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Identification and Distribution of Antibiotic Resistance Genes and Antibiotic Resistance Bacteria in the Feces Treatment Process: A Case Study in a Dairy Farm, China

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Abstract: The overuse of antibiotics has resulted in the prevalence of antibiotic resistance genes (ARGs) and antibiotic resistance bacteria (ARB) in the environment. High-density livestock farming is one of the major industries for antibiotic overuse. In this study, we sampled wastewater and manure at different stages of the feces treatment process from a dairy farm, as well as the soil in the farmland where the treated wastewater was being used for irrigation purpose. High-throughput Illumina sequencing was used to analyze the profiles of bacteria communities and ARGs. The results showed that the main ARG types were multidrug, aminoglycoside, glycopeptide, and tetracycline resistance genes, and Actinobacteria, Proteobacteria and Firmicutes were the main host bacteria phyla of these ARGs. The genus *Nocardioides* sp. and *Ornithinimicrobium* sp. were closely associated with the ARGs in the investigated samples. The relative abundances of ARGs in wastewater and manure were reduced by 68.5% and 62.1%, respectively, by the existing feces treatment process. Anaerobic fermentation and high-temperature fermentation were the most efficient treatment steps; the relative abundances of ARGs were reduced by 29.3% and 33.6% in the treated wastewater and manure, respectively. Irrigation with the treated wastewater significantly increased the abundance and diversity of ARGs and ARB in the surface soil of the farmland. The residual ARGs were found to transit through vertical gene transfer (VGT) and horizontal gene transfer (HGT) in soil. Therefore, the direct application of this inadequately treated wastewater and/or manure could risk spreading ARGs into the environment, and potentially impact human health. In order to effectively restrain the spread of ARGs, it is necessary to modify the wastewater and manure treatment processes and improve the regulations and guidelines of applying treated wastewater for irrigation.

Keywords: antibiotic resistance genes; antibiotic resistance bacteria; high-throughput sequencing; feces treatment; dairy waste

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1. Introduction

Antibiotics have been used widely in animal disease prevention and treatment, as well as husbandry breeding [1,2]. However, this resulted in the overuse of antibiotics in the livestock industries before regulations were put in place. The Chinese government implemented the Animal Drug Administration Regulations in 2004; however, more than 84,000 tons of antibiotics were consumed by livestock in 2013, accounting for 52% of the total

antibiotics used in China [3]. The veterinary antibiotics were poorly absorbed in the animal intestine, and nearly 90% of the antibiotics were excreted directly or as metabolites, which resulted in the abundance of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) [4–6]. Once entering the environment, ARGs can migrate and spread in various environmental media (such as soil, rivers, and groundwater) through the medium of mobile genetic elements (MGEs), causing more bacteria in the environment to develop antibiotic resistance [7]. The transmission pathways of ARGs mainly include vertical gene transmission (VGT) and horizontal gene transfer (HGT): VGT is the transmission of resistance genes (inherent in antibiotic-producing bacteria or produced by mutations) from parents to offspring; the resistance genes of HGT are induced by external environmental factors through plasmids, transposons, integrons/gene boxes, genomic islands, and other MGEs [8]. Extracellular DNA (secreted by living ARB or originated from the lysis of dead ARB) serves as a driver for HGT, a crucial mechanism enabling microorganisms to acquire beneficial genes for evolutionary adaptation [9]. The horizontal transfer of extracellular ARGs empowered microorganisms to acquire resistance genes [10]. Therefore, HGT is regarded as the most important factor for the current multiple resistance pandemic [11].

The use of antibiotics in dairy farms has increased as a result of increasing demand for beef and dairy products. Statistics have shown that full-grown milking cows weighing about 600 kg can discharge 40 to 50 kg of feces, 20 kg of urine, and 20 L of wastewater per day; the water usage ranges from 60 to 200 gal/cow/day for flush systems [12]. The daily amount of wastewater and manure produced in a medium or large farm (5000 to 10,000 cows) is staggering. Traditional wastewater and manure treatment processes mainly include sedimentation tanks, septic tanks, biogas digesters, etc. These methods are mainly designed for the removal of traditional pollutants, such as nitrogen, phosphorus, suspended solids, etc., but are not focused on the removal of ARGs, ARB, or residual antibiotics. Studies have shown that the overuse of ceftiofur in cattle farms contributes to the spread of ARGs and multidrug-resistant bacteria [13]. Sun et al. [14] found that the composition of ARGs in fecal samples from students during their stay at the farm was consistent with the composition of ARGs in the farm environment, suggesting that ARGs can enter the human gut in some way and influence the composition of ARGs in the human gut. Therefore, there is increased attention on the wide spread of ARB and ARGs in the environment [15–17]. Research on ARGs and ARB in livestock industry's waste treatment processes and the reuse of treated waste on farmland is vital to the identification and distribution of ARG and ARB sources and their pathways, and provides critical information for the assessment of the potential risks to human health and environment.

In this study, waste from different stages of the treatment process of a large dairy farm in Heze City, Shandong province, China, was investigated. The objectives were to explore the diversity and distribution of ARGs and ARB in the waste; analyze the effectiveness of the feces treatment processes in the removal of ARGs and ARB; and explore the impacts of the application of treated livestock wastewater on spread of ARGs and ARB on farmland.

2. Sampling and Methods

2.1. Waste Treatment Process

Cow manure excreted in the delivery room was first scooped up with a semi-mechanical cleaning device and flushed with recirculating water. A large volume of manure and wastewater mixture was discharged into a collection ditch, where the mixture was separated into liquid and solid by a screw-press liquid–solid separation (LSS) system. The separated manure residue entered the fermenter for high-temperature fermentation and was used as animal bedding after drying. The liquid part was anaerobically digested in an anaerobic tank and stored in the storage ponds, then diluted with water before application to farmland as a liquid fertilizer.

2.2. Sampling and Sample Preparation

Wastewater and manure samples from different stages of the treatment process were collected from a dairy farm in Heze City, Shandong province (latitude 34°85′ N, longitude 115°45′ E), in July 2022.

Figure 1 shows the sampling locations and information for livestock wastewater, manure, and soil samples. Five wastewater sampling locations were taken from the delivery room (sample CF), manure collection ditch (sample QD), LSS system (sample SL), anaerobic digestion tank (sample Y), and storage pond (sample HD). Four manure sampling locations were selected from the delivery room (sample CFS), manure collection ditch (sample QDS), LSS system (sample SLS), and fermenter (sample FJH). Wastewater and manure samples were collected using 800 mL brown glass bottles and 1 Kg aluminum foil collection bags, respectively. Three parallel samples were taken at each sampling location as duplicates for QA/QC purposes.

