogenic genera (*Pseudomonas* sp. and *Listeria* sp.). The transfer of ARGs due to manure application considerably increases human health risks. Heavy metals (26.9%) and microorganisms (23.7%) exerted relatively greater effects on the accumulation and a maintenance of ARGs in soil. Therefore, the harmless treatment of livestock manure and the reasonable manure application strategy are crucial for reducing the accumulation of pollutants and avoiding the occurrence of compound pollution in farmland soil.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems8030089/s1, Figure S1: The composition of the detected ARGs drug resistance mechanism in different soil layers. A, B, and C represent the 0–20 cm, 20–40 cm, and 40–60 cm soil layers, respectively. The numbers 0, 1, 2, 3, and 4 correspond to the application

of 0, 25, 50, 75, and 100% manure in the soil, respectively. JF: organic fertilizer (chicken manure after high-temperature composting). Figure S2: Relative abundance of ARGs in different soil layers. A: 0–20 cm soil layer, B: 20–40 cm soil layer. Figure S3: Heatmap of relative abundance of ARGs in different soil layers. A: 0–20 cm soil layer, B: 20–40 cm soil layer. Figure S4: Changes in pH (A), CEC (B), heavy metals (C), antibiotics (D), carbon (E), and nutrients (F) in different soil layers. A, B, and C represent the 0–20 cm, 20–40 cm, and 40–60 cm soil layers, respectively. The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively. JF: organic fertilizer (chicken manure after high-temperature composting). Figure S5: The relative abundance of microbial phylum in different soil layers (others represent all phyla with relative abundance less than 1%). A: 0–20 cm soil layer, B: 20–40 cm soil layer. Figure S6: Heatmap of microbial phylum in different soil layers. A: 0–20 cm soil layer, B: 20–40 cm soil layer. Figure S7: KEGG cluster analysis and functional analysis of microbial community genomes in chicken manure and different soil layers: Enzyme activity annotation of microbial communities in chicken manure and different soil layers (A); KEGG clustering of microbial community genomes in chicken manure and different soil layers (B). A, B, and C represent the 0–20 cm, 20–40 cm, and 40–60 cm soil layers, respectively. The numbers 0, 1, 2, 3, and 4 correspond to the application of 0, 25, 50, 75, and 100% manure in the soil, respectively. JF: organic fertilizer (chicken manure after high-temperature composting). Table S1: Pathogenic bacteria with ARGs and resistance mechanisms in soil.

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