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Antibiotic resistance genes in different animal manures and their derived organic fertilizer

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Abstract

Background: The prevalence of antibiotic resistance genes (ARGs) in animal manure poses a threat to environmental safety. Organic fertilizers fermented by livestock and poultry manure are directly applied to farmland and have the potential to cause outbreaks of bacterial resistance in agricultural environments. This study investigated the composition of ARGs in different animal manures and their derived organic fertilizers.

Results: The results showed that the abundance of several ARGs, such as *sul*2, *Tet*B-01, *Tet*G-01 and *Tet*M-01, in organic fertilizer samples was 12–96% lower than that in animal manure. However, the abundance of *Tet*K and *erm*C was higher in animal manure than in organic fertilizers. No correlation between ARGs and environmental factors such as pH, TN, and antibiotics was observed by redundancy analysis (RDA). Procrustes analysis revealed a significant correlation between bacterial community structures and ARG abundance ($r = 0.799$, $p < 0.01$). Nonmetric multidimensional scaling (NMDS) analysis suggested that microorganisms in organic fertilizer may be derived from animal manure. Additionally, the abundance of pathogenic bacteria (especially *Actinomadura*) would increase rather than decrease in manure compared to organic fertilizer.

Conclusion: The diversity and abundance of most ARGs significantly decreased from animal manure to organic fertilizer. Microorganisms in the prepared organic fertilizer may mainly be inherited from the animal manure. The results also showed that the pathogens in the prepared organic fertilizer would significantly reduce, but would still cause partial pathogen proliferation.

Keywords: Antibiotic resistance gene, Animal manure, Organic fertilizer, Pathogen

Background

Antibiotics are a class of medicines extensively used for promoting growth and controlling diseases on livestock and poultry farms [1]. However, up to 30–90% of the administered antibiotics are excreted through urine and manures, leading to the accumulation of residual antibiotics in the livestock environment [2–4]. Typical concentrations of antibiotics in livestock manure and poultry are generally in the range of 1–10 mg/kg and could be as high as 200 mg/kg [5]. According to previous studies, the content of enrofloxacin and ciprofloxacin in chicken manure were 61 300 μg/kg and 18 800 μg/kg, respectively [6]. High levels of residual antibiotics in manure provided selective pressure to the native microbial communities after the application of manure in soil [7–9], and bacteria could acquire antibiotic resistance genes (ARGs) via horizontal gene transfer or spontaneous mutation, thereby causing the proliferation of resistant bacteria [10, 11].

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ARGs could enter the environment via discharge of animal manure, leading to the contamination of soil, water and crops [12]. Most of the recent research focused on the quantitative detection of ARGs in manure and their surrounding environment [13]. For example, a total of 15 tetracycline resistance genes were detected in the soils around a pig farm [14]. The abundance of resistance genes was reported to be highly enriched in animal manures (121,000-fold in a farm in Beijing, 39,000-fold in a farm in Jiaxing and 57,000-fold in a farm in Pu Tian) [15]. Fang et al. study found that there were multidrug resistance (MDR) genes in stream water, which were disseminated from pig feedlot to surrounding stream via pig manure fertilization [16]; and Zhang et al. study described that cattle manure application increased the abundance of ARGs in plant root, while poultry manure application increased ARGs in rhizosphere, root endophyte and phyllosphere [17]. Thus, the continuous increase or high persistence of ARGs in livestock environments may pose potential threats to human health and the ecological environment [18, 19].

Composting is widely used to treat and re-utilize animal manures [20–22]. It has been reported that ARGs cannot be completely removed by fertilizer production during composting, which could even cause the proliferation of resistant bacteria [23, 24]. Although studies have shown a decrease in individual ARGs after composting, risks of ARG propagation still existed [25, 26]. To the best of our knowledge, no systematic evaluation is available on the comparison of ARG composition both in livestock manure and their derived organic fertilizers. The relationship of ARGs in different animal manures and their derived organic fertilizers is insufficient as well. This study is devoted to addressing this concern, with the following objectives: (1) investigate the ARG composition in different animal manure and fermented organic fertilizers; (2) explore the effect of bacterial communities on ARG changes, and (3) demonstrate the variations of antibiotic-resistant pathogens in manure and organic fertilizers.