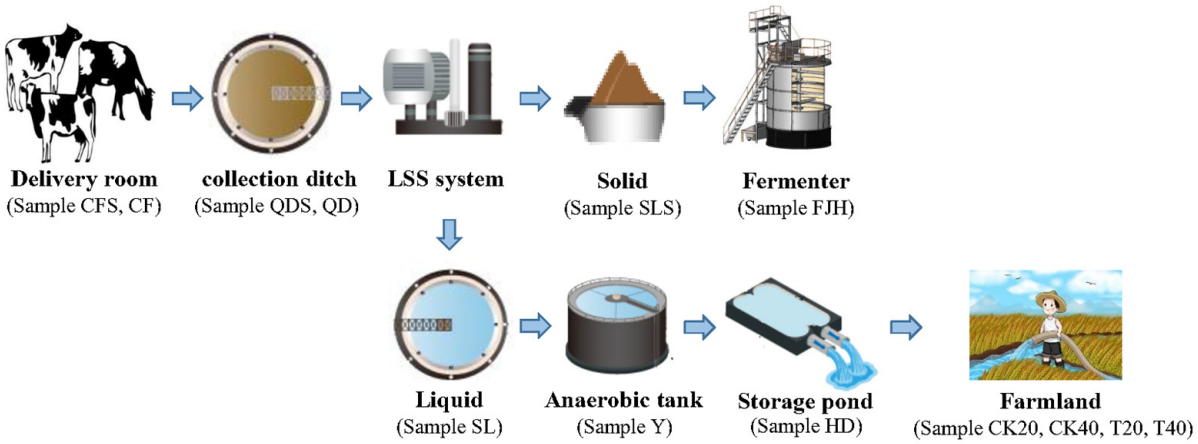

Figure 1. Sampling process for livestock wastewater, manure, and farmland soil samples.

To explore the effect of treated livestock wastewater on the spread of ARGs in the farmland soil, six sample locations were selected from the farmland in the vicinity of the dairy farm where treated wastewater had been applied for 5 years. The surficial soil sample, collected from 0 to 20 cm below the surface, was labeled T20, and the subsurface soil samples, collected from 20 to 40 cm, was labeled T40. Another block of farmland, separated by road and drainage trenches, which was not irrigated with the treated wastewater, was selected as the control. The control soil (surficial sample CK20 and subsurface sample CK40) samples were collected from the control farmland. In each sampling location, soil samples were collected using a five-point sampling method and homogenized to form a representative composite sample.

The collected wastewater, manure, and soil samples were dispensed into 10 mL sterile centrifuge tubes and sent to a commercial laboratory (Beijing Novogene Co., Ltd., Beijing, China) for high-throughput metagenomic sequencing using a foam box with dry ice. The other samples were transported back to the laboratory at 4 °C and preprocessed within 12 h. The manure samples were dried and passed through a 0.149 mm sieve. The treated manure samples and the remaining wastewater samples were then sent to Shiyanjia Laboratory for chemical testing.

2.3. Chemical Properties of the Samples

The total nitrogen (TN), total phosphorus (TP), and total organic carbon (TOC) in livestock wastewater were analyzed using the alkaline potassium persulfate digestion UV spectrophotometric method (UV-Visible Spectrophotometer TU-1810PC, Beijing Purkinje GENERAL Instrument Co., Ltd., Beijing, China), ammonium molybdate spectrophotometry (UV-Visible Spectrophotometer T6 New century, Beijing Purkinje GENERAL Instrument

Co., Ltd., China), and combustion oxidation nondispersive infrared absorption (Nondispersive infrared absorption TOC analyzer, DP-2000A, Beijing Yaou Depeng Technology Co., Ltd., Beijing, China), respectively. The TN of the manure was quantified using the Kjeldahl method (Burette). The TP of the manure was determined using the alkali fusion–Mo–Sb Anti-spectrophotometric method (UV-Visible Spectrophotometer TU-1810PC 752N, Beijing Purkinje GENERAL Instrument Co., Ltd., Beijing, China). The TOC of the manure was measured using the potassium dichromate oxidation spectrophotometric method (UV-Visible Spectrophotometer TU-1810PC 752N, Beijing Purkinje GENERAL Instrument Co., Ltd., Beijing, China). The measured data were processed using Excel (2016) and were expressed as mean ± SD.

2.4. Metagenomic Sequencing and Bioinformatics Analysis

2.4.1. Library Preparation and Sequencing

High-throughput sequencing was performed to analyze the composition of the bacterial community and the relative abundance of ARGs in the samples using the Illumina HiSeq platform. Total genomic DNA was extracted from fecal and soil samples using the Magnetic Soil and Stool DNA Kit (DP712, TIANGEN, Beijing, China), according to the manufacturer's instructions. The concentration and purity of the extracted DNA were determined with the Qubit® dsDNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). DNA extract quality was checked on 1% agarose gel. The qualified DNA samples were subjected to library construction and testing using the NEBNext® Ultra DNA Library Prep Kit for Illumina sequencing (NEB, Ipswich, MA, USA). The genomic DNA was first randomly sheared into fragments of approximately 350 bp in length using a Covaris ultrasonic fragmentation machine. The obtained fragments were end-repaired, A-tailed, and further ligated with an Illumina adapter. The library was then PCR-amplified, size-selected, and purified. Qubit 2.0 was used to initially quantify and dilute the library to 2 ng/μL, followed by Agilent 2100 for detecting the insert size of the library. Once the insert size met the expectations, qPCR was used to accurately quantify the effective concentration and the quality of the library (effective concentration of the library > 3 nM). Finally, the qualified libraries were sequenced using Illumina PE150 (Illumina China Scientific Equipment Co., Ltd., Beijing, China), generating approximately 10 Gb of metagenomic data per DNA sample. The sequences of the forty-five metagenomes were uploaded to the NCBI server at https://ncbi.nlm.nih.gov/. The accession numbers are PRJNA1112970.

2.4.2. Analysis of Sequencing Information

A certain percentage of low-quality data may exist in the raw data obtained from sequencing. To ensure the accuracy and reliability of subsequent information analysis results, Readfq (V8, https://github.com/cjfields/readfq, accessed on 12 July 2022) was used to perform quality control and host filtering of the raw data obtained from the Illumina HiSeq sequencing platform, and to obtain the clean data for subsequent analysis. The specific processing steps for data filtering are as follows: (1) reads with low-quality bases (default quality threshold ≤ 38) exceeding a certain proportion (default length 40 bp) were removed; (2) reads with N bases reaching a certain proportion (default length 10 bp) were removed; (3) reads that overlapped with adapters exceeding a certain threshold (default length 15 bp) were removed; (4) considering the possibility of host contamination in samples, clean data were blasted to the host database to filter out reads that may have come from the host origin. Bowtie2 (V. 2.2.4, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml, accessed on 12 July 2022) was used by default, with the following parameter settings: --end-to-end, --sensitive, -I 200, and -X 4. Eventually, Megahit (V. 1.0.4-beta) was used for assembly analysis of the clean data with the following assembly parameter settings: --presets meta-large (--end-to-end, --sensitive, -I 200, -X 400), and the assembled scaffolds were broken from the N junctions to obtain Scaftigs (continuous sequences within scaffolds). Single-sample Scaftigs fragments (≤500 bp) were filtered out for subsequent analysis.

