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Metagenomic Profiles of Yak and Cattle Manure Resistomes in Different Feeding Patterns before and after Composting

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ABSTRACT Antibiotic resistance is a global threat to public health, with antibiotic resistance genes (ARGs) being one of the emerging contaminants; furthermore, animal manure is an important reservoir of biocide resistance genes (BRGs) and metal resistance genes (MRGs). However, few studies have reported differences in the abundance and diversity of BRGs and MRGs between different types of animal manure and the changes in BRGs and MRGs before and after composting. This study employed a metagenomics-based approach to investigate ARGs, BRGs, MRGs, and mobile genetic elements (MGEs) of yak and cattle manure before and after composting under grazing and intensive feeding patterns. The total abundances of ARGs, clinical ARGs, BRGs, MRGs, and MGEs were lower in the manure of grazing livestock than in the manure of the intensively fed group. After composting, the total abundances of ARGs, clinical ARGs, and MGEs in intensively fed livestock manure decreased, whereas those of ARGs, clinical ARGs, MRGs, and MGEs increased in grazing livestock manure. The synergy between MGEs mediated horizontal gene transfer and vertical gene transmission via host bacteria proliferation, which was the main driver that altered the abundance and diversity of ARGs, BRGs, and MRGs in livestock manure and compost. Additionally, *tetQ*, IS*91*, *mdtF*, and *fabK* were potential indicators for estimating the total abundance of clinical ARGs, BRGs, MRGs, and MGEs in livestock manure and compost. These findings suggest that grazing livestock manure can be directly discharged into the fields, whereas intensively fed livestock manure should be composted before returning to the field.

IMPORTANCE The recent increase in the prevalence of antibiotic resistance genes (ARGs), biocide resistance genes (BRGs), and metal resistance genes (MRGs) in livestock manure poses risks to human health. Composting is known to be a promising technology for reducing the abundance of resistance genes. This study investigated the differences and changes in the abundances of ARGs, BRGs, and MRGs between yak and cattle manure under grazing and intensive feeding patterns before and after composting. The results indicate that the feeding pattern significantly affected the abundances of resistance genes in livestock manure. Manure in intensive farming should be composted before being discharged into the field, while grazing livestock manure is not suitable for composting due to an increased number of resistance genes.

KEYWORDS feeding pattern, antibiotic resistance gene, biocide resistance gene, metal resistance gene, composting, manure, yak

Antibiotic resistance is a global threat to public health, having caused an estimated 700,000 annual deaths; further, it is predicted to cause 10 million deaths by 2050 if left unchecked (1, 2). Antibiotic resistance genes (ARGs) are among the six emerging contaminants identified by the United Nations Environment Programme (UNEP) (3). The livestock industry is considered one of the main contributors to the spread of

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ARGs in the environment (4). Globally, the use of antibiotics in livestock is expected to increase by 67% by 2030 (5). In particular, China accounts for more than 46% of the world's total use; its primary function is to ensure growth and disease control in animal husbandry (6, 7). However, antibiotics are poorly absorbed by animals, with 70% of the parent compound excreted. On the one hand, animal excrement directly discharges drug-resistant bacteria into the environment; on the other hand, the residual antibiotics in the excreta persist in the natural environment for a long time, causing selective pressure on the bacteria, resulting in an increase in drug-resistant bacteria in the environment (8). Therefore, livestock manure is considered an important reservoir of ARG in the environment, which can be carried by commensal and pathogenic microorganisms to humans (9–11).

In addition, biocides (Cetrimide, HCl, H_2O_2) and heavy metals (Cu, Zn, As) are widely used in intensive feeding husbandry due to their bactericidal and animal growth-promoting effects. However, the biocides and heavy metals are poorly absorbed by animals, with 25 to 75% of the parent compound excreted (12–14). Therefore, animal manure has also become an important reservoir of biocide resistance genes (BRGs) and metal resistance genes (MRGs). Although their presence and spread in the environment do not directly affect human health, they can affect the efficiency of pesticides, disinfectants, and heavy metals used for preventive and therapeutic purposes in livestock and agricultural production (7, 14).

Aerobic composting, a bioremediation method, is widely used in the treatment of livestock and poultry manure to produce organic fertilizers. The mature product can provide nutrients for crops, improve the soil structure, and enrich the soil organic matter content (15). Previous studies have reported that aerobic composting might reduce the amount of ARGs that enter the environment. Qian et al. (16) reported that the abundance and diversity of ARGs are higher in chicken manure than in bovine or pig manure; however, industrial composting is more efficient in reducing the abundance of ARGs in chicken manure than in bovine and pig manure. Selvam et al. (17) found that after 42 days of pig manure composting, the abundance of *tet* and *sul* genes (10 ARGs in total) was below the limit of detection. In addition, while Qiu et al. (18) observed a decrease in *tetX* during poultry manure composting, an increase in *tetX* was detected during swine and cow manure composting. Moreover, Qian et al. (19) found that composting significantly decreases the abundance of *tetW*, *tetQ*, and *tetM* in cow manure while significantly increasing the abundance of *sul1*, *tetX*, *sul2*, and *tetC*. However, to the best of our knowledge, few studies have reported differences in the abundance and diversity of BRGs and MRGs between different types of animal manure and the changes in BRGs and MRGs before and after composting. In addition, few studies have reported the changes in ARGs, BRGs, and MRGs in grazing yak and cattle manure on the Qinghai-Tibet Plateau (QTP) before and after composting.

Therefore, in the present study, we sampled yak and cattle manure under grazing and intensive (high-density) feeding patterns to analyze ARGs, BRGs, MRGs, and mobile genetic elements (MGEs) using a metagenomics-based approach. The objectives of this study were to (i) compare the effects of intensive feeding and natural grazing patterns on ARGs, BRGs, and MRGs of livestock manure; (ii) investigate the effects of aerobic composting on ARGs, BRGs, MRGs, and MGEs from livestock manure under grazing and intensive feeding patterns; and (iii) explore the relationships between ARGs, BRGs, MRGs, MGEs, and bacterial communities. The results provide important theoretical support for the safe and rational utilization of manure in agriculture and the effective disposal of waste.

RESULTS

Diversity and abundance of ARGs, BRGs, MRGs, and MGEs in grazing and intensive feeding livestock manure before and after composting. A total of 21 ARG types and 414 subtypes were identified in all manure samples (Fig. 1A). ARG resistance to bata-lactam (131) was the most dominant type, followed by multidrug resistance (72), and macrolide-lincosamide-streptogramin (MLS) (46), tetracycline (42), and aminoglycoside (38) resistance. The subtype numbers and relative abundance of beta-lactam (*CfxA2*