Materials and methods

Sample collection and analysis

Animal manures and organic fertilizers were obtained from different organic fertilizer enterprises in China. Three kinds of animal manure, including chicken manure (JF1, JF2, JF3, JF4, JF5), cow manure (NF1, NF2, NF3, NF4, NF5), and sheep manure (YF1, YF2, YF3), were collected from different organic fertilizer factories. The different numbers indicate different sources of animal manure. These fertilizers were prepared by aerobic composting of the raw materials. Five types of organic fertilizers were derived from a single manure (JF1 → JM1, JF3 → JM3, JF4 → JM4), (YF1 → YM1, NF2 → NM2). The following organic compound fertilizers were derived from a mixture of various animal manures (NF4 + YF1 + JF5 → NYJM1, NF1 + YF2 → NYM1, YF2 + JF3 → YJM1, YF3 + JF2 → YJM2). Three samples were collected from manure/fertilizer at each site and combined to prepare one sample. The mixed sample was divided into two portions: one subsample was used for analysing physicochemical properties, and the second subsample was freeze-dried and stored at −80 °C for DNA extraction and microbial community structure analysis. Antibiotic concentrations were determined according to the approach by Li et al. [27].

DNA extraction

DNA extraction was carried out from 0.2 g animal manure and prepared organic fertilizer samples using a Power Soil DNA isolation kit (Omega Bio-Tek, Norcross, GA, USA) according to the kit manual. The extracted DNA was quantified with a Nano Drop spectrophotometer (Thermo Scientific) and stored at −80 °C until PCR analysis.

HT-qPCR

ARGs were quantitatively analysed with the Smart Chip Real-Time PCR System (WaferGen Biosystems Inc., Fremont, CA, USA) equipped with a high-throughput quantitative reaction platform (Qiyin Biological Technology Co., Ltd., Shanghai, China). A total of 51 primers targeting resistance genes and a pair of bacterial universal 16S rRNA gene primers were selected according to the literature [28]; five sulfonamide resistance genes (SRGs), 16 tetracycline resistance genes (TRGs), two fluoroquinolone resistance genes (FRGs), five aminoglycoside resistance genes, 11 β-lactamase resistance genes, seven macrolide–lincosamide–streptomycin B (MLSB) resistance genes, two vancomycin resistance genes, three other/efflux resistance genes, and the 16S rRNA gene were targeted. The experimental conditions and the data processing procedure were the same as those published in the literature [29].

16S high-throughput sequencing

The 16S V4 region was analysed by the Illumina HiSeq 2500 platform to study the bacterial community composition. The chimaeras were filtered using USEARCH, and the remaining sequences were clustered into 97% similarity operational classification units (OTUs). Each OTU sequence was selected by default, and the ribosomal database item classifier with the latest version of the green gene database with a confidence threshold of 80% was used to classify OTUs.

Data analysis

Weighted and unweighted UniFrac tests were performed using Mothur to determine the statistical significance of structural similarities among communities across different sampling locations. Ordination plotting with nonmetric multidimensional scaling (NMDS) was employed to visualize the beta-diversity information. Principal component analysis (PCA, based on Bray–Curtis distance) was used with STAMP software. R3.1.2 with Vegan 2.0 was used for the Mantel test and Procrustes analysis to determine the correlation between ARG data and bacterial communities. Additional, Network analysis showed the relationship between the host bacteria and ARGs using R 3.5.3 and Gephi 0.9.2. Redundancy analysis (RDA) was conducted using CANOCO 4.5 (Microcomputer Power, USA) to establish the underlying correlations between the ARGs and environmental factors.