The combined unigenes with sequences of bacteria, fungi, archaea, and viruses were extracted from NCBI's NR (V. 2018.01) database. The LCA algorithm was utilized to determine the abundance of each sample at various taxonomic levels (kingdom, phylum, order, family, genus, and species). To assess the diversity and abundance of the ARGs, the Resistance Gene Identifier (RGI) was used to obtain the annotation information by matching unigenes with the Comprehensive Antibiotic Research Database (CARD).

2.5. Metagenomic Sequencing Data Statistical Analysis

The abundance information for each gene in each sample was calculated based on the number of reads and gene length [18]. The relative abundance information of each gene in the sample was calculated by Formula (1):

$$G_k = \frac{r_k}{L_k} \cdot \frac{1}{\sum_{i=1}^{n} \frac{r_i}{L_i}} \quad (1)$$

where "r" is the number of reads for the gene on the comparison, "L" is the length of the gene, and "G" indicates the relative abundance.

Cluster trees were plotted based on a Bray–Curtis distance matrix; then, non-metric multi-dimensional scaling (NMDS) analysis was performed. The correlation and significance (* $p < 0.05$ and ** $p < 0.01$) between bacteria and ARGs were obtained by Spearman's correlation heatmap. The above statistical analyses were analyzed using the "vegan" package of R (V. 4.3.0).

3. Results

3.1. Chemical Properties of the Sampled Wastewater and Manure

The delivery room is the production site of wastewater and manure; the concentrations of TOC and TN were very high in samples CF and CFS (Table 1). QD samples were collected from the ditches after being washed using fresh water; therefore, the TOC and TN dropped significantly. The TOC increased significantly in sample SL, which was likely due to the release of organic matter from the manure after the physical LSS process. However, after the anaerobic process, the TOC, TN, and TP dropped dramatically by 12.4%, 52.9%, and 33.1% respectively.

Table 1. Chemical properties of livestock wastewater and manure samples.

Sample Type	Sample Name	TOC	TN	TP
Livestock wastewater (mg/L)	CF	2869.8 ± 85.4	1283.3 ± 7.9	147.7 ± 5.6
	QD	2428.2 ± 130.9	1040.7 ± 32.4	148.7 ± 4.7
	SL	3403.8 ± 188.7	1058.0 ± 25.7	235.3 ± 43.7
	Y	2983.2 ± 156.0	1173.0 ± 20.0	157.4 ± 34.0
	HD	2295.0 ± 79.0	552.0 ± 29.2	119.1 ± 24.6
Manure (mg/kg)	CFS	54,550.0 ± 4450.0	829.3 ± 8.8	525.5 ± 78.5
	QDS	38,100.0 ± 2400.0	722.0 ± 162.0	535.5 ± 11.5
	SLS	78,800.0 ± 5800.0	671.7 ± 31.5	498.5 ± 85.5
	FJH	57,300.0 ± 4300.0	676.0 ± 45.0	513.7 ± 80.4

The TOC concentrations in manure sample CFS from the delivery room were also very high, but decreased from 54,550 ± 4450 mg/kg (mean ± SD) to 38,100 ± 2400 mg/kg (mean ± SD) after being washed with fresh water (Table 1). The TOC concentration in SLS was more than two times higher than sample QDS after mechanical dehydration. Then, the TOC concentration was reduced by 27.28% (sample FJH) after high-temperature fermentation in the fermenter. However, the physical LSS process and high-temperature fermentation had no significant influence on the concentrations of the TN and TP in the manure.

3.2. Metagenome Assembly Results

In this study, 15 livestock wastewater samples, 12 manure samples, and 18 soil samples were sequenced using the Illumina Hiseq platform. The metagenomic raw data (wastewater: 10,624.9 ± 387.88 Mbp, manure: 11,103.13 ± 1901.16 Mbp, soil: 10,481.17 ± 462.97 Mbp) were filtered to obtain the clean data (wastewater: 159,224.34 ± 387.2 Mbp, manure: 133,151.72 ± 1899.9 Mbp, soil: 10,474.63 ± 462.07 Mbp). The means of the total number of Scaftigs of the wastewater, manure, and soil samples were 510.3 ± 35 Kbp, 508.4 ± 82.5 Kbp, and 356.1 ± 70.6 Kbp, respectively. The lengths of the longest Scaftigs of the wastewater, manure, and soil samples were 411,249 bp, 241,110 bp, and 70,877 bp, respectively (Table S1). These data were used for subsequent microbial species annotation, functional annotation, and analysis of the variation in bacteria communities and ARGs of the livestock wastewater and manure.

3.3. Metagenomic Sequencing Results

3.3.1. Diversity and Composition of Bacteria in the Wastewater and Manure Samples

The clustering tree was constructed based on Bray–Curtis distance matrix clustering analysis (Figure 2). The main compositions of the bacteria in livestock wastewater samples Y and HD were similar, but exhibited significant differences from the samples of QD, SL, and CF. The bacterial communities of manure samples QDS and SLS were similar to those of samples CFS and FJH. The NMDS visualized the Bray–Curtis distance between samples, which was consistent with the clustering tree results (Figure S1). There were highly diverse bacterial communities in livestock wastewater and manure. The LSS system affected the distribution of microorganisms in the solid and the liquid fractions. Notably, anaerobic digestion and high-temperature fermentation caused significant changes in the bacterial community structure.

