Prevalence of Antibiotic Resistance Genes and Bacterial Pathogens in Long-Term Manured Greenhouse Soils As Revealed by Metagenomic Survey

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Prevalence of Antibiotic Resistance Genes and Bacterial Pathogens in Long-Term Manured Greenhouse Soils As Revealed by Metagenomic Survey

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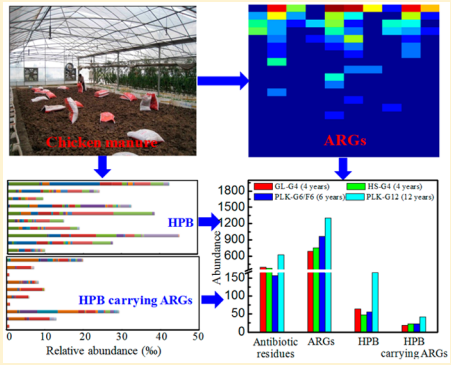
Supporting Information

ABSTRACT: Antibiotic resistance genes (ARGs), human pathogenic bacteria (HPB), and HPB carrying ARGs pose a high risk to soil ecology and public health. Here, we used a metagenomic approach to investigate their diversity and abundance in chicken manures and greenhouse soils collected from Guli, Pulangke, and Hushu vegetable bases with different greenhouse planting years in Nanjing, Eastern China. There was a positive correlation between the levels of antibiotics, ARGs, HPB, and HPB carrying ARGs in manures and greenhouse soils. In total, 156.2−5001.4 μg/kg of antibiotic residues, 22 classes of ARGs, 32 HPB species, and 46 species of HPB carrying ARGs were found. The highest relative abundance was tetracycline resistance genes (manures) and multidrug resistance genes (greenhouse soils). The dominant HPB and HPB carrying ARGs in the manures were *Bacillus anthracis*, *Bordetella pertussis*, and *B. anthracis* (sulfonamide resistance gene, *sul1*), respectively. The corresponding findings in greenhouse soils were *Mycobacterium tuberculosis* and *M. ulcerans*, *M. tuberculosis* (macrolide-lincosamide-streptogramin resistance protein, MLSRP), and *B. anthracis* (*sul1*), respectively. Our findings confirmed high levels of antibiotics, ARGs, HPB, and HPB carrying ARGs in the manured greenhouse soils compared with those in the field soils, and their relative abundance increased with the extension of greenhouse planting years.



INTRODUCTION

Antibiotics have been widely used in China since the early 1990s as food additives at subtherapeutic doses in livestock and poultry breeding to prevent diseases in animals and improve production performance.[1] Approximately 30−90% of the antibiotics fed to animals can be excreted by feces or urine as parent compounds or metabolites. Subsequently, these residual antibiotics can enter the soil environment following the land application of animal wastes at the level of 15 000−150 000 kg/ha per year in the cultivation of greenhouse vegetables in China, which accounts for 85% of global total greenhouse cultivation area.[2−5] Antibiotic residues in greenhouse soils are usually low (i.e., μg/kg to mg/kg) because of their adsorption, biodegradation, photolysis, and transport.[6] However, repeated applications of manure can still result in their "persistent" pollution.[3]

Manure carries antibiotic resistance genes (ARGs) and incorporates antibiotic residues into soils, and these residues even at low concentrations exert a selective pressure on the microbial community and induce the emergence of diverse ARGs or multidrug resistance (MDR) genes.[4,7] The occurrence of *E. coli* carrying *aadA* and *tetB* was significantly more frequent in the manured soil samples compared with swine manure.[8] The abundance of sulfonamide ARGs clearly increased due to repeated applications of sulfonamide-contaminated pig manure in arable soils.[9] In addition, Zhu et al.[10] reported that the abundance of the top 63 ARGs subtypes of the detected 149 ARGs increased 192−28 000-fold in swine manures compared with antibiotic-free swine manures and control soils.

ARGs are readily captured by human pathogenic bacteria (HPB) to form superbugs such as *Salmonella*, *Bacteroidales*, *Campylobacter*, *Shigella*, and *E. coli* O157:H7.[11−13] Micallef et al.[14] found that eight *Enterococcus* species with resistance to ciprofloxacin, rifampicin, and levofloxacin were the prevalent opportunistic pathogens in tomato farm soil from the Mid-Atlantic United States. Yang et al.[15] reported that the *Bacteroidales* bacteria *Myroides ordoratimimus* (antibiotic resistant bacteria) and *Sphingobacterium* spp. (MDR bacteria) were related to human clinical opportunistic pathogens in chicken manure. These HPB species confer antibiotic resistance and pathogenicity and easily infect humans by contact or via the consumption of raw vegetables (e.g., radishes, tomatoes, strawberries, raspberries, and lettuce),[16] which in turn poses a