Results and discussion

Diversity and abundance of ARGs in animal manures and organic fertilizer

The abundance and diversity of 51 target ARGs in animal manures and their prepared organic fertilizer were obtained through HT-qPCR. The results showed that the ARG diversity in the organic fertilizer samples was relatively lower than that in the animal manure samples ($p < 0.05$) (Fig. 1a). The absolute abundance of ARGs in the animal manure and organic fertilizer samples was in the range of 2.1×10^5 to 7.8×10^5 copies/g and 1.6×10^5 to 7.3×10^5 copies/g, respectively, which was similar to the findings by Zhang et al. [30]. A previous study also found that composting could reduce the abundance and diversity of ARGs in animal manure, likely due to the inactivation of some microorganisms by high temperature [31].

The types of ARGs in the animal manure from different breeds were significantly different due to the differences in manure properties and microbial composition (Fig. 1b) [32]. The detection rates of ARG subtypes from a single kind of animal manure to manufactured organic fertilizer samples (JF1 → JM1 and NF2 → NM2) were reduced by 42% and 34.6%, respectively. However, the detection rates of ARG subtypes from multiple animal manures (mixing of NF and YF, YF and JF) to compound organic fertilizer samples increased by 28% (NF1 + YF2 → NYM1) and 24% (YF2 + JF3 → YJM1), respectively (Fig. 1a). These results indicated that the composting process could reduce the release of partial ARG subtypes from single manure to organic fertilizer, which were consistent with the study of previous observations [33]. It is noteworthy that the composite organic fertilizer fermented by multiple manures would increase the diversity of ARGs, highlighting the importance of manure management concerning the fate of ARGs [34] (Additional file 1: Table S1).

The chicken manure had the highest absolute abundance of ARGs (2.8×10^5–7.8×10^5 copies/g), which was 2–4 times higher than those in cow manure (2.1×10^5–3.3×10^5 copies/g) and sheep manure (2.2×10^5–5.1×10^5 copies/g) (Fig. 1c). Previous studies have shown that the difference in ARG levels between poultry manure and cattle manure may be related to the difference in antibiotic use patterns and microorganisms in manure among different species of livestock [35, 36]. The removal efficiencies of ARGs in different animal manures and/or different treatment processes were also reported [15, 37]. Most TRGs (*tet*B-01, *tet*G-01 and *tet*M-01) decreased by 12–96% after composting (Fig. 1c), which was confirmed by previous findings [38, 39]. The abundance of *tet*X was higher than those of other ARGs in all samples, likely due to the broad range of potential hosts of *tet*X [34]. Some TRGs decreased, while others persisted or significantly increased (including *tet*K and *tet*X) after thermophilic composting [40]. Compared with the raw response of animal manure, the abundance of individual ARGs (*tet*K) increased 2–216 times in the organic fertilizer. Ezzariai et al. reported that the abundance of *tet*X in swine manure decreased exponentially under anaerobic conditions [37]. The reason for this opposite trend is still being explored, since the resistance mechanism of *tet*X is still unknown [15, 41].

SRGs were predominant in all samples in terms of absolute abundance. From JF1 to JM1, the levels of the *sul*1 and *sul*2 genes decreased from 1.1×10^5 copies/g to 10^0 copies/g and from 1.2×10^5 copies/g to 1.58×10^1 copies/g, respectively. Similar results were found in the study of Wolters et al. [33], that the level of *sul*2 dramatically decreased during composting. The abundance of MLSB resistance genes (*erm*A, *erm*B, *erm*F and *erm*X) significantly decreased by 10–98% from manure to organic fertilizer (Fig. 1c), lower than the abundances of TRGs and SRGs. The abundance of MLSB resistance genes was related to the low use of macrolides during feeding. The response of ARGs varied in composting due to ecological complex microbial processes. This result suggested that the composting process may cause individual ARGs proliferation.

Fresh manure composting is a feasible approach to decrease the level of certain ARGs before their application to farmland. However, most ARGs remain or even proliferate after composting, which may be caused by differences in external conditions, such as raw materials, environmental factors or microbial communities [42]. A recent study also showed that variations in microbial communities may have an impact on ARGs in composting [31].