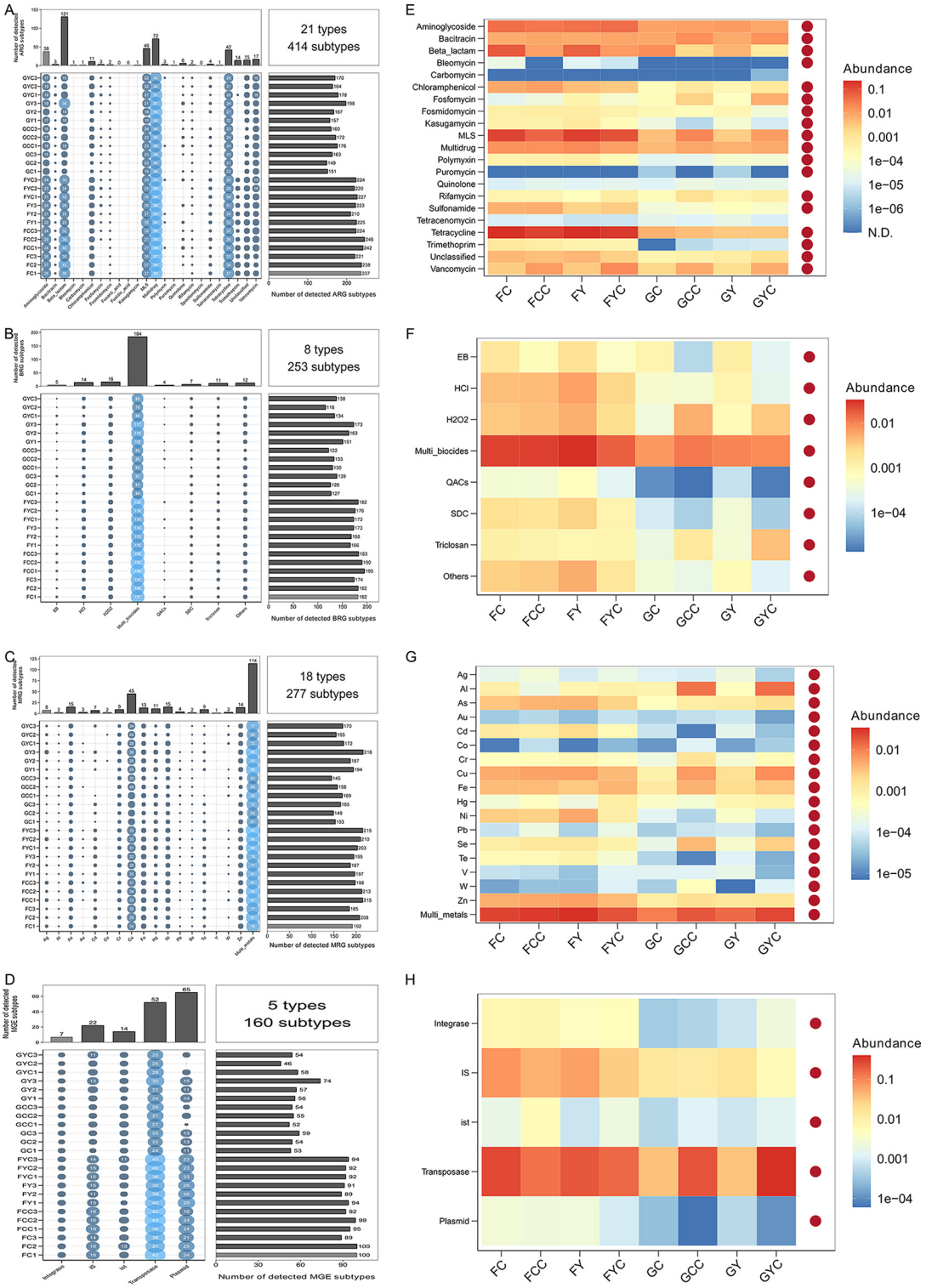

FIG 1 Number of ARG (A), BRG (B), MRG (C), and MGE (D) subtypes detected in different manure and composting samples. Comparison of the abundance (copies per 16S) of all detected ARG (E), BRG (F), MRG (G), and MGE (H) types among the different groups. The red dots on the right represent significant differences in the abundance of the ARG, BRG, MRG, and MGE types among different groups ($P < 0.05$). FC, intensive feeding cattle manure; FCC, intensive feeding cattle manure compost; FY, intensive feeding yak manure; FYC, intensive feeding yak manure compost; GC, grazing cattle manure; GCC, grazing cattle manure compost; GY, grazing yak manure; GYC, grazing yak manure compost.

and *CfxA3*), MLS (*VatB* and *ermB*), and tetracycline (*tetW*, *tetQ*, *tet32*, *tet44*, *tetO*, *tetM*, *tetX2*, and *tet40*) resistance genes in intensive feeding livestock were higher than those in grazing livestock ($P < 0.05$) (Fig. 1E; see also Fig. S1 and S5 in the supplemental material). A total of 8 BRG types and 253 subtypes were detected (Fig. 1B). BRG resistance to multiple biocides (184) was the most dominant type, followed by H_2O_2 (16) and Hcl (14). The subtype numbers and relative abundance of multibiocide (*qacE*, *cpxA*, *robA*, *cpxR*, and *soxR*) resistance genes in intensive feeding livestock were higher than those in grazing livestock ($P < 0.05$) (Fig. 1F; see also Fig. S2 and S6 in the supplemental material). Meanwhile, a total of 18 MRG types and 277 subtypes were detected (Fig. 1C). Genes resistant to multiple metals (114), Cu (45), As (15), Ni (15), and Zn (14) were dominant. The subtype numbers and abundance of Cu (*pstA*, *pstC*, and *pstS*) resistance genes in intensive feeding livestock were higher than those in grazing livestock ($P < 0.05$) (Fig. 1G; see also Fig. S3 and S7 in the supplemental material). A total of 5 MGE types and 160 subtypes were observed (Fig. 1D). Plasmids (65), transposases (52), and insertion elements (IS) (22) were the main MGE types detected. The subtype numbers and abundance of IS (IS*91* and IS*679*), transposase (*tnpA* and *tnpA5*), and plasmid (*IncFIC* and *IncFIB*) resistance genes in intensively fed livestock were markedly higher than those in grazing livestock ($P < 0.05$) (Fig. 1H; see also Fig. S4 and S8 in the supplemental material).

The total abundances of ARGs (Fig. 2A), clinical ARGs (see Fig. S9A in the supplemental material), BRGs (Fig. 2B), MRGs (Fig. 2C), and MGEs (Fig. 2D) were lower in grazing manure than in intensive-feeding manures ($P < 0.05$). Moreover, the total abundance of ARGs (Fig. 2E), clinical ARGs (Fig. S9B), and MGEs (Fig. 2H) decreased in yak and cattle manure under intensive feeding patterns after composting ($P < 0.05$). However, the total abundance of ARGs (Fig. 2E), clinical ARGs (Fig. S9B), MRGs (Fig. 2G), and MGEs (Fig. 2H) increased in yak and cattle manure under grazing patterns after composting ($P < 0.05$).

Correlation of ARGs, BRGs, MRGs, MGEs, and bacterial phyla before and after composting. According to the principal coordinates analysis (PCoA), the microbial communities of yak and cattle manure were clustered together under grazing patterns and completely separated from those of manure under intensive feeding patterns (Fig. 3A). *Bacteroidetes* and *Actinobacteria* were the dominant bacterial phyla in all manure samples before and after composting (Fig. 3B). The relative abundances of *Actinobacteria*, *Chloroflexi*, and *Thermotogae* increased in all manure samples after composting ($P < 0.05$) (Fig. 3B). In addition, the composting process decreased the relative abundance of *Verrucomicrobia* in grazing livestock manure, while it increased that of *Acidobacteria* and *Chloroflexi* ($P < 0.05$) (Fig. 3B).

According to the network, under intensive feeding patterns, the ARGs, BRGs, and MRGs were positively correlated with the relative abundance of *Chlorobi* and *Actinobacteria* before composting (Fig. 3C) and with that of *Chloroflexi* after composting (Fig. 3D). Under grazing patterns, the ARGs, BRGs, and MRGs were positively correlated with the relative abundance of *Chlorobi*, *Acidobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Tenericutes*, and *Chloroflexi* before and after composting (Fig. 3E and F). In addition, under intensive feeding patterns, MGEs were positively correlated with the relative abundances of *Chlorobi*, *Verrucomicrobia*, and *Cyanobacteria* in livestock manure before compositing (Fig. 3C) and that of *Chloroflexi* after composting (Fig. 3D). Meanwhile, under grazing patterns, MGEs were positively correlated with the relative abundance of *Verrucomicrobia* before composting (Fig. 3E) and *Verrucomicrobia*, *Acidobacteria*, and *Chloroflexi* after composting (Fig. 3F).