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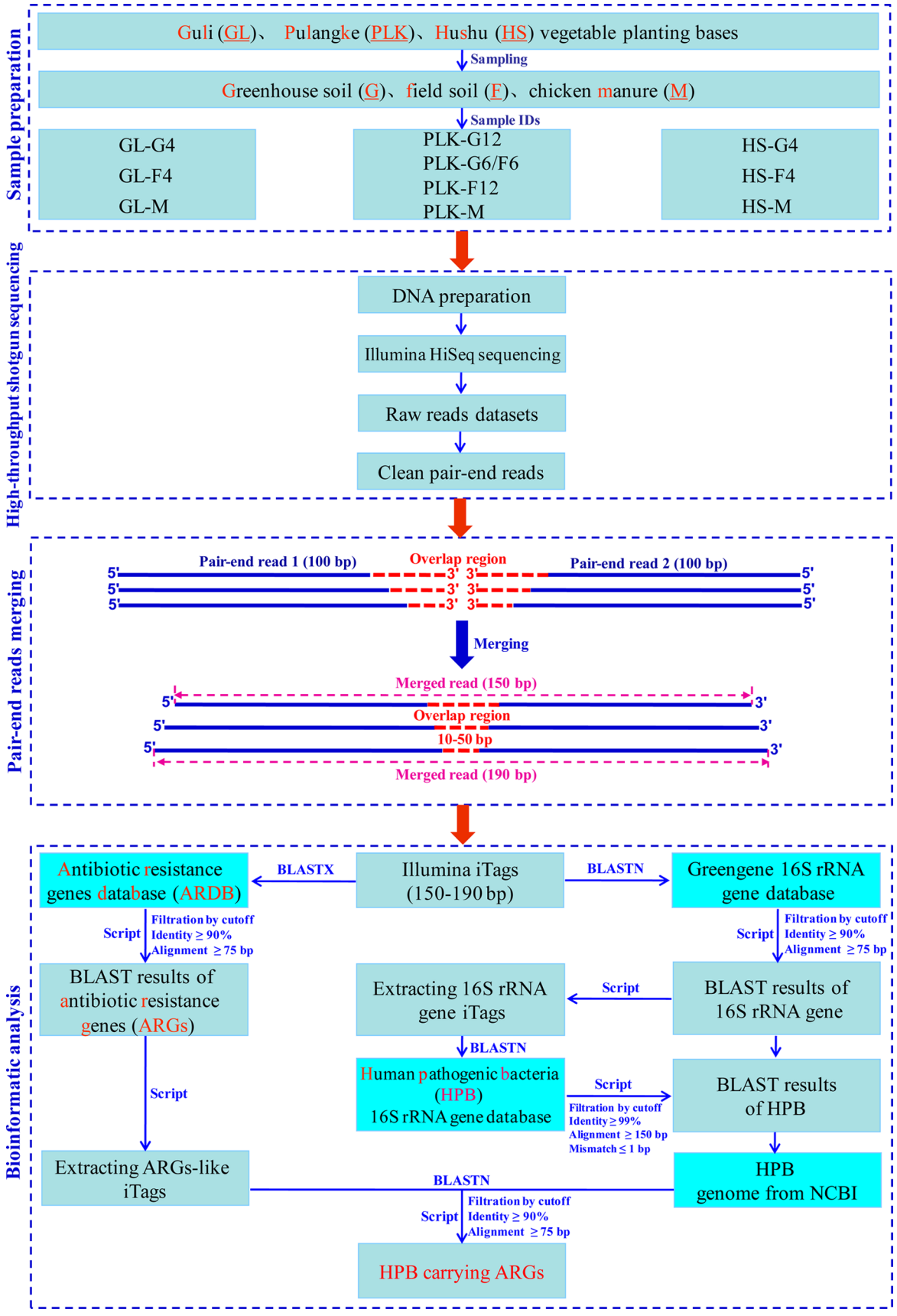

Figure 1. Flowchart of the metagenomic analysis for antibiotic resistance genes (ARGs), human pathogenic bacteria (HPB), and HPB carrying ARGs in chicken manures and soils.

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serious threat to public health.[17] Although the diversity and abundance of ARGs in manures and farm soils have been investigated in several studies,[10,18] little is known about the diversity and abundance of ARGs, HPB, and especially HPB carrying ARGs in greenhouse soils following long-term applications of manure.

The present study examines the diversity and abundance of ARGs, HPB, and HPB carrying ARGs in manure-amended greenhouse soils from different planting years by metagenomic analysis using an Illumina high-throughput shotgun sequencing technique. The objectives of this study were (1) to determine the residual amounts of different classes of antibiotics; (2) to detect the diversity and abundance of ARGs, HPB, and HPB carrying ARGs; (3) to reveal the correlations between antibiotic residues, ARGs, HPB, HPB carrying ARGs, and greenhouse planting years. These findings will contribute to a more comprehensive and accurate evaluation of the ecological risks associated with manure application in a greenhouse soil environment.

MATERIALS AND METHODS

Chemicals. Technical grade antibiotics were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and included: tetracycline (97.0%, TC), oxytetracycline (96.5%, OTC), chloroteracycline (92.5%, CTC), sulfadiazine (99.0%, SDZ), sulfadimidine (99.0%, SDD), sulfamethoxazole (99.0%, SMX), lincomycin (98.0%, LCC), norfloxacin (99.5%, NOR), ciprofloxacin (95.0%, CIP), enrofloxacin (98.5%, ENR), and chloramphenicol (98.5%, CPC). These antibiotics are divided into five classes: tetracyclines (TC, OTC, and CTC), sulfonamides (SDZ, SDD, and SMX), fluoroquinolones (NFC, OTC, and CTC), lincosamides (LCC), and chloramphenicols (CPC). Analytical grade and chromatographic grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

Manure and Soil Sampling. Chicken manure samples and corresponding manure-amended greenhouse soil samples (0−15 cm) were collected from three representative vegetable cultivation bases in the Guli (GL), Pulangke (PLK), and Hushu (HS), located in Nanjing suburbs in Eastern China. The three chicken manures originated mainly from three local chicken farms in the surrounding counties and were used as organic fertilizer. Field soil samples (0−15 cm) were collected from a vegetable field adjacent to the greenhouse and used as the controls (no history of manure application). Soil collected from five sampling sites within each vegetable greenhouse and was thoroughly mixed to obtain a composite sample. Detailed information on the three representative vegetable cultivation bases and three chicken manures is summarized in Tables S1 and S2 in the Supporting Information. Leaf vegetables (Chinese cabbage, pakchoi, bokchoi, spinach, and lettuce) and fruit vegetables (cucumber and tomato) were cultivated in these bases for 4−12 years. Three chicken manure samples and seven soil samples were designated as follows: GL chicken manure (GL-M), PLK chicken manure (PLK-M), and HS chicken manure (HS-M), GL greenhouse soil for 4 years (GL-G4), GL field soil for 4 years (GL-F4), PLK greenhouse soil for 12 years (PLK-G12), PLK greenhouse for 6 years followed by an uncovered shed for 6 years (PLK-G6/F6), PLK field soil for 12 years (PLK-F12), HS greenhouse soil for 4 years (HS-G4), and HS field soil for 4 years (HS-F4). The physicochemical properties of the manures and soils are summarized in Table S3 in the Supporting Information. All samples were individually transferred into a plastic bag and transported immediately to the laboratory within 2 h. Subsequently, each sample was sieved (2 mm) to remove stones and debris and was stored at −20 °C until further analysis. Each treatment was replicated three times for the determination of antibiotic residues.