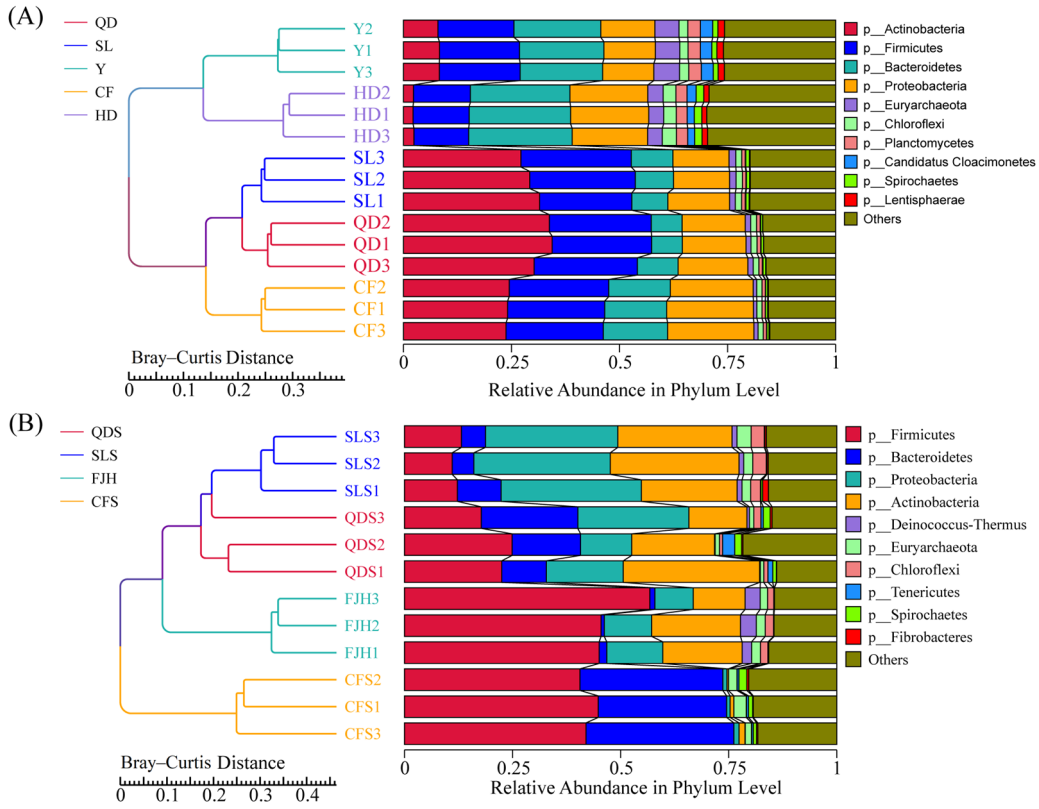

Figure 2. Clustering analysis of the bacteria at phylum level. (**A**) Livestock waste water samples; (**B**) manure samples.

The bar graph shows the differences in bacterial community composition between groups at the phylum level (Figure 2). The dominant phyla in livestock wastewater were Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria, accounting for 56.6%~81.0% of the total abundance. The relative abundances of Actinobacteria (28.93% vs. 8.22%) and Firmicutes (23.77% vs.18.27%) in sample Y were significantly reduced compared with sample SL, while the relative abundance of Bacteroidetes (8.92% vs. 19.57%) increased significantly. In addition, there was a similar trend at the genus level. The relative abundances of *Corynebacterium*, *Nocardioides*, and *Luteimonas* in sample Y were lower than the samples of CF, QD, and SL, but the relative abundance of *Methanothrix* increased (Figure S2a).

The dominant phylum in manure was consistent with that of the livestock wastewater, accounting for about 76.6% to 78.2% of the total abundance. Firmicutes (42.46%) and Bacteroidetes (32.32%) were the abundant microbes in manure sample CFS. The relative abundances of Firmicutes (21.71% vs. 12.15%) and Bacteroidetes (16.16% vs. 6.89%) in sample SLS decreased significantly, while Proteobacteria (18.45% vs. 31.54%) showed significant enrichment, compared with sample QDS. The relative abundances of Bacteroidetes (6.89% vs. 1.22%), Proteobacteria (31.54% vs. 10.94%), and Actinobacteria (26.12% vs. 16.96%) were significantly reduced after high-temperature fermentation, compared with samples SLS and FJH. However, Firmicutes was noteworthy: the relative abundance was highly enriched after high-temperature fermentation (12.15% vs. 49.08%). Figure S2b shows that the dominant bacterial genera in CFS and SLS were *Bacteroides* and *Luteimonas*, respectively. *Luteimonas* sp. was significantly reduced after high-temperature fermentation; conversely, *Bacillus* was significantly enriched (manure sample FJH).

3.3.2. Abundance and Diversity of ARGs in the Wastewater and Manure Samples

The annotation profiles of ARGs were analyzed using the Illumina Hiseq platform. A total of 609 ARG subtypes out of 29 ARG types were identified from livestock wastewater, and a total of 635 ARG subtypes out of 27 ARG types were identified in the manure. Subtypes of ARGs were classified according to the type of resistance (Figure 3), and due to the large number of subtypes of ARG with a proportion of less than 2%, they were classified differently. The main types of ARGs detected in livestock wastewater were multidrug (24.96%), aminoglycoside (14.94%), glycopeptide (6.9%), tetracycline (6.73%), macrolide (4.27%), peptide (2.96%), and phenicol (2.46%) resistance genes (Figure 3A). The types and proportions of the main ARGs detected in manure were generally consistent with those in livestock wastewater, with multidrug (23.78%), aminoglycoside (14.33%), glycopeptide (6.46%), tetracycline (5.35%), peptide (3.46%), macrolide (3.31%), and phenicol (3.15%) resistance genes (Figure 3B).

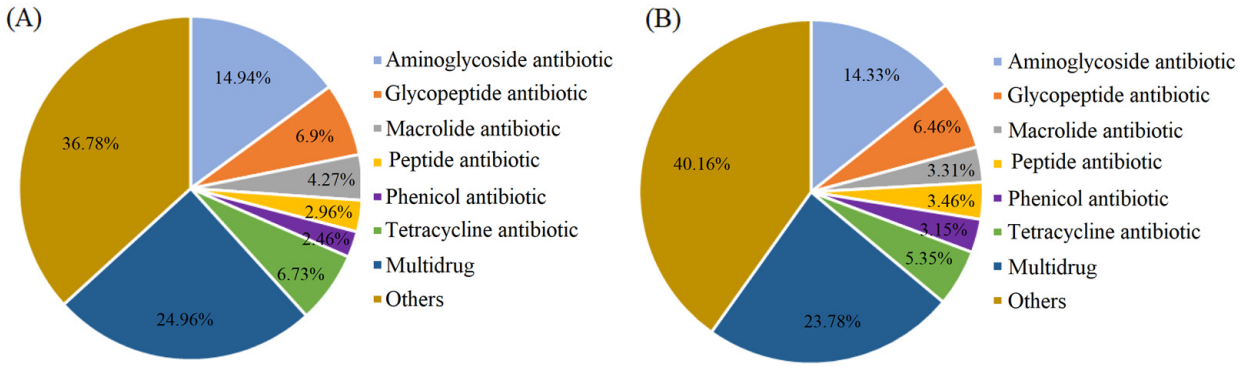

Figure 3. The main ARG categories detected in the livestock wastewater samples (**A**) and the manure samples (**B**).