Horizontal and vertical transmission of ARGs, BRGs, and MRGs in livestock manure and composting samples under different feeding patterns. Among the ARG, BRG, MRG, and MGE, subtypes were found to be significantly correlated (Fig. 4A to F). However, the correlations between ARG, BRG, MRG, and MGE subtypes and bacterial richness were weak (see Fig. S10 in the supplemental material). Moreover, Mantel tests and Procrustes analyses revealed that the ARG, BRG, MRG, and MGE subtype compositions exhibited significant goodness of fit (Fig. 4G to L). Similarly, significant goodness of fit was observed between ARG, BRG, MRG, and MGE subtypes and the bacterial community composition (see Fig. S11 in the supplemental material).

VPA was performed to further explore the main contributors of MGEs and bacterial communities to the ARGs, BRGs, and MRGs. VPA demonstrated that 93.7%, 89.6%, and

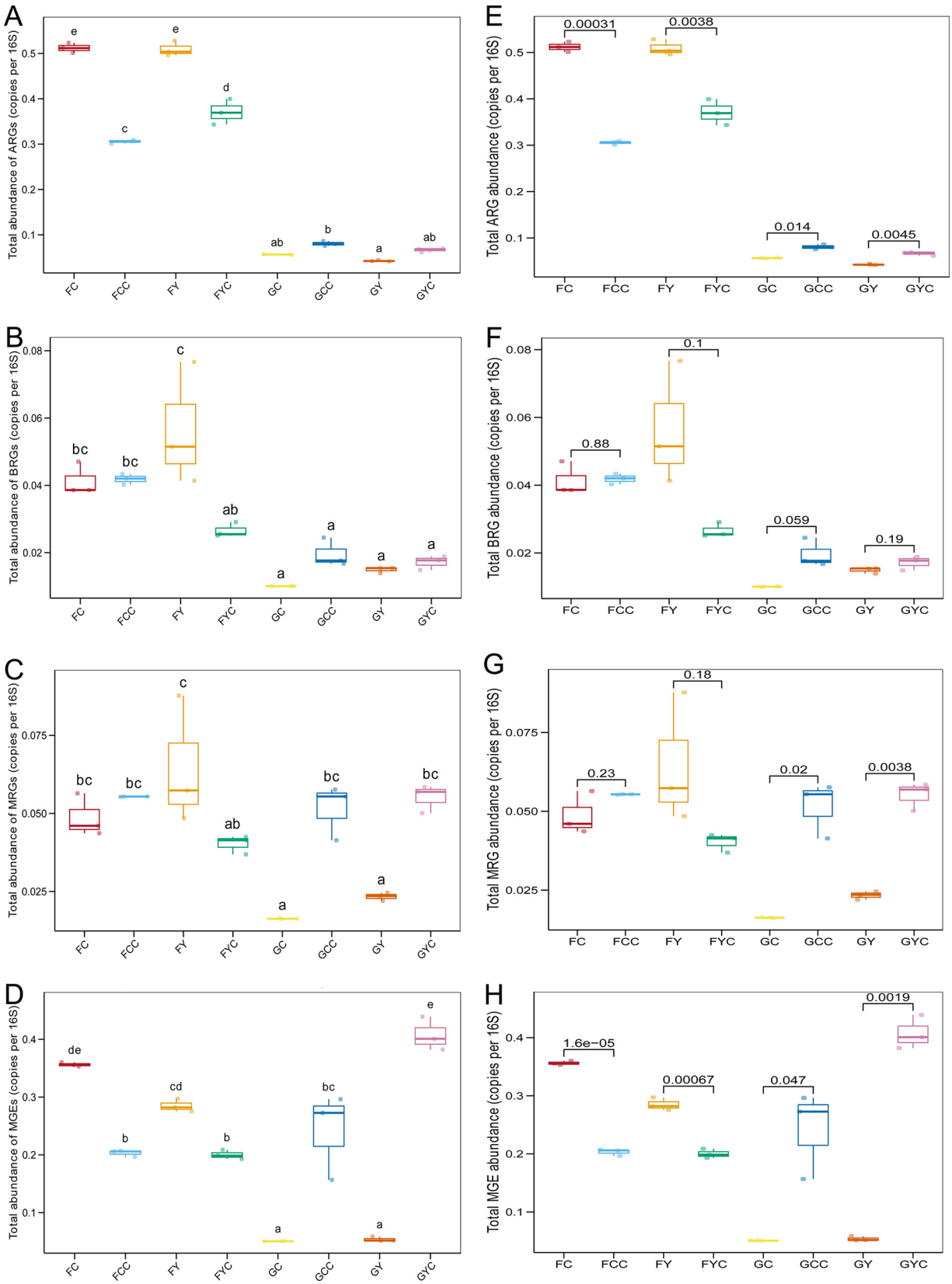

FIG 2 Comparison of the total abundance of ARGs (A), BRGs (B), MRGs (C), and MGEs (D) among the different groups. Different letters above the name of each treatment represent significant differences between groups ($P < 0.05$). Distribution of the total abundances of ARGs (E), BRGs (F), MRGs (G), and MGEs (H) in the four groups of manure before and after composting. Figure ($P < 0.05$) on the bar indicates a statistically significant difference. Figure ($P > 0.05$) on the bar indicates no significant difference.