Extraction and Determination of Antibiotics. The residues of the 11 antibiotics in the manures and soils were extracted from the chicken manure and soil samples following the method described by Fang et al.[19] and quantified according to the method described by Ho et al.[20] using an ultra performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS, Waters). The analytical conditions, limit of detection (LOD), and limit of quantitation (LOQ) of the 11 antibiotics are summarized in Table S4 in the Supporting Information. To evaluate the effectiveness of the antibiotic extraction method, a recovery experiment was conducted. Three replicated standard concentrations (0.1, 1, and 10 mg/kg) of the 11 antibiotics were mixed together with 2 g (dry weight equivalent) of either manure or soil samples and processed as described above.

DNA Extraction and Sequencing. Total DNA was extracted from 1.0 g of each manure or soil sample using a FastDNA SPIN Kit for Soil (MP Biomedicals, CA) according to the manufacturer's instructions. The DNA extracted from three technical replicates of each sample was pooled into one DNA sample to minimize any potential DNA extraction bias. The concentration and quality of the extracted DNA were determined using spectrophotometry (NanoDrop ND-1000, Wilmington, DE). Prepared DNA samples were sent to Novegene (Beijing, China), and approximately 5 μg of the each DNA samples was used for shotgun library construction. Subsequently, Illumina high-throughput sequencing was performed with the HiSeq 2000 platform using a PE101 + 8+101 cycle (Paired-end sequencing, 101-bp reads and 8-bp index sequence) sequencing strategy. Approximately 5 Gb of metagenomic data were generated for each DNA sample. Each manure and soil sample was sequenced for three technical replicates.

Quality Filtering. The metagenomic data sets were filtered using a self-written script to remove the reads containing three or more ambiguous nucleotides and those with a length less than 100 bp. Next, the 100 bp paired-end raw reads were paired-merged using a self-written script to screen for 10−50 bp overlap paired-end reads and to assemble them into 150−190 bp iTags (Illumina tags). Finally, the number of iTags was normalized to 10 000 000 in each metagenomic data set using a self-written script for downstream bioinformatic analysis. The obtained clean iTag data sets for all samples were uploaded to the MG-RAST server (http://metagenomics.anl.gov/, and the MG-RAST IDs are summarized in Table S5 in the Supporting Information).

Bioinformatic Analysis. A detailed flowchart for data analysis is shown in Figure 1. Four bioinformatic analyses were conducted in this study:

(i) ARGs: A widely accepted ARGs database was downloaded from the Antibiotic Resistance Database (ARDB, http://ardb.cbcb.umd.edu/).[21] The redundant sequences from the downloaded database were removed using a self-written script. The resulting database retained 2998 nonredundant sequences of 7797 original sequences from the ARDB. A total of 22 subdatabases were established for the ARG subtypes (Table S6 in the Supporting Information). The metagenomic iTags from each sample were searched against the non-

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redundant ARDB using BLASTX with an *E*-value < 1×10^{-5}. An iTag sequence was annotated as an ARG-like sequence if its best hit in the nonredundant ARDB had ≥90% amino acid identity and an alignment length ≥25 amino acids (75 bp).

(ii) 16S rRNA gene (16S): The Greengenes 16S database (version 2013) was downloaded directly from the Greengenes Web site (http://greengenes.lbl.gov/).[22] The metagenomic iTags from each sample were searched against the Greengenes 16S database using BLASTN with an *E*-value <1×10^{-20}. The Greengenes 16S hit iTags were extracted from the metagenomic iTags data sets.

(iii) HPB: An HPB 16S database was constructed based on the taxonomic list derived from the HPB virulence factor database (http://www.mgc.ac.cn/VFs/)[23] and other references.[24,25] All of the selected HPB 16S are publicly available from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) because their complete genomes have already been sequenced. As shown in Table S7 in the Supporting Information, a total of 708 16S sequences were retrieved and assigned to 61 human pathogenic bacterial species. The 16S iTags from each sample were searched against the HPB 16S database using BLASTN with an *E*-value <1×10^{-20}. The BLAST hit outputs were further filtered to annotate the HPB using the strict criteria of amino acid identity ≥99%, alignment length ≥150 bp, and mismatch ≤1 bp.

(iv) HPB carrying ARGs: To reveal the diversity and abundance of HPB carrying ARGs, the genome sequences of the BLAST hit HPB were directly downloaded from the NCBI GenBank and then searched against the above ARG-like sequences for each sample. The BLAST hit outputs were further filtered to annotate the HPB carrying ARGs using strict criteria with amino acid identity ≥90% and alignment length ≥25 amino acids.

Statistical Analysis. Univariate analysis of covariance was conducted between antibiotic residues, ARGs, HPB, and HPB carrying ARGs in manures and soils using SPSS 19.0 (SPSS Inc., Chicago, IL). The averages and standard deviations of all data were processed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA). To distinguish the differences in diversity and abundance of ARG subtypes, a heat map of each dominant ARG class was visualized using Matlab 7.0 (The MathWorks, Natick, MA).