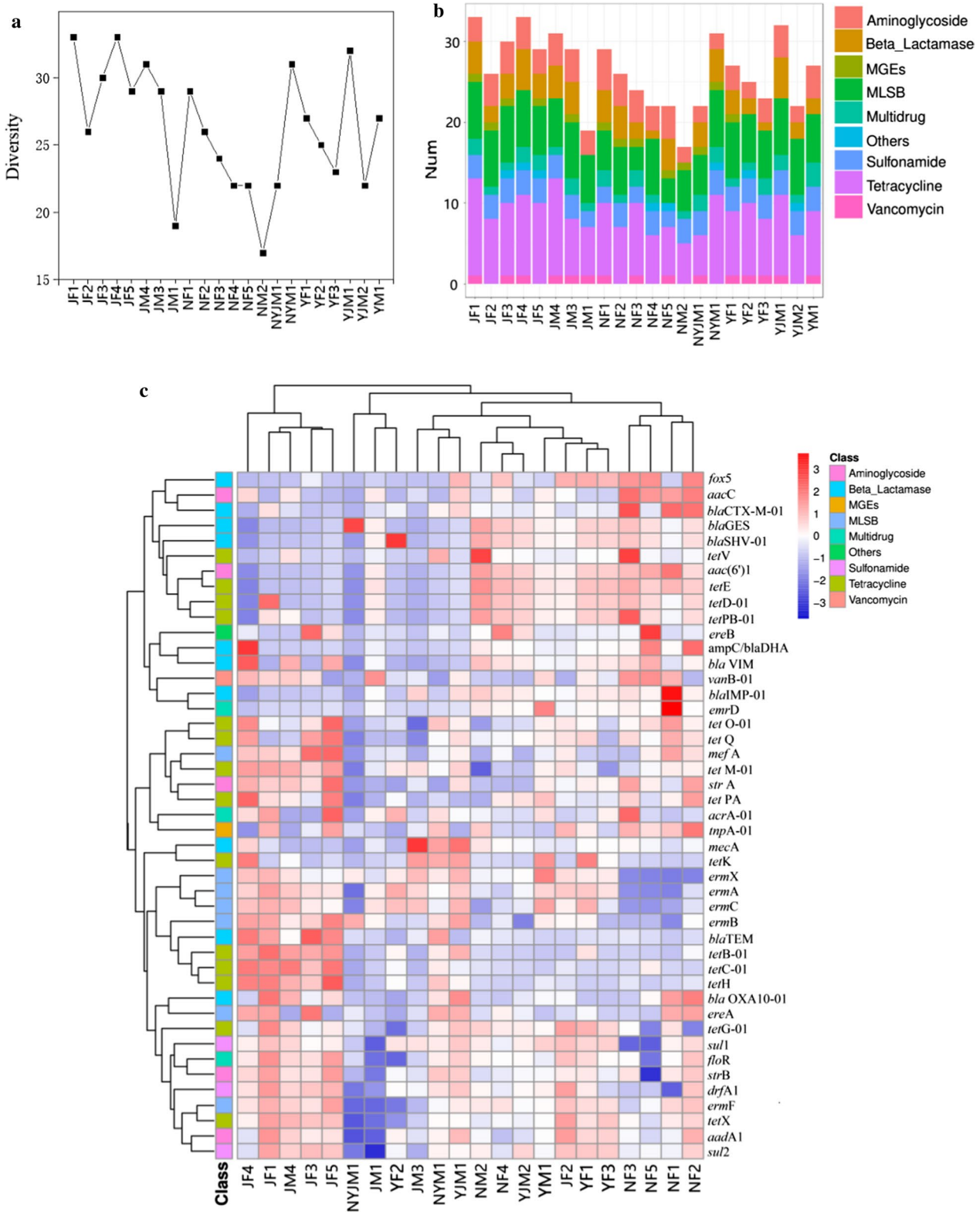

Fig. 1 **a** The diversity of ARGs in different animal manure and organic fertilizers. **b** The diversity of ARG subtypes in different animal manure and organic fertilizers. **c** Heatmap of the relative abundance of ARGs in animal manure and organic fertilizer

Microbial communities in animal manure and organic fertilizer

As shown in Fig. 2, no significant correlation between the ARGs and the environmental factors was observed in this study. Previous studies have found that ARGs were subjected to relatively smaller impacts from the environmental factors than microbial community [31].