The diversity and relative abundance of the ARGs in livestock wastewater at different treatment stages are shown in Figure 4A and Table 2. The main ARGs with high relative abundance in the wastewater samples were *tetW/N/W*, *sul1*, *lsaE*, *tetM*, etc. The diversity

of ARGs in sample SL was the highest, followed by sample CF (Figure 4A). However, the mean relative abundance of ARGs in sample CF was the highest (Table 2), which was about 2–3 times higher than the other wastewater samples. The wastewater treatment process significantly reduced the diversity and relative abundance of ARGs. In the first manure cleanup step, the relative abundance of ARGs decreased by 57.62% after flushing using water (sample QD). In the anaerobic digestion step, the diversity and relative abundance of the ARGs were all significantly reduced (686.3 ppm vs. 455.4 ppm). Among them, the relative abundances of *lsaE*, *lnuC*, *AAC6-Ie-APH2-Ia*, *ANT9-Ia*, *aadA*, *sul1*, *tetW/N/W*, and *ErmF* were reduced the most. In contrast, the relative abundances of *tetM* and *adeF* increased by 70.64% and 66.08%, respectively. Overall, the relative abundance of ARGs in livestock wastewater was reduced by a total of 68.5% from the delivery room to the anaerobic pond (Table 2).

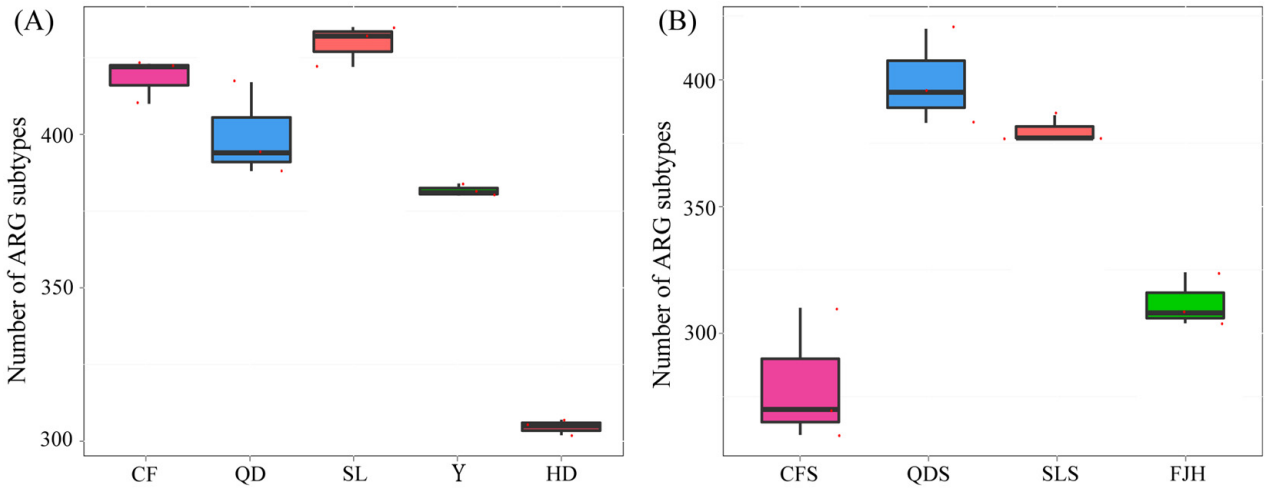

Figure 4. Boxplot of the detected ARG subtypes number in the livestock wastewater samples (**A**) and the manure samples (**B**).

Table 2. Relative abundance of the ARG subtypes in the livestock wastewater samples.

Sample	*tetW/N/W*	*sul1*	*lsaE*	*tetM*	*AAC6-Ie-APH2-Ia*	*adeF*	*lnuC*	*ErmF*	*ANT9-Ia*	*aadA*	Others	Total ARGs
CF	63.3 ± 2.3	101.5 ± 4.4	59.3 ± 1.8	53.2 ± 2.6	51.2 ± 1.4	15.3 ± 0.5	45.5 ± 2.3	39.5 ± 1.4	36.6 ± 1.8	32.2 ± 2.5	947.7 ± 12.4	1445.3 ± 10.1
QD	59.1 ± 0.6	22.5 ± 0.2	24.6 ± 1.3	9.7 ± 0.6	3.9 ± 0.5	12.0 ± 1.0	27.1 ± 3.0	8.4 ± 1.1	15.4 ± 0.8	7.6 ± 0.1	422.2 ± 8.1	612.6 ± 12.5
SL	62.6 ± 4.0	28.8 ± 2.1	34.1 ± 6.8	13.5 ± 1.8	6.6 ± 1.5	11.6 ± 0.7	26.1 ± 2.4	9.0 ± 1.0	19.8 ± 3.8	8.9 ± 0.9	465.1 ± 11.9	686.3 ± 30.3
Y	37.7 ± 3.1	18.3 ± 0.5	22.7 ± 0.7	23.1 ± 1.6	2.5 ± 0.2	19.3 ± 0.4	8.9 ± 0.3	6.6 ± 1.0	9.5 ± 0.9	6.7 ± 0.5	300.1 ± 6.9	455.3 ± 10.1
HD	18.7 ± 0.5	23.6 ± 0.6	22.8 ± 1.3	24.1 ± 1.5	2.4 ± 0.1	45.9 ± 3.1	5.2 ± 0.4	7.5 ± 0.3	7.3 ± 0.2	6.7 ± 0.6	326.9 ± 6.1	491.1 ± 8.0

The diversity of ARGs and their relative abundances in manure samples were showed in Figure 4B and Table 3. The main ARGs with high relative abundance in manure were *lnuC*, *CfxA2*, *tetW/N/W*, etc. The diversity of the ARGs in sample CFS were the lowest, but the relative abundance was the highest (841.9 ppm): 1.5 to 2.6 times higher than the other samples. In the first manure cleanup step, the relative abundance of ARGs in manure decreased by 33.25%. The relative abundance of *tetW/N/W*, *tetW*, *ANT6-Ia*, *Mycobacterium tuberculosis rpsL*, *lnuC*, *CfxA2*, and *adeF* were significantly reduced after high-temperature fermentation. In contrast, some ARGs had strong tolerance to high-temperature; the relative abundances of *QnrB74* (0.06 ppm vs. 28.37 ppm), *vanTC* (0.06 ppm vs. 12.9 ppm), and *lnuG* (9.8 ppm vs. 19.6 ppm) increased significantly after fermentation. Notably, the whole relative abundance of the ARGs was reduced by 33.7% and 29.3% by liquid anaerobic digestion and solid high-temperature fermentation, respectively. The relative abundance of ARGs in manure samples was reduced by a total of 62.1% from the delivery room to the fermenter.

Table 3. Relative abundance of the ARG subtypes in the manure samples.