RESULTS

Residual Levels of Antibiotics in the Manures and Soils. The recoveries of the 11 antibiotics at three concentrations of 0.1, 1.0, and 10.0 mg/kg were 60.1−83.5% with relative standard deviations (RSDs) < 4.3% in the chicken manures and 62.3−91.1% with RSDs < 3.5% in the soils. The LOD and LOQ of the 11 antibiotics in all samples were 0.1−5.0 μg/kg and 0.5−15.0 μg/kg, respectively (Table S4 in the Supporting Information). These results indicate that our extraction method was suitable for antibiotic residue analysis. As shown in Figure 2 and Table S8 in the Supporting Information, the residual concentrations of the antibiotics, expressed as the sum of tetracyclines, sulfonamides, fluoroquinolones, LCC, and CPC, were 2526.0, 5001.4, 4722.1 μg/kg in PLK-M, GL-M, and HS-M, respectively, and 631.7, 156.2, 395.7, 384.1 μg/kg in PLK-G12, PLK-G6/F6, GL-G4, and HS-G4, respectively. However, antibiotic residues were under the LOD in PLK-F12, GL-F4, and HS-F4. These results show that several classes of antibiotic residues were present in the chicken manures and greenhouse soils. The residual levels of antibiotics

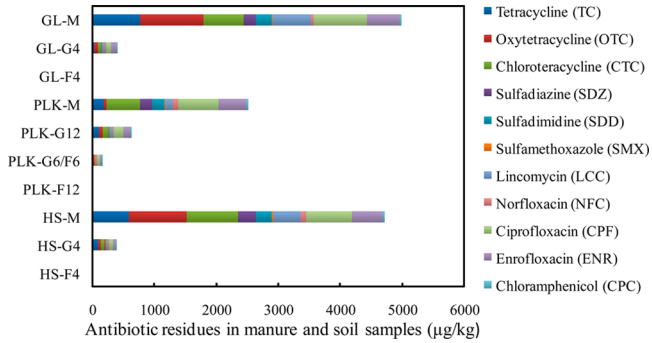

Figure 2. Residual levels of antibiotics in chicken manures and soils. Each value is the mean of three replicates.

in the chicken manures were considerably higher than those found in the greenhouse soils. Furthermore, the residual levels of tetracyclines and fluoroquinolones were higher than those of other antibiotics in both the chicken manures and soils. In addition, the individual antibiotic level in PLK-G6/F6 (cultivation in the greenhouse for 6 years and then cultivation in an uncovered shed for 6 years) decreased to 11.6−64.5% of the corresponding antibiotic level in PLK-G12 (cultivation in the greenhouse for 12 years), indicating that the uncovered shed significantly decreased antibiotic residues in soils.

As shown in Table S9 in the Supporting Information, significant ($P \leq 0.01$) positive correlations were observed in the levels of antibiotics between the chicken manures and the greenhouse soils using univariate analysis of covariance, e.g., PLK-M and PLK-G12 ($R = 0.785$), GL-M and GL-G4 ($R = 0.771$), and HS-M and HS-G4 ($R = 0.794$). A gradual accumulation of antibiotic residues in the greenhouse soils was significantly and positively correlated ($R = 0.680$, $P \leq 0.05$) with an extension of the greenhouse planting years (Figure S1 and Table S12 in the Supporting Information).

Diversity and Abundance of ARGs in the Manures and Soils. The relative abundance (i.e., the ARGs hit number divided by metagenomic iTags number in each sample) of ARGs in each chicken manure and soil sample is shown in Figure 3a. The relative abundance of ARGs varied from 0.01% to 0.23% in the chicken manures, 0.007% to 0.015% in the greenhouse soils, and 0.0009% to 0.0015% in the field soils. These results show that the ARGs abundance in the chicken manures and greenhouse soils was 9.9−220.1 and 5.5−8.9 times higher, respectively, than those in the field soils.

In total, 22 classes of ARGs were found in all samples (Figure 3b). Tetracycline resistance (TCR) genes were the most abundant ARGs in the chicken manures, followed by those encoding resistance to CPC, sulfonamide, aminoglycoside, and purine. However, the most abundant ARGs in the greenhouse soils were those encoding MDR, followed by ARGs encoding resistance to macrolide-lincosamide-streptogramin (MLS), acridine, tetracycline, and fosmidomycin. A higher abundance of MDR genes and a lower abundance of other ARG classes were observed in the greenhouse soils compared with the chicken manures (Figure 3b). The relative abundance of MDR genes in the greenhouse soils was 4.7−12.5 times greater than that in the field soils, and no significant ($P \leq 0.05$) difference was found among the GL-F4, PLK-F12, and HS-F4. Additionally, the relative abundance of MDR genes in PLK-G6/F6 was only 67.6% of that in PLK-G12, which suggests that uncovering a shed noticeably decreased the abundance of MDR genes.

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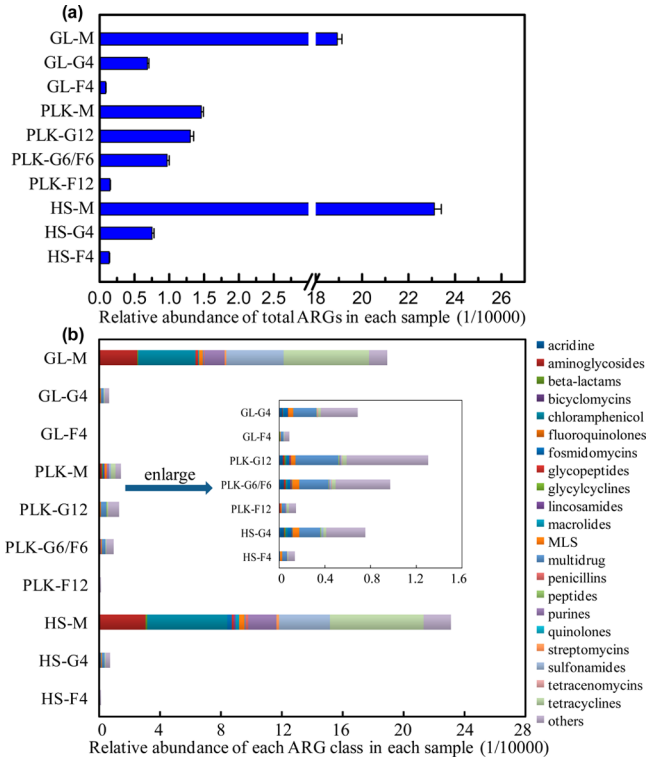

Figure 3. Relative abundance of total antibiotic resistance genes (ARGs) in chicken manures and soils (a). The diversity and abundance of different classes of ARGs in both manures and soils (b). All ARGs were categorized according to the classes of antibiotics. The number of iTags in each sample was normalized to the same size (10 000 000). The relative abundance of ARGs was defined as the ARGs hit number divided by metagenomic iTags number in each sample. "1/10 000" indicates one ARG-like iTag in 10 000 Illumina iTags. Error bars represent standard deviation of the mean. MLS: macrolide-lincosamide-streptogramin.