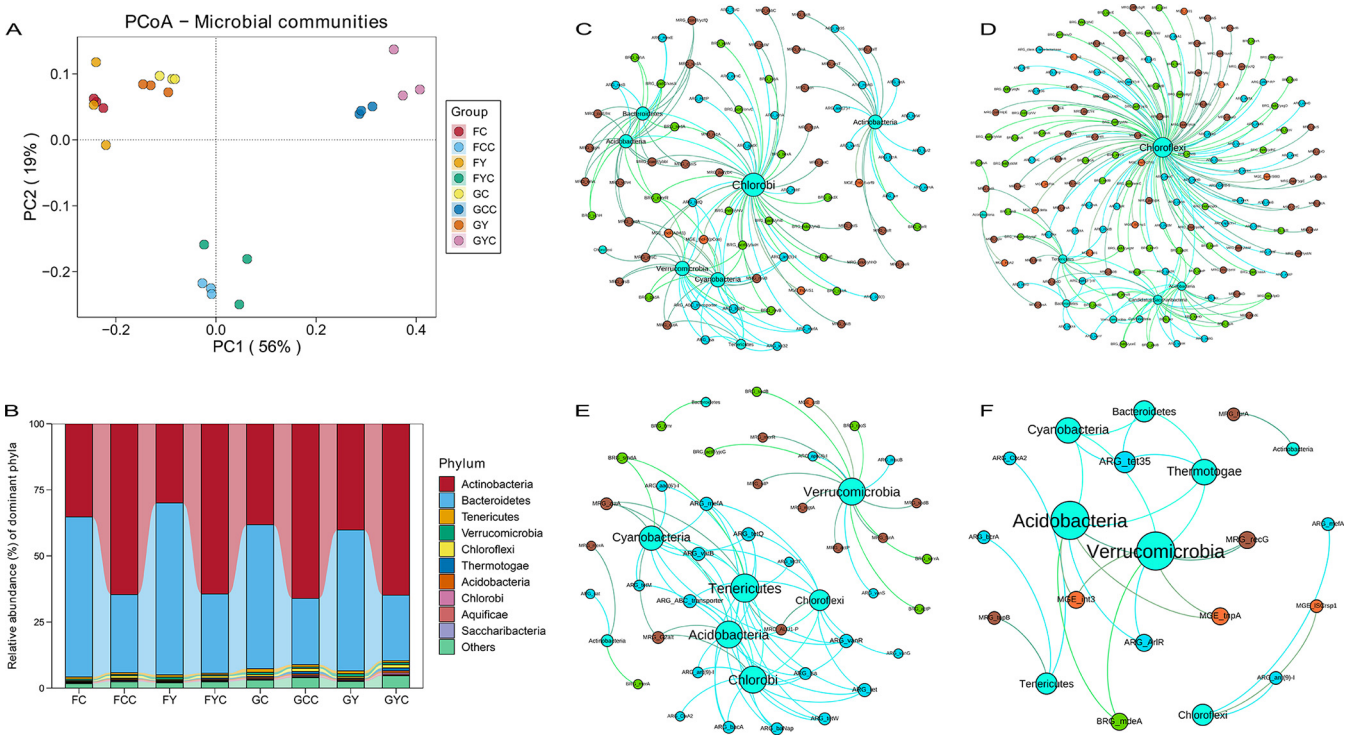

FIG 3 Network analysis of correlation between the ARGs, BRGs, MRGs, MGEs, and bacterial community. (A) Principal coordinate analysis (PCoA) of the microbiota of yak and cattle manure before and after composting under grazing and intensive feeding patterns. (B) Composition of dominant bacterial communities of yak and cattle manure before and after composting under grazing and intensive feeding patterns. Relationships between ARGs, BRGs, MRGs, MGEs, and bacteria (at the phylum level) based on Pearson's correlation coefficients ($P < 0.05$) before (C) and after (D) composting under grazing patterns. Relationships between ARGs, BRGs, MRGs, MGEs, and bacteria (at the phylum level) based on Pearson's correlation coefficients ($P < 0.05$) before (E) and after (F) composting under intensive feeding patterns. The nodes are colored according to ARGs, BRGs, MRGs, MGEs, and phylum, and the node size is dependent on the number of connections to other nodes (degree). Each connection represents a significant correlation ($P < 0.05$).

88.7% of variance in ARGs, BRGs, and MRGs, respectively, could be explained by variations in MGEs and bacterial communities (see Fig. S12 in the supplemental material). In addition, for ARGs, BRGs, and MRGs, there was little difference in the proportion of variation explained by the MGEs and bacterial communities. For ARGs, BRGs, and MRGs, the proportion of variation explained by MGEs was 13.1%, 20.2%, and 19.0%, respectively, and that explained by the bacterial community was 15.5%, 11.3%, and 13.4%, respectively.

Coselection of ARGs, BRGs, and MRGs mediated by MGEs from livestock manure and composting samples in different feeding patterns. A clustering analysis was conducted based on correlations among the abundance of ARGs, BRGs, MRGs, and MGEs, resulting in seven clusters (Fig. 5). According to the different MGE types, ARG, BRG, and MRG types were grouped as follows: integrase was correlated with trimethoprim, sulfonamide, and chloramphenicol resistance genes; ist was correlated with Hg, quinolone, tetracycline, V, Au, Fe, vancomycin, Pb, and Ag resistance genes; transposase was correlated with triclosan, fosfomycin, Cu, Al, bacitracin, Se, W, rifamycin, Cr, Co, and H_2O_2 resistance genes; and IS and plasmids were correlated with tetracycline, MLs, aminoglycoside, and fosmidomycin resistance genes.

Clinical ARG, BRG, MRG, and MGE indicators from livestock manure and composting samples in different feeding patterns. In total, 121, 112, 132, and 38 clinical ARG, BRG, MRG, and MGE subtypes, respectively, were considered the core subtypes in livestock manure and composting samples under different feeding patterns on the QTP (Fig. 6A to D). Although these core subtypes occupied a small fraction of those detected, they accounted for up to 90%, 80%, 80%, and 90% of the total abundance of clinical ARGs, BRGs, MRGs, and MGEs, respectively (see Fig. S13 to S16 in the supplemental material). The changes in abundance of core clinical ARGs, BRGs, MRGs, and MGEs were similar to the total abundance results (Fig. S13 to S16). Linear regression analysis revealed a significant linear correlation between the abundance of core clinical

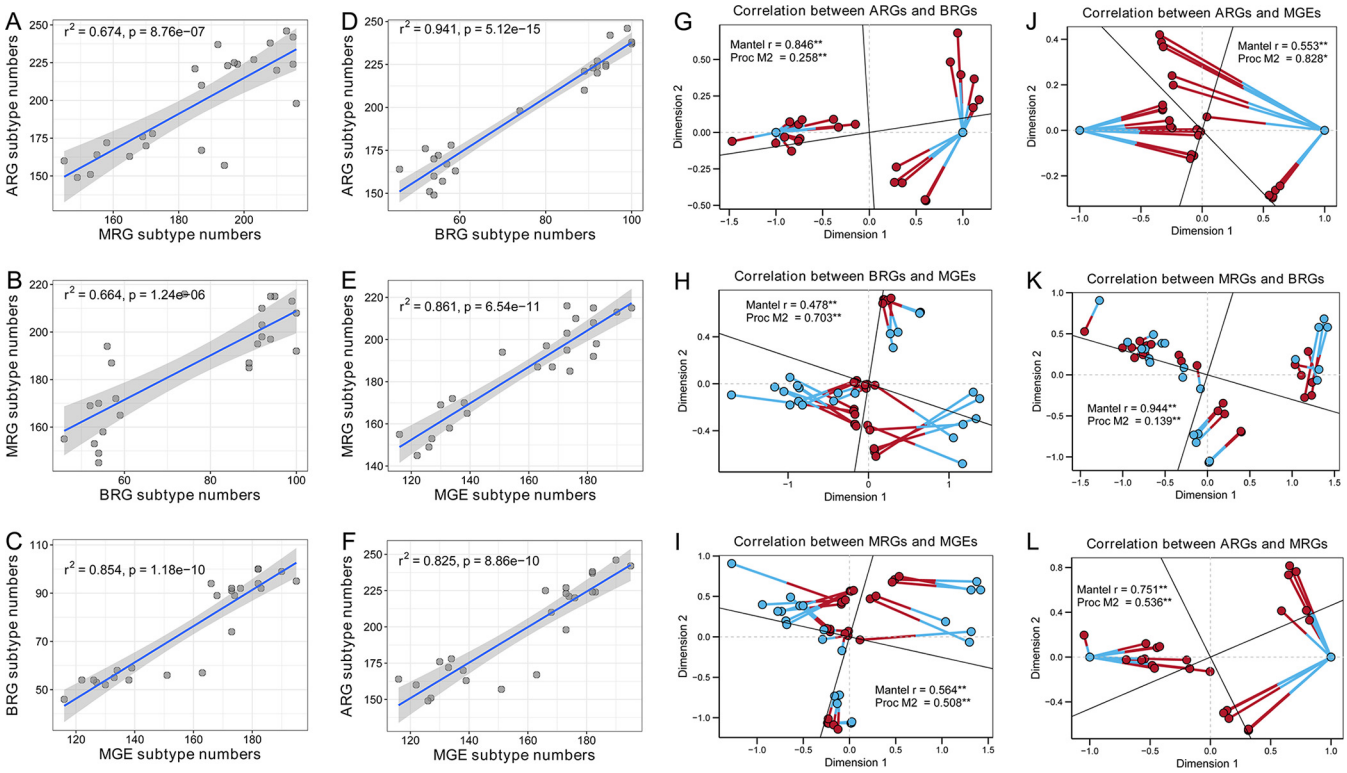

FIG 4 Correlations among the detected number and total abundance of ARGs, BRGs, MRGs, and MGEs. ARGs with MRGs (A), MRGs with BRGs (B), BRGs with MGEs (C), ARGs with BRGs (D), MRGs with MGEs (E), and ARGs with MGEs (F). Procrustes analyses revealing correlations among the ARGs, BRGs, MRGs, and MGEs as follows: ARGs with BRGs (G), BRGs with MGEs (H), MRGs with MGEs (I), ARGs with MGEs (J), MRGs with BRGs (K), and ARGs with MRGs (L).