Sample	*lnuC*	*CfxA2*	*tetW/N/W*	*adeF*	*QnrB74*	*ANT6-Ia*	*Mycobacterium tuberculosis rpsL*	*vanTC*	*tetW*	*lnuG*	Others	Total ARGs
CFS	386.2 ± 53.2	87.1 ± 5.3	41.2 ± 7.2	0.7 ± 0.6	0.0 ± 0.0	1.3 ± 0.6	0.3 ± 0.0	0.6 ± 0.6	22.3 ± 5.2	0.6 ± 0.4	301.6 ± 24.5	841.9 ± 44.4
QDS	38.6 ± 7.2	15.4 ± 3.6	31.7 ± 6.9	12.0 ± 5.4	0.1 ± 0.1	27.0 ± 7.6	16.4 ± 5.0	0.1 ± 0.1	7.8 ± 1.6	17.2 ± 6.5	395.7 ± 32.3	562.0 ± 37.8
SLS	7.8 ± 2.0	0.8 ± 0.7	20 ± 4.5	42.6 ± 1.1	0.1 ± 0.1	18.0 ± 2.9	27.4 ± 4.7	0.1 ± 0.1	4.1 ± 0.4	9.8 ± 1.5	320.6 ± 15.4	451.2 ± 19.4
FJH	2.6 ± 0.3	0.1 ± 0.0	8.1 ± 0.4	19.7 ± 3.3	28.4 ± 9.8	10.2 ± 2.9	22.1 ± 5.1	12.9 ± 11.9	2.9 ± 0.5	19.6 ± 1.3	192.4 ± 20.9	319.1 ± 26.0

3.3.3. Abundance and Diversity of the ARGs in the Surrounding Farmland Soil

The effect of the long-term application of livestock wastewater on the spread of the ARGs in farmland soil is demonstrated in Figure 5. The main types of ARGs detected in the soil were multidrug (24.29%), aminoglycoside (12.59%), glycopeptide (6.91%), tetracycline (6.03%), macrolide (3.90%), carbapenem (3.01%), and fluoroquinolone (2.30%) resistance genes (Figure 5A). This was similar to the type of ARGs in livestock wastewater. The subtypes of ARG in surface soil treated with livestock wastewater differed significantly from the untreated soils, while the diversity of ARGs in the subsurface soil (sample T40) did not exhibit significant differences from the other soil (Figure 5B).

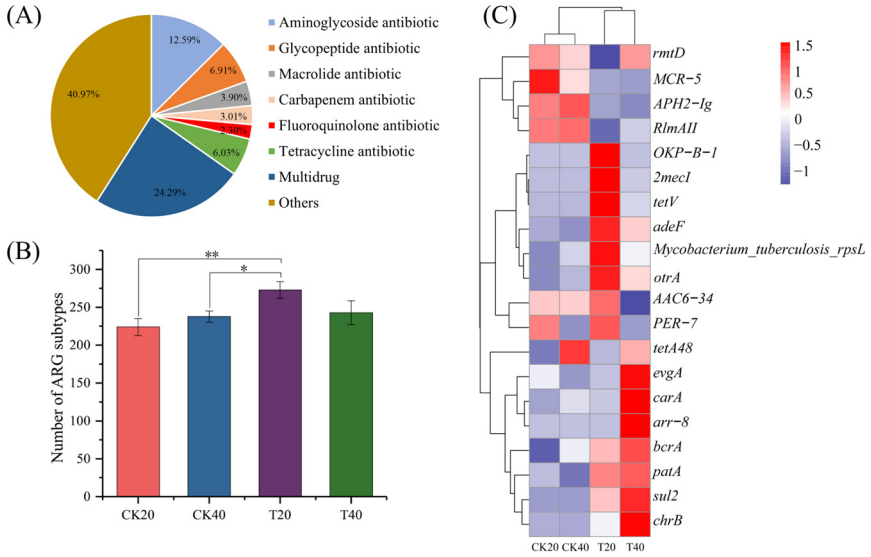

Figure 5. Effect of the application of livestock wastewater on the spread of the ARGs in farmland soil. (**A**) Main ARG categories. (**B**) Number of ARG subtypes. (**C**) Clustering heatmap analysis of the top 20 ARGs (* $p < 0.05$ and ** $p < 0.01$).

The application of livestock wastewater mainly affected the diversity of ARGs in topsoil. The top 20 ARGs were selected for clustering heatmap analysis based on their relative abundances (Figure 5C). The ARGs with high relative abundance in sample T20 were *OKP-B-12*, *mecI*, *tetV*, *adeF*, *Mycobacterium tuberculosis rpsL*, and *otrA*. The relative abundances of *evgA*, *carA*, *arr-8*, *sul2*, and *chrB* in sample T40 were significantly higher than the other groups. *MCR-5* was the only high-relative-abundance ARG found in the top control soil of CK20, and *tetA48* was the only high-relative-abundance ARG found in the subsurface soil of CK40.

3.3.4. Correlation Analysis between the Bacterial Communities and the ARGs

The top 20 bacterial genera and the top 20 ARGs were selected to analyze the correlations between the ARGs and the bacterial communities. A Spearman correlation heatmap was drawn using R (V. 4.3.0) (Figure 6). The result showed that 19 out of 20 bacteria in livestock wastewater samples, 18 out of 20 bacteria in manure samples, and 15 out of 20 bacteria in soil samples were significantly and positively correlated with the main ARGs.