A comparison of the diversity and abundance of the eight dominant ARG classes in the manures and soils is shown in Figure 4. These dominant ARGs included MDR (20 subtypes), TCR (25 subtypes), beta-lactam resistance (10 subtypes), CPC resistance (11 subtypes), fosfomycin resistance (3 subtypes), aminoglycoside resistance (16 subtypes), acridine resistance (5 subtypes), and MLS resistance (8 subtypes) genes. The dominant subtypes of ARGs in the greenhouse soils were *mexF* and *bpeF* (MDR), which had a higher relative abundance than those in the chicken manure and field soils (Figure 4). Simultaneously, the abundant subtypes of ARGs in the chicken manures were *tetA(G)*, *tetX2*, *tetA*, *tetX*, *tetA(33)* (TCR), followed by aminoglycoside acetyltransferase (AAT), *aadA*, *aphD* (aminoglycoside ARGs), and *cmx* (CPC ARGs).

Significant ($P \leq 0.05$) positive correlations were found in the ARGs between the chicken manures and greenhouse soils using univariate analysis of covariance, e.g., GL-M and GL-G4 (R = 0.809), PLK-M and PLK-G12 (R = 0.996), PLK-M and PLK-G6/F6 (R = 0.985), and HS-M and HS-G4 (R = 0.993) (Table S9 in the Supporting Information). Interestingly, significant ($P \leq 0.05$) positive correlations were observed between the residual levels of tetracyclines, sulfonamides, lincosamides, fluoroquinolones, and CPC and their corresponding relative abundance of ARGs class with high correlation coefficients of 0.809, 0.815, 0.752, 0.890, 0.734, and 0.853, respectively (Table S10 in the Supporting Information). The relative abundance of ARGs in the greenhouse soils gradually increased with the extension of greenhouse planting years with a highly positive correlation coefficient (R = 0.786, $P \leq 0.05$) (Figure S1 and Table S12 in the Supporting Information).

Diversity and Abundance of HPB in the Manures and Soils. The diversity and relative abundance (i.e., the HPB 16S hit number divided by the Greengenes 16S hit number in each sample, and Greengenes 16S hit number in each metagenomic iTags data set is shown in Figure S2 in the Supporting Information) of HPB in the chicken manures and soils is shown in Figure 5. A total of 32 pathogenic bacteria were found. *Mycobacterium tuberculosis* and *M. ulcerans* were the dominant HPB species in the soils, followed by *Bordetella pertussis*, *Bacillus anthracis*, *Brucella melitensis*, *Corynebacterium diphtheria*, *Bartonella quintana*, and *M. leprae*. The relative abundance of *M. tuberculosis* and *M. ulcerans* in the greenhouse soils was 1.7−14.0 and 1.6−2.4 times higher, respectively, compared with the field soils. The relative abundance of these two HPB in PLK-G6/F6 was 45.9% and 34.7% of the PLK-G12, respectively. The dominant HPB species in the chicken manures were *B. anthracis* and *B. pertussis*, followed by *Staphylococcus aureus*, *C. diphtheria*, *Enterococcus faecalis*, *B. melitensis*, *M. tuberculosis*, *C. jeikeium*, and *M. ulcerans* (Figure 5). The mean relative abundance of HPB in the chicken manures was considerably higher than that in the greenhouse and field soils, and the highest relative abundant HPB was *B. pertussis* in GL-M, *E. faecalis* in PLK-M, and *B. anthracis* in HS-M. Significant ($P \leq 0.01$) positive correlations were found in the HPB between the chicken manures and greenhouse soils, e.g., GL-M and GL-G4 (R = 0.691), PLK-M and PLK-G12 (R = 0.970), PLK-M and PLK-G6/F6 (R = 0.899), and HS-M and HS-G4 (R = 0.964) (Table S9 in the Supporting Information). Similarly, a significant positive correlation (R = 0.693, $P \leq 0.01$) was also found between HPB abundance in the greenhouse soils and greenhouse planting years (Figure S1 and Table S12 in the Supporting Information).

HPB Carrying ARGs in the Manures and Soils. Figure 6 shows the diversity and relative abundance (i.e., the HPB carrying ARGs hit number divided by the Greengenes 16S hit number in each sample) of HPB carrying ARGs in the chicken manures and soils. As shown in Figure 6 and Table S13 in the Supporting Information, a total of 46 HPB carrying ARGs were found, and the ARGs harbored in the HPB contained 25 subtypes, such as *sul1*, *cmx*, *tetT*, *adeB*, *OXA-53*, *vanRG*, *aac*, *msrA*, *tetM*, etc. The most dominant HPB carrying ARGs in the chicken manures was *B. anthracis* harboring sulfonamide *sul1* followed by *M. tuberculosis* (MLSRP), *C. diphtheriae* (sulfonamide dihydropteroate synthase), *C. jeikeium* (CPC *cmx*), *S. aureus* (aminoglycoside phosphotransferase), *E. faecalis* (aminoglycoside phosphotransferase), and *S. aureus* (glycopeptide resistance protein). The mean relative abundance of HPB carrying ARGs in the chicken manures was 1.9 and 23.4 times higher, respectively, than that in the greenhouse and field soils. As shown in Figure 6, both *M. tuberculosis* (MLSRP) and *B. anthracis* (*sul1*) were the most dominant HPB carrying ARGs in the soils, with a higher abundance inside the greenhouses compared with the fields. The relative abundance of these HPB carrying ARGs in PLK-G6/F6 decreased significantly to 57.3% and 47.8%, respectively, of the relative abundance in PLK-G12. In this study, the MLSRP subtype was found in *M. tuberculosis*, *S. flexneri*, *S. dysenteriae*, and *S. agalactiae*. The MDR gene can be harbored by some pathogenic bacteria such as *Acinetobacter baumannii*, *Salmonella enteric*, *Yersinia enterocolitica*, *Pseudomo-*