ARGs, BRGs, MRGs, and MGEs and their total abundance in each sample, with a correlation coefficient of ~1 (see Fig. S17 in the supplemental material). This further confirms that the core clinical ARGs, BRGs, MRGs, and MGEs closely represent the overall levels.

Random forest analysis results demonstrate that *vanR*, *tolC*, *tetQ*, *tet44*, *sitC*, *rosB*, and *floR* resistance genes significantly influenced the total abundance of clinical ARGs. Meanwhile, IS*91*, *tnpA*, *tolC*, *vanR*, *pstB*, and *int3* resistance genes significantly influenced the total abundance of BRGs, and *arsC*, *baeR*, *copA*, *mdtF*, *smdA*, and *vanS* resistance genes significantly influenced the total abundance of MRGs. Finally, *vatB*, *tnpA3*, *tetO*, *smdA*, IS*91*, *fabK*, and *copA* resistance genes significantly influenced the total abundance of MGEs (Fig. 6E). Additionally, linear regression analyses indicated that the abundances of *tetQ*, IS*91*, *mdtF*, and *fabk* were significantly correlated with the total abundance of clinical ARGs, BRGs, MRGs, and MGEs, respectively (Fig. 6F to I).

DISCUSSION

Previous studies have reported various abundant ARGs in pig, chicken, and cattle manure samples, indicating that livestock manure is an important reservoir of ARGs (4, 16, 20). The farming of chicken, pig, and cattle involves a high-density feeding pattern, which suggests that the ARGs in the manure of these animals are affected by the selective pressure of antibiotics. In the present study, a comparative metagenomic approach was applied to analyze the ARGs, BRGs, and MRGs in the manure and compost from yak and cattle under grazing and intensive feeding patterns to explore the effects of human intervention on these factors. According to the subtype numbers and the total abundance of ARGs and MGEs, lower total subtype numbers and total abundances of ARGs and MGEs were observed in the manure from grazing livestock compared with that in the manure from intensively fed animals. This is consistent with the report of Wang et al. (21), wherein the numbers and relative abundances of ARGs in the gut of grazing yaks were lower than those in intensively fed beef and dairy cattle. Notably, the changes in

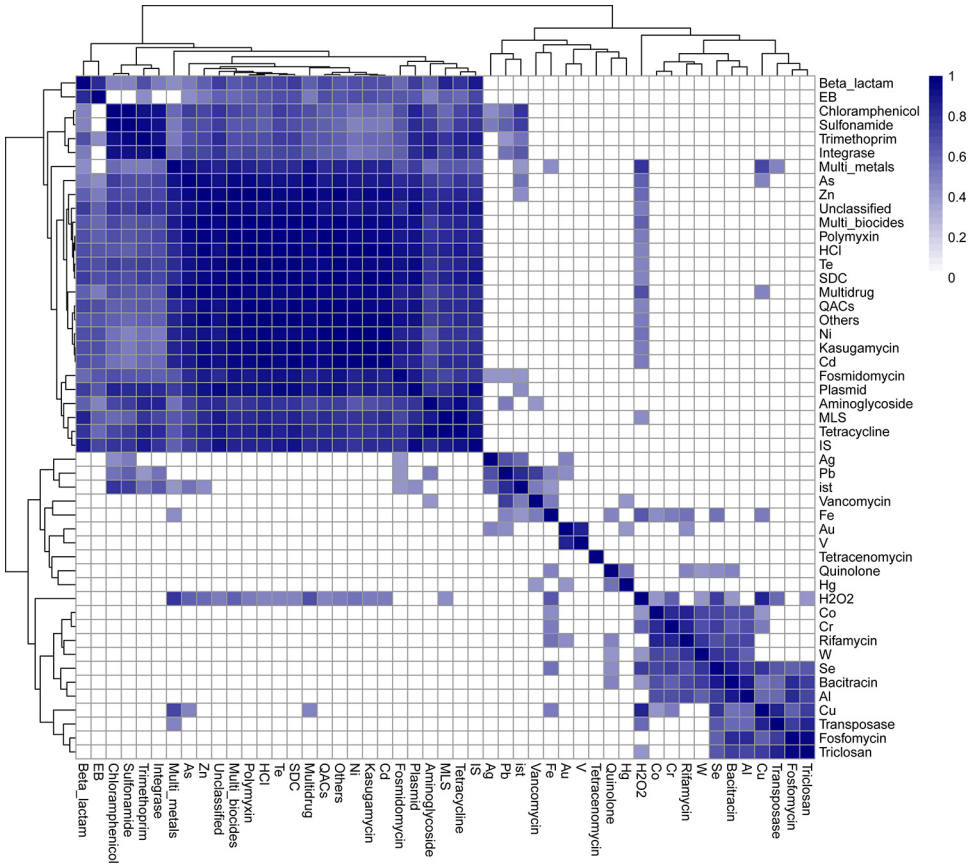

FIG 5 Clustering based on the abundance of ARG, BRG, MRG, and MGE types.

the resistance genes of yak and cattle manure before and after composting were the same under both grazing and intensive feeding patterns. This result further confirms that the feeding pattern, rather than the breed of the livestock, heavily influences the generation of resistance genes in the manure.

With the rapid decline in the cost of antibiotic production and the increasing use of antibiotics, an increasing number of subtherapeutic doses of antibiotics are being used in intensive feeding to promote livestock growth and disease prevention (22). The intensive feeding farms that were included in this study commonly use antibiotics (such as tetracyclines, quinolones, and beta-lactams) as feed additives. Therefore, the higher abundance of ARGs in raw manure under intensive feeding patterns was affected by antibiotic selective pressure. In addition, this is the first study to investigate the differences in BRG and MRG abundances in raw manure between grazing and intensive feeding patterns. The total number of subtypes and total abundances of BRGs and MRGs were also lower in grazing manures than in intensive feeding manures. This may be related to the diet and management of livestock under different feeding modes; that is, the intensive feeding farms in our study commonly use heavy metals as feed additives and regularly sterilize the enclosure with a fungicide. In addition, the feed used in intensive farming is artificially planted; the planting and foraging processes for corn can lead to the transmission of pesticide residues and heavy metal enrichment to livestock, thereby causing selective pressure for heavy metals and pesticides (23–26). It is worth noting that, the manure of grazing livestock on the QTP harbor ARGs, BRGs, and MRGs. This was demonstrated by D'Costa et al. (27), who detected ARGs in 30,000-year-old permafrost sediments. Meanwhile, Allen et al. (28) and Lang et al. (29) found ARGs in undisturbed Alaskan soil, indicating that resistance genes occur naturally (8).