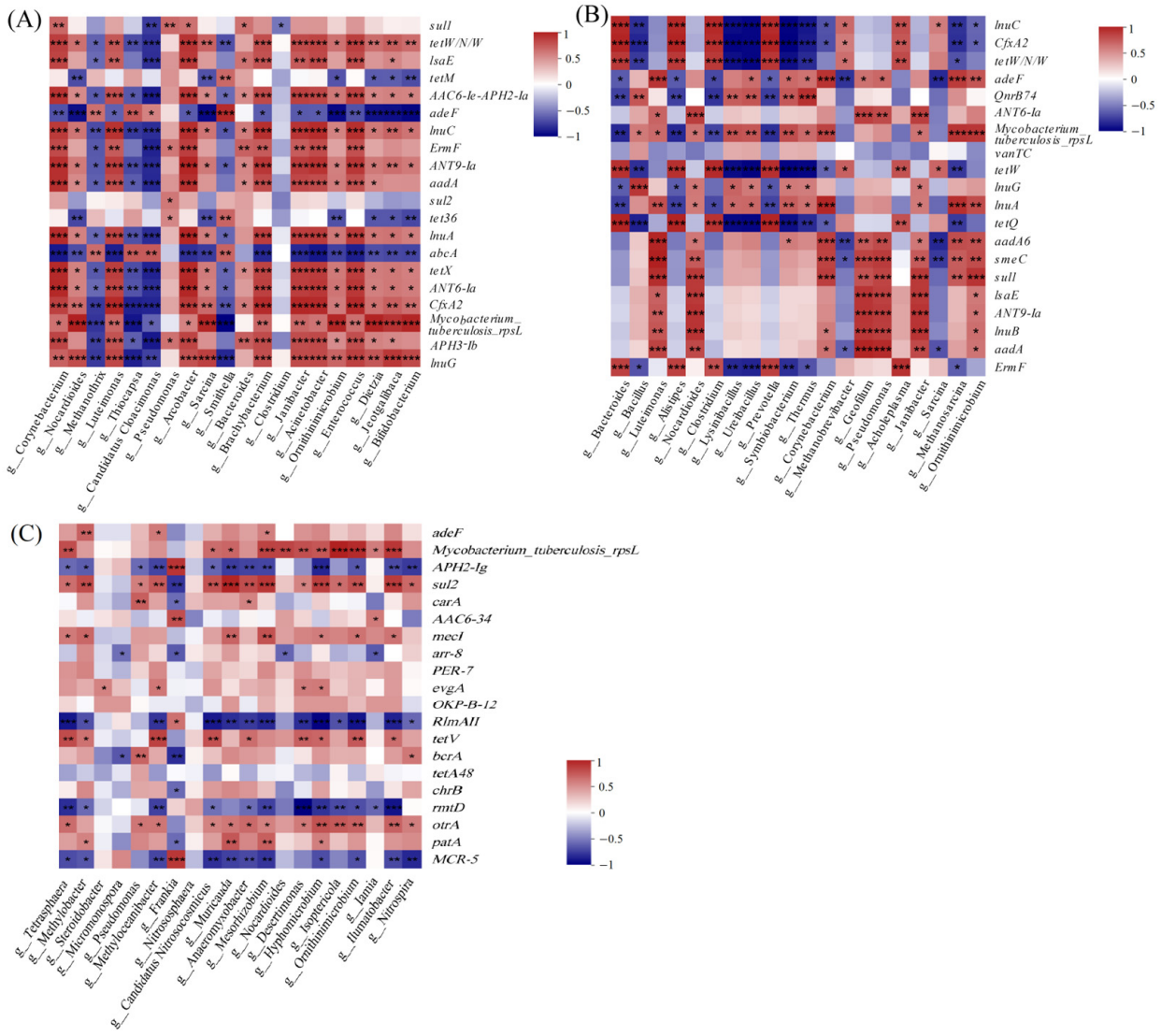

Figure 6. Correlation heatmap analysis between the top 20 bacteria community and the top 20 ARGs in the livestock wastewater samples (**A**), the manure samples (**B**), and the soil samples (**C**). (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Corynebacterium sp., *Luteimonas* sp., *Arcobacter* sp., *Brachybacterium* sp., *Acinetobacter* sp., *Janibacter* sp., and *Enterococcus* sp. were strongly and positively correlated with most of the ARGs in the livestock wastewater (Figure 6A). These bacteria were potential multidrug-resistant bacteria considering their close relationship with multiple ARGs. In the manure samples, the distribution of different ARGs in host bacteria showed obvious differentiation (Figure 6B). *Luteimonas* sp., *Nocardioides* sp., *Geofilum* sp., *Pseudomonas* sp., *Janibacter* sp., and *Ornithinimicrobium* sp. were the main host bacterial genera of the ARGs *aadA6*, *smeC*, *sul1*, *lsaE*, *ANT9-Ia*, *lnuB*, and *aadA* in the manure. *Bacteroides* sp., *Alistipes* sp., *Clostridium* sp., *Prevotella* sp., and *Acholeplasma* sp. were the host bacteria of *lnuC*, *CfxA2*, *tetW/N/W*, *tetW*, *tetQ*, and *ErmF*. *Corynebacterium* sp., *Methanosarcina* sp., and *Ornithinimicrobium* sp. showed highly significant positive correlations ($p \leq 0.01$) with *adeF*, *Mycobacterium tuberculosis rpsL*, *aadA6*, *smeC*, *sul1*, and *lnuA* (Figure 6B). In the soil samples, *Ilumatobacter* sp., *Ornithinimicrobium* sp., *Isoptericola* sp., *Hyphomicrobium* sp., *Desertimonas* sp., and *Mesorhizobium* sp. were significantly positively correlated with *Mycobacterium tuberculosis rpsL* and *sul2* ($p \leq 0.01$) (Figure 6C).

The comparative analysis showed that *Pseudomonas* sp., *Nocardioides* sp., and *Ornithinimicrobium* sp. were the three bacterial genera in the samples of livestock wastewater, manure, and soil, respectively. *Mycobacterium tuberculosis rpsL* and *adeF* were the two ARGs that were detected in all three types of samples, but the correlations of *Mycobacterium tuberculosis rpsL* with the host bacteria were stronger than *adeF* (Figure 6).

4. Discussion

4.1. Occurrence and Distribution of the ARGs in Livestock Farms

Antibiotics are often used in the delivery room to treat cow diseases (such as mastitis), which leads to the accumulation of antibiotics residues and the further enrichment of drug-resistant microorganisms. We have identified that the microbial community structures and the number of ARGs in excreta in the delivery room were significantly higher than the other locations. Furthermore, the amounts of antibiotics used in different types of livestock farms also vary greatly [19–21]. The most frequently detected ARGs in cattle manure are *tet* (tetracycline), *sul* (sulfonamides), and *erm* (erythromycin) genes [22,23]. In the present study, we also detected multiple and abundant ARGs in the dairy livestock wastewater and manure; *tetW/N/W*, *sul1*, *lsaE*, *tetM*, and *lnuC* were the abundant ARGs in livestock wastewater; and *lnuC*, *CfxA2*, and *tetW/N/W* were the dominant ARGs in manure (Tables 2 and 3). The main ARGs in this dairy farm were the resistant genes of sulfonamides, tetracycline, and lincoamide. The researchers in this study conducted inquiries and statistics on the usage records of antibiotics used in the daily production process, and found that the antibiotics we inferred based on the types of ARGs were consistent with those commonly used in daily production. These antibiotics have stable structures and are difficult to degrade, which may lead to the accumulation of relevant resistant bacteria.