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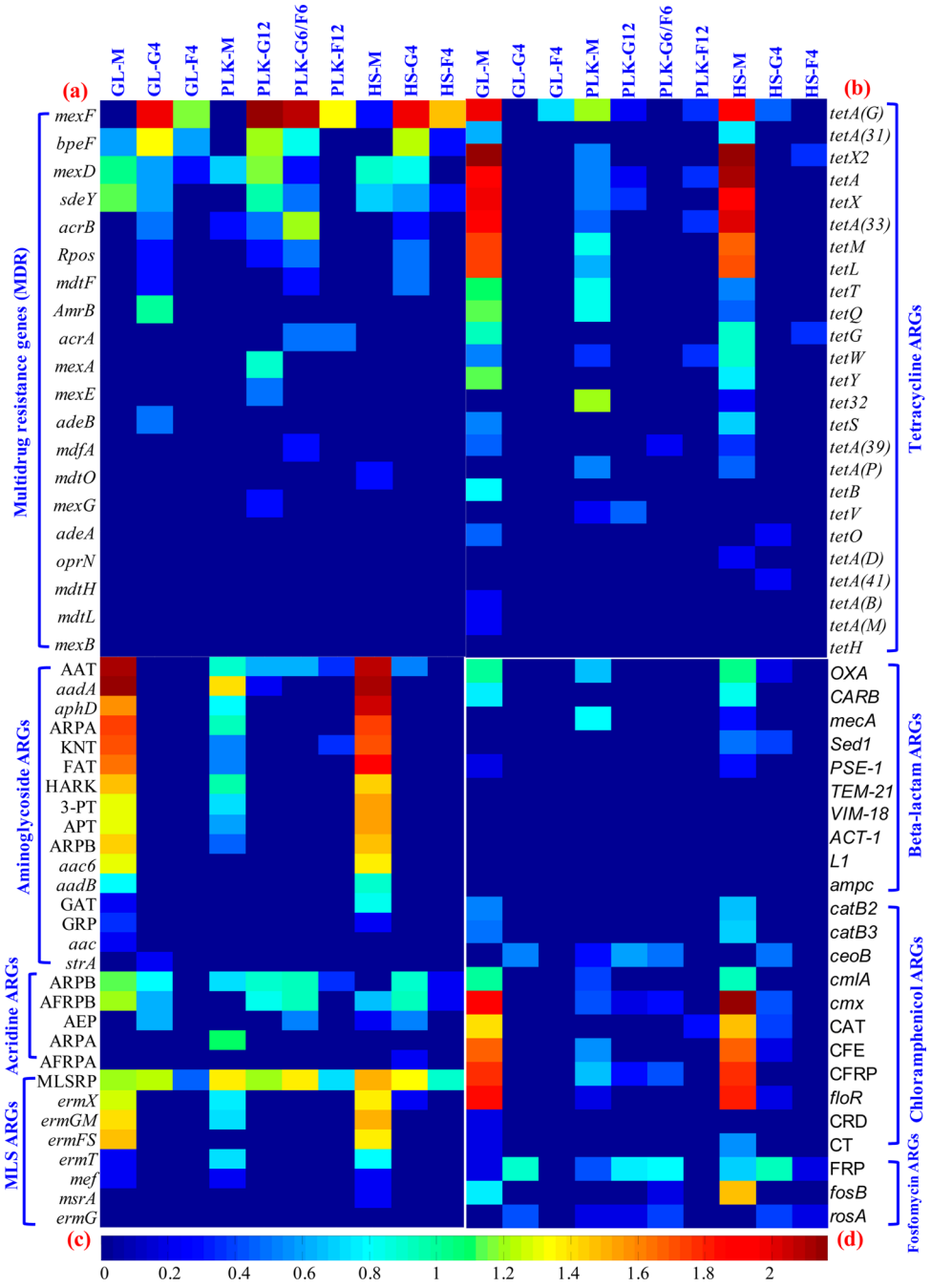

Figure 4. Heat maps of the dominant ARG subtypes in chicken manures and soils: (a) multidrug resistance genes (MDR); (b) tetracycline resistance genes; (c) aminoglycoside (AG) resistance genes, acridine resistance genes, and macrolide-lincosamide-streptogramin (MLS) resistance genes; (d) beta-lactam resistance genes, chloramphenicol (CPC) resistance genes, and fosfomycin resistance genes. The color intensity in each panel shows the common logarithm value of the ARG subtypes hit number in each normalized metagenomic iTags data set (10 000 000), referring to the color bar below. AAT, AG acetyltransferase; ARPA, AG resistance protein A; KNT, kanamycin nucleotidyltransferase; FAT, fused AG 3′-adenyltransferase-AG 6′-acetyltransferase; HARK, hydroxyurea antibiotic resistant kinase; 3-PT, AG 3′-phosphotransferase; APT, AG phosphotransferase; ARPB, AG resistance protein B; GAT, gentamicin acetyltransferase; GRP, gentamicin resistance protein; ARPB, acridine resistance protein B; AFRPB, acriflavin resistance protein B; AEP, acridine efflux pump; ARPA, acridine resistance protein A; AFRPA, acriflavin resistance protein A; MLSRP, MLS resistance protein; CAT, CPC acetyltransferase; CFE, CPC and florfenicol (FFC) exporter; CFRP, CPC and FFC resistance protein; CRD, CPC resistance determinant; CT, CPC transporter; FRPB, fosfomycin resistance protein B.

nas aeruginosa, *Shigella boydii*, and *S. dysenteriae*. Additionally, *E. faecalis* can carry diverse ARGs such as aminoglycoside phosphotransferase, sulfonamide dihydropteroate synthase, lincosamide nucleotidyltransferase, *tetT*, and *vanRG*.