The total abundance of ARGs decreased in yak and cattle manure under intensive feeding patterns after composting, which is consistent with reports of previous studies

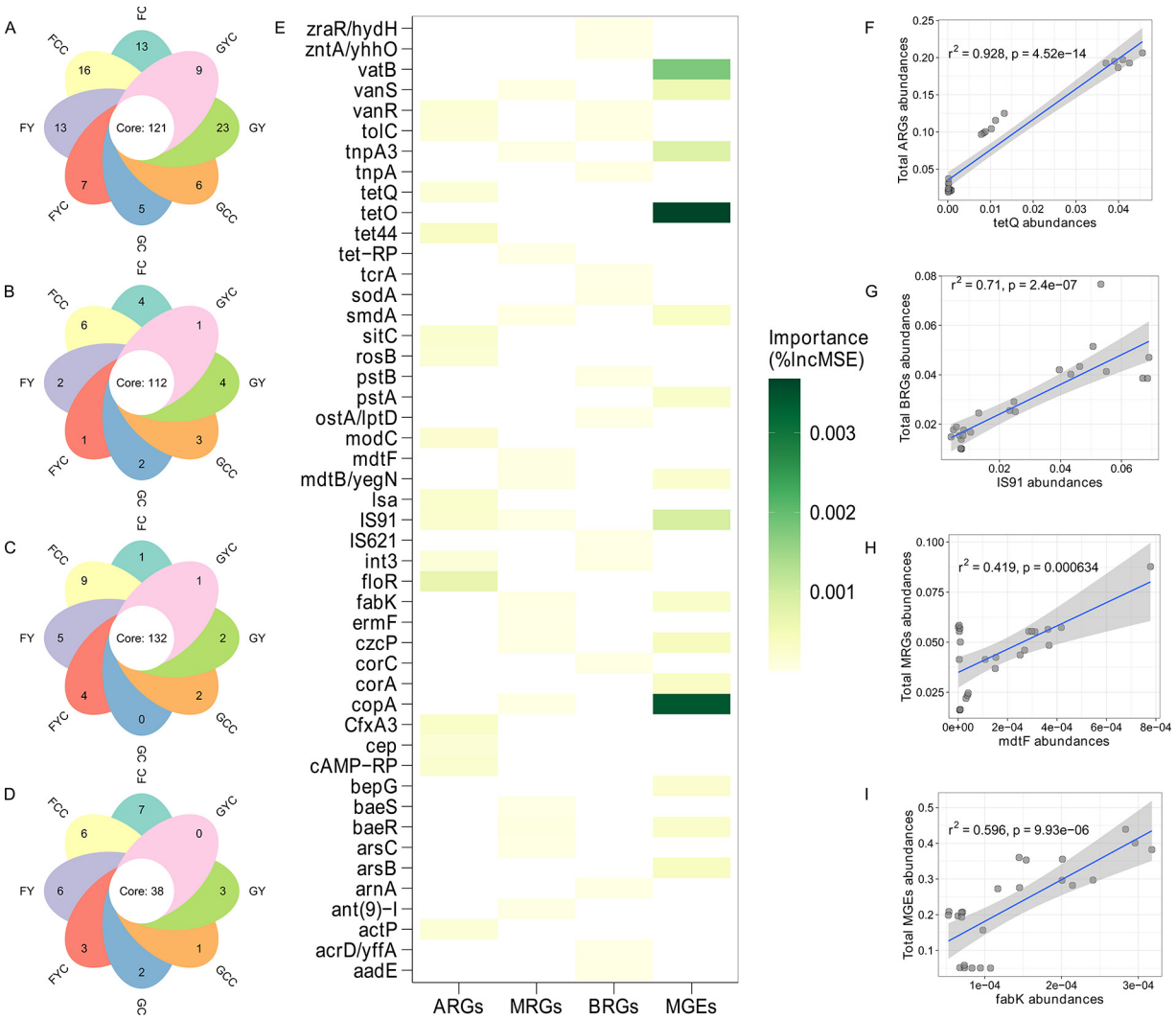

FIG 6 Core gene numbers of clinical ARGs (A), BRGs (B), MRGs (C), and MGEs (D) that are shared in all manure and composting samples. (E) Random forest analysis of genes that significantly influence the total abundance of clinical ARGs, BRGs, MRGs, and MGEs. Linear regression analysis between the abundance of indicator genes and the total abundance of clinical ARGs (F), BRGs (G), MRGs (H), and MGEs (I).

showing that composting decreases the abundance of ARGs (16, 30–33). In intensive livestock manure feeding, most ARGs are selected using large amounts of antibiotics in a short period of time, being less persistent and therefore easier to eliminate when the selection pressure is released during composting (16). However, notably, the total abundance of ARGs increased in yak and cattle manure under grazing patterns after composting, which might be explained by the increased abundance of MGEs in yak and cattle manure under grazing patterns after composting. MGE-mediated horizontal gene transfer (HGT) plays an important role in the development of multidrug resistance and spread of ARGs (8, 34). Wang et al. (21) reported that the abundance of integrons is higher in grazing yak gut than in dairy and beef cattle under intensive feeding patterns, indicating that the higher abundance of MGEs in the yak may have a stronger ability to transfer and spread ARGs. In the present study, transposase abundance increased in yak and cattle manure under grazing patterns after composting. Transposons, which are important elements involved in resistance formation in MGEs, can spread ARGs horizontally in bacteria through site-specific recombination (35, 36). Therefore, during the composting of grazing yak and cattle manure, the increased abundance of transposons resulted in the horizontal transfer of resistance genes, which increased the abundance of ARGs after composting. Conversely, MGEs and ARGs decreased in yak and cattle manure under intensive

feeding patterns after composting. In addition, composting did not reduce the abundance of BRGs or MRGs in yak and cattle manure under intensive feeding patterns, indicating that the composting process had little effect on the abundances of BRGs and MRGs in manure under intensive feeding patterns. Similar to that of ARGs, the abundance of MRGs increased in the yak and cattle manure under grazing patterns after composting. These results indicate that, although the abundances of ARGs and MRGs in yak and cattle manure were significantly lower under the natural grazing pattern than under intensive feeding, the abundances of these resistance genes increased markedly after composting. Accordingly, manure under the grazing pattern can be directly returned to the field.

Furthermore, the types, subtypes, and abundance of ARGs, BRGs, MRGs, and MGEs were identified in all manure samples to evaluate differences in resistance gene composition between the manures of livestock under grazing and intensive feeding patterns. For ARGs, the subtype number and relative abundance of beta-lactam, MLS, and tetracycline resistance genes in intensive-feeding livestock were higher than those in grazing livestock, consistent with the results of Wang et al. (21). In our study, the intensive-feeding farms widely use antibiotics, and their selective pressure results in a significant increase in intensive feeding livestock manure (37). For BRGs, the subtype number and relative abundances of multibiocide resistance genes in intensive-feeding livestock were clearly higher than those in grazing livestock. Given that the pens in our study farms were disinfected once per week (Cetrimide, HCl, H_2O_2), using fungicides, the high percentage of multibiocide resistance genes may be caused by biocide selective pressure during intensive feeding husbandry (38). For the MRGs, the subtype numbers and abundances of Cu resistance genes in intensive-feeding livestock were clearly higher than those in grazing livestock. Within the current study farms, heavy metals (Cu and Zn) are often used as feed additives due to their ability to promote animal growth and prevent diseases (4). Therefore, the high percentage of Cu resistance genes may be caused by heavy metal selective pressure during intensive feeding husbandry. Indeed, Guo et al. (39) reported that copper induces the expression of antibiotic resistance systems, as in *Escherichia coli* increasing tetracycline resistance when exposed to trace amounts of Cu. This partially explains the markedly higher subtype numbers and relative abundance of tetracycline resistance genes in intensively fed livestock compared with grazing livestock. In general, when compared with grazing patterns, artificially added antibiotics and feed additives have resulted in the resistance of animal gut microbes to a variety of harmful components under intensive feeding patterns. Resistance genes in manure enter the environment through fertilization or enter livestock and poultry again through feed, thereby forming a vicious circle. Therefore, it is necessary to reduce the transmission of resistance genes in feces by composting.