4.2. ARGs Removal Efficiency of Current Livestock Wastewater and Manure Treatment Process

Contemporary livestock wastewater and manure treatment technologies are anaerobic digestion, oxidation ponds, composting, wetlands, etc. These technologies are not specifically designed for the removing of ARB and ARGs. Anaerobic digestion and high-temperature fermentation are widely used techniques in large-scale farms [24–26]. In the present dairy farm, the anaerobic digestion process and high-temperature fermentation process reduced the relative abundances of ARGs in wastewater and manure by 33.6% and 29.3%, respectively. Yang et al. [27] and Zhang et al. [28] found that the ARG abundance could be reduced by 20–25% after anaerobic digestion treatment. Lu et al. [29] constructed 16 lab-scale swine manure composting treatments, and found that the composting process in the optimized conditions could reduce ARGs and mobile genetic elements by 45% and 27.3%, respectively. However, anaerobic fermentation had different removal efficiency rates on different ARG subtypes [30,31]. The relative abundances or concentrations of some ARG subtypes and antibiotics were even higher than before anaerobic fermentation treatment [32]. Similar patterns were found in the present study, where the relative abundances of *tetM* and *adeF* increased after the anaerobic digestion of livestock wastewater samples (Table 2); the relative abundances of *QnrB74*, *lnuG*, and *vanTC* increased significantly after the high-temperature fermentation of manure (Table 3). There are several possible reasons for this phenomenon: the first factor may be due to the chemical stability of the antibiotics themselves [33]; the second factor may be that the enrichment of these genes is due to the aggregation of resistance genes on mobile genetic elements [34,35]; and the third factor may be that the host bacteria containing these ARGs are thermophilic microbes or facultative anaerobic fermentation bacteria [36,37]. In summary, the waste treatment process in this study was able to remove 29.3–33.6% of ARGs and ARB; hence, there could be an potential risk of spreading ARGs and ARB to the farmland if this treated wastewater is used for irrigation.

4.3. Relationship between ARB and ARGs in Livestock Wastewater, Manure, and Farmland Soil Samples

Microbes are an important carrier of ARGs. Microbes carrying different ARGs may amplify or attenuate under different environmental conditions, thus altering the distribution of ARGs in different environments. ARG dissemination is primarily attributed to VGT and HGT [38–40]. The variation in ARG abundance is closely associated with the discrepancy of bacterial community [41,42]. In the present study, we found that significant positive correlations existed between ARGs and some bacteria; most of the bacteria in the livestock wastewater were affiliated to Actinobacteria, Proteobacteria, and Firmicutes. Zhang et al. [43] also discovered that these bacteria were responsible for the carrying and spreading of ARGs. Moreover, the present study found that *lnuC* had 13 host bacteria in livestock wastewater, including *Corynebacterium* sp., *Luteimonas* sp., *Arcobacter* sp., *Brachybacterium* sp. and *Janibacter* sp. *SmeC* and *sul1* in manure samples had eight of the same host bacteria, including *Luteimonas* sp., *Nocardioides* sp., *Corynebacterium* sp., *Geofilum* sp., *Pseudomonas* sp., *Janibacter* sp., *Methanosarcina* sp., and *Ornithinimicrobium* sp. This suggests that the different ARGs may have the same host bacteria, and each particular ARG may also have many potential host bacteria. Taking *Mycobacterium tuberculosis rpsL* as an example, many new host bacteria (*Ilumatobacter* sp., *Isoptericola* sp., *Hyphomicrobium* sp., *Desertimonas* sp., *Mesorhizobium* sp., and *Tetrasphaera* sp.) were detected in addition to its two original host bacteria (*Nocardioides* sp. and *Ornithinimicrobium* sp.) after the wastewater was applied to the soil. This result indicated that *Mycobacterium tuberculosis rpsL* might be transferred to farmland soil through vertical and horizontal pathways. Liu et al. [44] showed that the long-term application of biogas slurry after anaerobic digestion may aggravate the HGT of ARGs between different bacterial hosts in soil. You et al. [45] also revealed that the residual ARGs in feces could be transferred to soil bacteria through the HGT process, thereby increasing the species and abundance of its host bacteria. Therefore, VGT and HGT are all important transmission modes of ARGs in livestock wastewater and manure.

4.4. Resource Utilization and Risk of Livestock Wastewater and Manure

Zhu et al. [46] revealed that long-term swine wastewater irrigation introduced potential bacteria hosts and increased the spread of ARGs through HGT. Research by Pu et al. [31] showed that the relative abundances of ARGs in the vegetable soil treated using wastewater over 10 years were 21-fold higher than the control soil. In the present study, the application of livestock wastewater significantly increased the number and abundance of ARGs in the farmland soil. The application of wastewater could lead to an increase in antibiotics, thereby enhancing the spread and distribution of ARGs through the soil–plant–food system.

In terms of the manure, high-temperature fermentation treatment reduced the water content, odor, and the abundance of ARB and ARGs of the residue. The treated manure residue was mainly used as padding material for the cow bed. However, there were still large amounts of ARGs and ARB remaining in the manure residue. Lu et al. [29] revealed that the abundance of *aph3iia*, *aph6id*, *sul1*, *sul2*, *ermf*, *mefa*, *tet36*, *tetM*, *tetQ*, and *tetW* were still in high abundances even after high-temperature fermentation. The residual harmful bacteria, such as *Klebsiella* spp., *Escherichia coli*, *Streptococcus* spp., and *Staphylococcus* spp., could cause mastitis and affected the production and quality of milk [47–49]. The present study showed that there were still 312 ARG subtypes in the manure residue after high-temperature fermentation, as well as a large number of Firmicutes and Actinobacteria.

The reuse of treated wastewater and manure may be helpful to solve the waste disposal issues for the high-density livestock and poultry industries; however, the presence of antibiotics, ARB, and ARGs in the treated waste could pose a series of environmental and human health risks.

5. Conclusions

Conventional livestock wastewater treatment processes cannot efficiently remove ARGs and ARB, and treated feces is a potential source of ARG/ARB pollution. The abundance and diversity of ARGs in the wastewater, manure, and farmland soil samples were explored using the high-throughput sequencing technique. Actinobacteria, Proteobacteria, and Firmicutes were the main host bacteria phyla of the ARGs, and the genera *Nocardioides* sp. and *Ornithinimicrobium* sp. were closely associated with the ARGs in the investigated samples. The relative abundances of ARGs in wastewater and manure were reduced by 68.5% and 62.1%, respectively, due to the conventional feces treatment process. Anaerobic fermentation and high-temperature fermentation were the most efficient steps in removing ARGs and ARB, despite significant proportions still existing after the treatment process. Irrigation with the inadequate treated wastewater significantly increased the abundance and diversity of ARGs and ARB in the surface soil of the farmland. The direct application of the inadequate treated wastewater and/or manure could lead to potential risk of spreading ARGs into the environment, and potentially threaten the ecosystem and human health. To effectively restrain the spread of ARGs, it is necessary to modify the process of ARG removal in wastewater and manure treatment, and improve the regulations and guidelines on the applications of dairy farm waste for farmland irrigation.

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