Significant ($P \leq 0.01$) positive correlations were found in the HPB carrying ARGs between all chicken manures and all greenhouse soils, such as PLK-M and PLK-G12 ($R = 0.960$), PLK-M and PLK-G6/F6 ($R = 0.849$), HS-M and HS-G4 ($R = 0.626$), and GL-M and GL-G4 ($R = 0.751$) (Table S9 in the Supporting Information). There were significant ($P \leq 0.01$) positive correlations between all HPB carrying ARGs and all ARGs ($R = 0.901$) and between all HPB carrying ARGs and all

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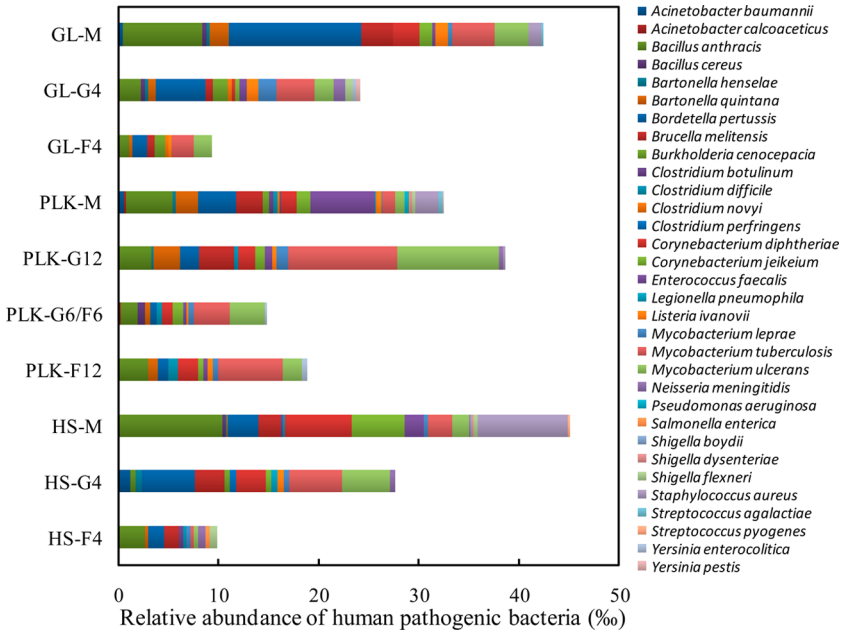

Figure 5. Diversity and relative abundance of human pathogenic bacteria (HPB) in chicken manures and soils. The relative abundance of HPB is defined as the HPB 16S rRNA gene hit number divided by Greengenes 16S rRNA gene hit number.

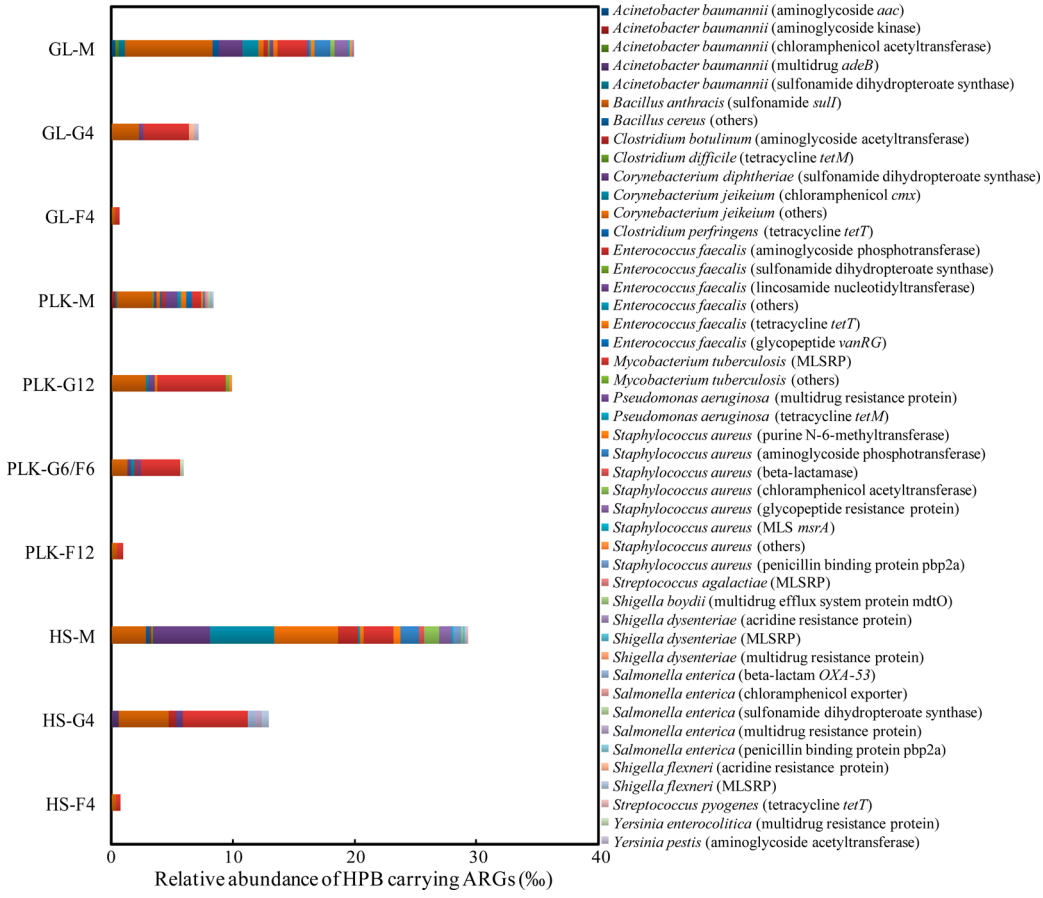

Figure 6. Diversity and relative abundance of human pathogenic bacteria (HPB) carrying antibiotic resistance genes (ARGs) in chicken manures and soils. The relative abundance of HPB carrying ARGs is defined as the HPB carrying ARGs hit number divided by Greengenes 16S rRNA gene hit number in each sample). MLSRP: macrolide-lincosamide-streptogramin resistance protein.

HPB (R = 0.870) in all samples (Table S11 in the Supporting Information). Additionally, the relative abundance of HPB carrying ARGs in the greenhouse soils gradually increased with an extension of the greenhouse planting years, and a significant positive correlation (R = 0.756, $P \leq 0.05$) is presented in Figure S1 and Table S12 in the Supporting Information.