Bacteria are carriers of ARGs, BRGs, and MRGs; however, different bacteria exhibit varying abilities to receive and transfer these genes. Bacterial community characteristics may also determine the presence of ARGs, BRGs, and MRGs in manure (4, 40–42). Hence, the diversity and composition of microbial communities in the manure and compost samples were evaluated in the present study. *Bacteroidetes* and *Actinobacteria* were the dominant bacterial phyla in all of the manure samples before and after composting. This result is consistent with findings of previous studies that also reported *Actinobacteria* and *Bacteroidetes* as the dominant phyla throughout the composting process, although the composting materials and conditions differed (43–48). Moreover, Liu et al. (37) reported that *Actinobacteria*, *Thermotogae*, and *Chloroflexi* are thermophilic microorganisms that are abundant throughout the thermophilic stage. This is consistent with our results, wherein the relative abundances of *Actinobacteria*, *Chloroflexi*, and *Thermotogae* significantly increased in all manure samples after composting. In addition, Wang et al. (49) and Guo et al. (50) reported that the total abundance of *Actinobacteria* or composting temperature ($>$55°C) reflects the maturity of the compost, indicating that all manure samples in our study were ripe.

Network analysis was performed to examine the correlation between ARG, BRG, MRG, and MGE subtypes and potential host bacteria in manure under grazing and intensive

feeding patterns before and after composting (51). Results showed that the potential host bacteria positively correlated with ARG, BRG, and MRG types differed significantly in livestock manure under intensive feeding patterns after composting. More specifically, the potential host bacteria changed from *Chlorobi* and *Actinobacteria* to *Chloroflexi*. Qian et al. (19) reported that *Chloroflexi* may carry ARGs during composting of cow manure. In the present study, *Chloroflexi* were the main potential host bacteria for resistance genes in livestock manure after composting. However, fewer changes were observed in the host bacteria that were positively correlated with ARG, BRG, and MRG types in grazing livestock manure after composting. That is, *Chlorobi*, *Acidobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Tenericutes*, and *Chloroflexi* were the primary host bacteria in the grazing manure before and after composting. However, the dominant phylum was not necessarily the potential host of ARGs, BRGs, MRGs, or MGEs. In fact, MGEs exhibited positive correlations with the relative abundances of *Chlorobi*, *Verrucomicrobia*, and *Cyanobacteria* in livestock manure under intensive feeding patterns before compositing, whereas the MGEs were primarily concentrated in *Chloroflexi* after composting. It was further demonstrated that *Chloroflexi* was the main carrier of resistance genes and MGEs in livestock manure under intensive feeding patterns during the composting process. In grazing livestock manure, MGEs were positively correlated with the relative abundance of *Verrucomicrobia* before composting. Meanwhile, after composting, the number of bacterial species carrying MGEs increased in the manure of grazing livestock and became predominated by *Verrucomicrobia*, *Acidobacteria*, and *Chloroflexi*. In addition, the composting process decreased the relative abundance of *Verrucomicrobia* in grazing livestock manure while increasing that of *Acidobacteria* and *Chloroflexi*. The elevated abundance of *Acidobacteria* and *Chloroflexi* carrying MGEs further revealed the increased abundance of ARGs, BRGs, and MRGs in grazing livestock manure after composting. Previous studies have shown that increasing or decreasing MGEs plays an important role in the enrichment and removal of ARGs, BRGs, and MRGs, which is consistent with our results (4, 52).

ARGs, BRGs, and MRGs in the environment are disseminated through HGT and the proliferation of bacteria, which maintain or increase abundance (30, 32, 36). Zhou et al. (31) reported that the effects of physical and chemical properties on bacterial communities are the major drivers of ARGs in compost from intensive feeding farms. In contrast, MGE-mediated HGT is a key determinant of ARG propagation during chicken manure composting with the addition of biochar and zeolite (53). Additionally, the combined effects of heavy metal concentration, transposon abundance, and microbial community composition drive the propagation of ARGs in the livestock industry (16). Owing to differences in composting materials and conditions, the relative importance of MGE-mediated HGT and vertical transmission of resistance genes via host bacteria proliferation differed in these studies. In the current study, the composition of ARGs, BRGs, and MRGs in all manure and composting samples exhibited minimal differences in the proportion of variation explained by MGEs and bacterial communities, indicating that synergy between MGE-mediated HGT and vertical transmission via proliferation of host bacteria were the drivers shaping the abundance and diversity of ARGs, BRGs, and MRGs in livestock manure and composting samples on the QTP.

Coselection includes coresistance and cross-resistance, depending on whether ARGs, BRGs, and MRGs are located in the same MGE (e.g., integrase, ist, transposase, IS, and plasmid) (54). Importantly, the identification of correlations among ARGs, BRGs, MRGs, and MGEs in our study can provide new insights into their coselection. Previous studies have suggested that integrase, ist, transposase, IS, and plasmids are significantly positively correlated with many ARGs, BRGs, and MRGs, which is consistent with the results of this study (34, 53–57). The coselection phenomenon was first discovered in heavy metal-contaminated environments (58), highlighting the possibility of coselecting antibiotic resistance with other functional genes. In particular, the coselection of ARGs and MRGs with heavy metals has been observed during swine manure composting, where multiple genes encoding ARGs and MRGs were situated in the same MGEs (i.e., cross-resistance and coresistance) (4). Furthermore, the coselection of ARGs

and BMRGs has been observed in subtropical estuaries, where multiple genes encoding ARGs and biocide and metal resistance genes (BMRGs) are situated in specific MGEs (i.e., coresistance) (53). In the present study, using clustering analysis based on correlations of multiple ARGs, BRGs, and MRGs with the same MGEs, we conjectured that the coselection of ARGs, BRGs, and MRGs from livestock manure and composting samples in different feeding patterns on the QTP may include both cross-resistance and coresistance.

By evaluating only a few indicator genes to assess the total abundance of ARGs, BRGs, MRGs, and MGEs in the environment, it is possible to track resistance gene pollution and evaluate their risk in the environment (16, 35, 53). Early studies proposed seven potential resistance genes (*sul1*, *oprD*, *tetM*, *ermB*, *intI1*, *catB3*, and *tnpA*) as indicators of environmental antimicrobial resistance status (51, 53, 59, 60). However, proper indicators for the status of clinical ARGs, BRGs, MRGs, and MGEs in livestock manure and composting ecosystems on the QTP are lacking. To bridge this gap, we used core resistance genes and random forest analysis to identify potential indicators to forecast resistance gene levels in livestock manure and composting ecosystems in the QTP. Based on the most significant dominant predictors for clinical ARGs, BRGs, MRGs, and MGEs, *tetQ*, IS*91*, *mdtF*, and *fabK* genes were identified as potential indicators to evaluate the pollution levels of clinical ARGs, BRGs, MRGs, and MGEs, respectively, in manure and composting samples of livestock on the QTP. Moreover, linear regression analyses demonstrated that the abundance of *tetQ*, *IS91*, *mdtF*, and *fabK* was significantly correlated with the total abundance of clinical ARGs, BRGs, MRGs, and MGEs, respectively. Our results further demonstrate that *tetQ*, IS*91*, *mdtF*, and *fabK* are powerful indicators for assessing clinical ARG, BRG, MRG, and MGE contamination levels in different feeding patterns of livestock manure and compost samples.

However, when analyzing metagenomics data, a number of detected resistance genes may not be resistance genes *per se* or may require additional mutations to mediate resistance; this represents a limitation of the current study. In particular, there is a large number of less defined genes for resistance to biocides and metals, various regulatory genes with minor or negligible roles in resistance can be detected, and various transporter genes may have no, or limited, roles in resistance despite "multidrug resistance" nomenclatures. Therefore, these resistance genes must be further verified.

Conclusions. Yak and cattle manure under grazing patterns on the QTP harbor ARGs, BRGs, and MRGs; however, the abundance of resistance genes increases after the composting period. In particular, the abundances of ARGs, BRGs, and MRGs of yak and cattle manure under intensive feeding patterns are notably higher than those under grazing patterns, and the abundance of ARGs decreases after composting. Notably, the resulting resistance gene profiles of yak and cattle manure before and after composting are highly similar under grazing and intensive feeding patterns. In addition, the changes in the abundance of resistance genes in manure after composting are consistent with the changes in the MGEs. Meanwhile, according to the network, the microorganisms that correlate with the resistance genes of manure also become significantly altered after compositing. Therefore, we recommend that manure from grazing livestock be returned directly to the field, whereas manure from intensively fed livestock should be returned to the field after composting.