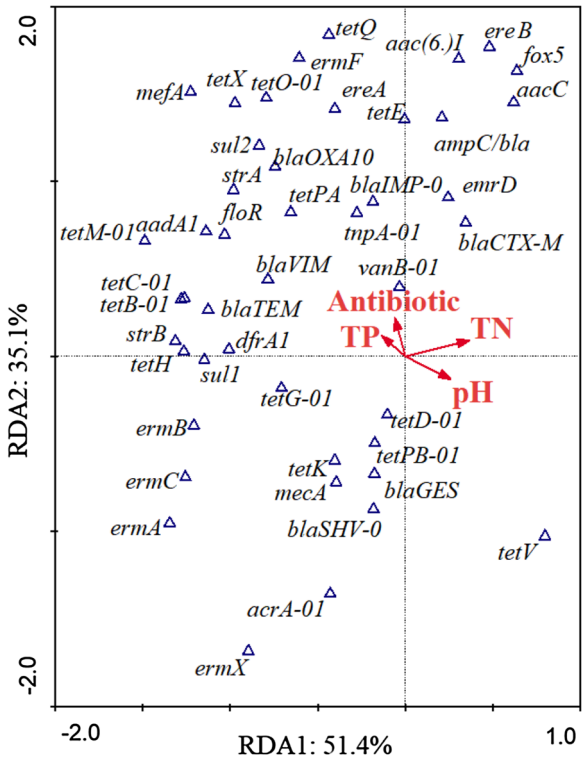

Fig. 2 Redundancy analysis (RDA) for correlation analysis between ARGs and environmental factors

And we focused on the microbial community structures in animal manure and organic fertilizer samples. The results are shown in Fig. 3a, b. The composition and abundance of microbial communities varied greatly in different sample classifications. The compound organic fertilizer NYJM had the highest microbial diversity (Fig. 3a), and the samples of different animal manures and organic fertilizer were clustered into different categories (Fig. 3b). From the sorted map of the microbial community in Fig. 3b, the manure samples partially overlapped with the aggregated organic fertilizer samples (such as NF and NM), suggesting that the bacterial community structures in organic fertilizer were similar to those in manure from the corresponding composting source.

Compared with their derived fertilizer, the abundance of microorganisms decreased in cow manure; conversely, an increase in microbial abundance was observed in chicken manure, which might be related to composting conditions and manure nutritional structure (Fig. 3a) [43, 44]. In particular, the abundance of microorganisms among JM samples varied dramatically, which might originate from the condition of livestock farms as well as individual differences in animals (including age and species) [45]. As shown in Fig. 3b, the composition of the microbial community between animal manure and their derived organic fertilizer (e.g., YF → YM and JF → JM) was significantly different. Interestingly, the Bray–Curtis distance between YF and YM and between JF and JM was closer than that between the YF or JF samples and other organic fertilizer samples. The overlap between NF and NM indicated that the microbial community structures of NF and NM were more similar than those of the other samples. The intersections between the YJM, JM, JF, and YF