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DISCUSSION

Antibiotic residues in soil have been shown to be correlated with manure type, manure application rate, soil type, vegetable species, cultivation method, and environmental conditions.[26] In this study, several classes of antibiotics were found in chicken manures and greenhouse soils, which may have resulted from the long-term fertilization of antibiotic-contaminated chicken manure in the greenhouse soils.[27] Similarly, the residual concentrations of tetracyclines, sulfonamides, and CPC were 4.5−24.7, 5.9−33.4, and 3.3−17.9 mg/kg, respectively, in manures (swine, bird, and cattle) and soils collected from Shanghai, Eastern China.[28] In addition, Huang et al.[29] reported that the residual levels of TC, OTC, CTC, ENR, CIP, and ofloxacin ranged from 189.8 μg/kg to 2668.9 μg/kg in farmland soils from four coastal cities in Fujian, Eastern China. In the current study, the observed lower concentrations of antibiotics in the greenhouse soils compared with the chicken manures may be due to the adsorption, biodegradation, photolysis, and infiltration of antibiotics in the soil.[19] Nevertheless, the long-term repeated application of chicken manure can still lead to the persistent contamination of greenhouse soils with antibiotics. Meanwhile, it is noteworthy that higher residual levels of antibiotics were observed in PLK-G12 (organic vegetable cultivation) compared with GL-G4 and HS-G4 (traditional vegetable cultivation) (Figure 2), which may be attributed to the high application of chicken manure in the organic vegetable base (Table S1 in the Supporting Information). Similarly, the absence of antibiotic residues in the field soils may be attributed to the fact that chicken manure was not applied to these soils.

In this study, the long-term application of chicken manure led to a noticeable increase in ARGs diversity and abundance in the greenhouse soils. Other studies have reported that different types of manure resulted in a marked increase in ARGs abundance in soil,[9,30] such as *ermF*, *sul1*, and *sul2*.[18] Cook et al.[31] reported that the abundance of sulfonamides, streptomycins, and tetracyclines ARGs increased up to 3 orders of magnitude in soil after poultry litter application. Several pathways have been identified as potential contributors to the diversity and abundance of ARGs in greenhouse soils: (i) inherent ARGs in the natural environment; (ii) ARGs carried by chicken manure; (iii) ARGs induced by antibiotic residues; (iv) horizontal gene transfer (HGT) of ARGs among soil bacteria. Pruden et al.[32] demonstrated that ARGs can be transferred between nonpathogens, pathogens, and even distantly related organisms (Gram-positive and Gram-negative bacteria) by mobile genetic elements such as class 1 integrons (intl1), plasmids, insertion sequences, transposons, and phages. In this study, an integron database was constructed based on 411 intl1 sequences (Table S14 in the Supporting Information). The relative abundance of intl1 was considerably higher in the chicken manures than that in the greenhouse soils, and no intl1 sequence was observed in the field soils (Figure S3 and Table S15 in the Supporting Information). The results show that chicken manures contained a large number of intl1 sequences, and furthermore, these sequences may be transported into greenhouse soils following land application.

Following the long-term application of chicken manures, the accumulation of MDR genes was observed in the greenhouse soils compared with the field soils, which may be due to the presence of different classes of antibiotics. A similar finding was reported by Heuer et al.,[9] who found an accumulation of sulfonamide ARGs in arable soils due to repeated applications of pig manure containing SDZ residues. In the current study, the dominant MDR subtypes, such as *mexF*, *bpeF*, and *mexD*, encode an efflux pump that export intracellular antibiotics out of cells, which is an important mechanism of resistance in MDR genes.[33] Meanwhile, the most abundant gene found in chicken manures was the TCR gene, which is in agreement with those findings reported for piggery manure,[34] swine manure,[35] and cow manure.[36]

The findings of this study revealed that the levels of antibiotic residues, ARGs, HPB, and HPB carrying ARGs varied greatly with sample type and sampling location, and their abundance in the greenhouse soils was highly positively correlated with greenhouse planting years. The most dominant ARG classes in the chicken manures and greenhouse soils were the TCR and MDR genes, respectively. The most dominant HPB species were *B. anthracis* and *B. pertussis* in the chicken manures and *M. tuberculosis* and *M. ulcerans* in the greenhouse soils. The most highly abundant species of HPB carrying ARGs were *B. anthracis* (*sul1*) in the chicken manures and *M. tuberculosis* (MLSRP) and *B. anthracis* (*sul1*) in the greenhouse soils. A good positive correlation was found in antibiotic residues, ARGs, HPB, and HPB carrying ARGs between chicken manures and greenhouse soils, and their abundance in the greenhouse soils increased with an extension of the greenhouse planting years. Further metatranscriptomic analyses are required to reveal the expression levels of ARGs (particularly ARGs harbored in the HPB) in soil microbial communities.

ASSOCIATED CONTENT

Supporting Information

Increased abundance of antibiotic residues, ARGs, HPB, and HPB carrying ARGs with the extension of greenhouse planting years (Figure S1); relative abundance of the Greengenes 16S hit number in metagenomic iTag data sets (Figure S2); relative abundance of class 1 integrons in chicken manures and soils (Figure S3); information on three vegetable bases (Table S1); information on three manure samples (Table S2); physicochemical properties of all samples (Table S3); optimal analysis conditions of antibiotics (Table S4); metagenome MG-RAST IDs and sample sizes (Table S5); composition of the ARDB database (Table S6); composition of the HPB database (Table S7); antibiotic residues in all samples (Table S8); analysis of covariance on antibiotic residues, ARGs, HPB, and HPB carrying ARGs between chicken manures and greenhouse soils (Table S9); analysis of covariance between antibiotic residues and ARGs in all samples (Table S10); analysis of covariance between ARGs, HPB, and HPB carrying ARGs in all samples (Table S11); analysis of covariance between environmental pollutants (antibiotic residues, ARGs, HPB, and HPB carrying ARGs) in the greenhouse soils and greenhouse planting years (Table S12); diversity and abundance of HPB carrying ARGs in chicken manures and soils (Table S13); list of class 1 integrons (Table S14); and abundance and diversity of class 1 integrons in chicken manures and soils (Table S15) This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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