MATERIALS AND METHODS

Ethics approval. This experiment was approved and supervised by the Animal Ethics Committee of Lanzhou University in Lanzhou, Gansu, China (file no. 2010-1 and 2010-2).

Study sites and animal management. We selected three intensive feeding farms and three pasture lands on the QTP. Each intensive-feeding farm had approximately 300 livestock (male and female), including cattle and yaks. Each pasture land had more than 200 livestock (male and female), including yaks and cattle. The grazing areas were densely covered with crisscrossing streams with abundant clean and unpolluted water that served as important sources of drinking water for local herders and livestock. The grazing livestock (yaks and cattle) in this study grazed on natural pastures year-round, did not receive supplemental feed or feed additives (e.g., antimicrobials, biocides, or heavy metals), and drank stream water. Feeding livestock (yaks and cattle) came from local intensive feeding farms, fed with total mixed ration throughout the year and free access to drinking water. In addition, in our study, the intensive feeding farms used antibiotics (tetracyclines, 120 to 180 mg/kg of body weight; quinolones, 160 to

210 mg/kg; and beta-lactams, 180 to 240 mg/kg) and heavy metals (Cu, 3.2 to 4.6 mg/kg; Zn, 4.5 to 6.5 mg/kg; As, 6.2 to 8.6 mg/kg) as feed additives for livestock growth promotion and disease control. Antibiotics and heavy metals were preadded to the total mixed ration according to dosage requirements. The pens were disinfected once per week with biocides (Cetrimide, 0.1%; HCl, 1%; H_2O_2, 3%).

Manure and composting. The site was cleaned before composting and a double layer of 1.2-mm rainproof cloth was used as a water barrier to prevent the compost leachate from seeping down. The entire site was fenced off to prevent disturbance by humans and animals. Due to the low temperatures and lack of labor resources in the alpine grazing areas of the Tibetan plateau, we used static aeration and insulation measures for open-air strip pile rotting, with piles measuring 2.3 m by 2.3 m by 1.5 m (length by width by height) and 3 m between piles. Static aeration was achieved by means of a ventilation tube embedded in the pile, while thermal insulation was achieved by the greenhouse effect of the double plastic film outside the pile. The temperature of the pile was measured using a temperature sensor (LM75).

Sample collection. Feces were collected from the pens of intensive feeding or grazing livestock. Before composting, yak and cattle manure in each intensive feeding farm and pasture were evenly mixed separately. A 500-g sample of each yak and cattle manure mixture from each intensive feeding farm and pasture was collected as a base sample. The manure mixtures were then divided into three piles, which were used as replicates. According to the U.S. Environmental Protection Agency (USEPA) standard, the composting process comprised the maintenance of a temperature of $\geq$55°C for more than 3 days. In this study, the composting average temperatures reached a maximum of 58.6°C. Different parts of the pile were sampled to ensure homogeneity of the sample and to reduce the variability within the layers. In the later stage of composting, 6 samples were collected from different layers of each pile; subsequently, the 18 samples of yak or yellow cattle manure from the same intensive farm or pasture were mixed into one sample to represent the compost samples of yak and yellow cattle under different feeding methods. A total of 12 fresh livestock manure samples and their corresponding compost samples were collected from three intensive feeding farms and three herders on the QTP. Each sample was placed in a 100-mL cryovial, immediately placed in a liquid nitrogen tank, and transported to the laboratory for storage at −80°C prior to DNA extraction. Samples from the uncomposted manure were designated as GY (grazing yak), GC (grazing cattle), FY (intensive feeding yak), and FC (intensive feeding cattle); composted manure samples were designated GYC, GCC, FYC, and FCC, respectively.

DNA extraction and sequencing. DNA was extracted from each sample using a FastDNA spin kit for soil (MP Biomedicals, CA, USA), following the manufacturer's recommendations. The purity and quality of the genomic DNA were assessed on 1% agarose gels using a NanoDrop spectrophotometer (Thermo Scientific, USA). Sequencing library generation and metagenomic sequencing on the Illumina NovaSeq 6000 platform (Microeco, Shenzhen, China) were performed as described by Zhou et al. (61). After sequencing, the raw data were filtered using KneadData (version 0.7.4). Approximately 6 Gbp of clean metagenomic data were generated per sample.

To assess the changes in the bacterial community during composting and the relationship between the bacterial community and ARGs, BRGs, MRGs, and MGEs, the V3-V4 region of the 16S rRNA gene was amplified and sequenced using 341F/806R primers on the Illumina HiSeq 2500 platform according to the PE250 strategy (61). The clean sequences were clustered using USEARCH at a 97% similarity level after quality and filtration using QIIME (version 1.9.0). Each representative operational taxonomic unit (OTU) was assigned a taxonomic annotation according to the SILVA (SSU132) database (62).

Annotation and analysis of ARGs, BRGs, MRGs, and MGEs. ARGs-OAP v2.0 was used for the annotation and quantification of ARGs, BRGs, MRGs, and MGEs. The database referenced for ARGs was SARG (63). The database referenced for the BRGs and MRGs was reconstructed using BacMet (64). The database referenced for the MGEs was the latest released database of MGEs (65). Briefly, the threshold for gene annotation was set at a similarity of >90%, and a length of reads matching the reference sequence was >25 amino acids (61). Species taxonomic annotation and quantification of clean reads were obtained by metagenomic sequencing using the Karken2 application under the default parameters (66). According to the annotation results, we obtained the normalized abundance of ARGs, BRGs, MRGs, and MGEs in each sample (i.e., the number of ARG copies per 16S rRNA gene).

Identification of clinical ARGs. The ARG reference sequence in the SARG database was aligned with the PathoSystems Resource Integration Center (PATRIC) database using BLAST. The threshold value of the alignment was set at a consistency of >80% and matching length of >80%. The matched SARG reference sequences were clinical ARGs (42, 67). Next, the reference sequence level gene abundance table obtained by the ARG-OAP tool was matched with the identified clinical ARGs, and the gene abundance table of the clinical ARGs in the sample was obtained.

Statistical analysis. All statistical analyses were performed using R v4.1.0. The *t* test (two groups, base package) or Tukey's honestly significant difference (HSD) test (multiple groups, multcomp package) were used to compare the total detected numbers, type and subtype abundances, and total abundance of ARGs, BRGs, MRGs, and MGEs in different groups. The Mantel test, Procrustes analysis (vegan package), and linear regression (base package) were used to assess the correlation between bacterial communities and ARGs, BRGs, MRGs, and MGEs. Spearman rank correlation analysis was conducted to determine the abundance of each ARG, BRG, MRG, and MGE type, and the results were clustered to identify the relationship between ARGs, BRGs, MRGs, and MGEs (linkET package). Through random forest analysis, important predictors representing variation in the resistome were identified from the core resistome (randomForest package). Linear regression was used to assess the accuracy of the indicator genes in predicting pollution levels in the resistome (base package). The correlation of ARGs, BRGs, MRGs, MGEs, and potential host bacteria was analyzed by network analysis using Gephi (version 0.9.2) with the Fruchterman Reingold placement algorithm.

Variance partitioning analysis (VPA) was conducted to evaluate the contributions of bacterial communities and MGEs to ARGs, BRGs, and MRGs (vegan package).

Data availability. Raw reads were deposited at NCBI (under BioProject accession number PRJNA830819).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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