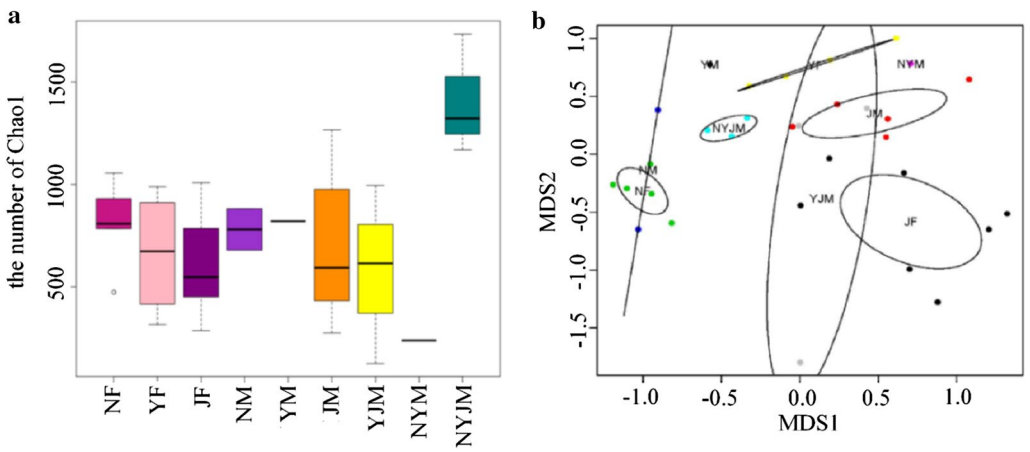

Fig. 3 **a** Alpha diversity of Chao 1 for each group sample; **b** nonmetric multidimensional scaling (NMDS) plot of all samples

samples suggested that there was a correlation between the microbial composition of animal manure and their derived organic fertilizer.

Relationship between the bacterial communities and ARGs

The results from the Mantel test indicated that ARG abundance was substantially correlated with bacterial community structures based on Bray–Curtis distance. Procrustes analysis showed that clustering based on the abundance of ARGs and 16S OTUs exhibited a goodness-of-fit (based on Bray–Curtis distance, sum of squares $M^2 = 0.476$, $r = 0.799$, $p < 0.01$, 999 permutations), indicating that the bacterial community structures exerted a significant influence on ARG abundance. This finding was consistent with a previous study showing that the change in the microbial community structure was the major factor driving the variation of ARG profiles in animal manure and organic fertilizer [46, 47]. The PCA results also confirmed that ARGs within different animal manures and organic fertilizers showed a similar distribution pattern of the bacterial communities (Fig. 4). It was concluded that the variation in ARGs from animal manure to organic fertilizer was strongly correlated with the microbial community. The shift of the bacterial communities played a key role in the direct change of the ARG patterns [34]. It was noted that the presence of pathogens in the microbial community would not only decrease the productivity of livestock and poultry breeding, but also increase the risk of ARGs spreading from organic fertilizer to the agricultural environment.

The fate of pathogens from animal manure to organic fertilizer

The variability in the relative abundance of the top 20 genera was determined to evaluate the risk of pathogens from manure to organic fertilizer (Fig. 5). It is worth noting that most of them were pathogens in the top 20 most abundant genera. *Corynebacterium*-1, *Virgibacillus*, *Streptomyces* and *Actinomadura* were the major genera in the animal manure and organic fertilizer samples. After composting, the relative abundance of pathogens was altered, but the dominant genus was still *Corynebacterium*-1; compost usually has a heterogeneous population composed of human and animal pathogens that could cause disease in livestock [48]. The relative abundance of *Virgibacillus* was significantly reduced in animal manure compared to prepared organic fertilizer (especially in JF-JM). In addition, the abundance of *Actinomadura* in organic fertilizer was 2–300 times higher than that in animal manure. These two genera were carriers of antibiotic resistance genes (such as *ermX* and *tetPA*), and their abundances were found to have a similar reduction trend as ARGs after composting. The genera *Actinomadura* and *Virgibacillus* belong to the phylum *Actinobacteria* and are opportunistic pathogens that cause disease in animals and plants [49]. Lv et al. identified that *Actinobacteria* were prominent in the thermophilic stage, and the groups in the study could probably carry and disseminate ARGs [50]. The abundance of *Pseudomonas* (phylum *Proteobacteria*), which was found to be an opportunistic pathogen carrying most ARGs with multiple resistance, was significantly increased from animal manure

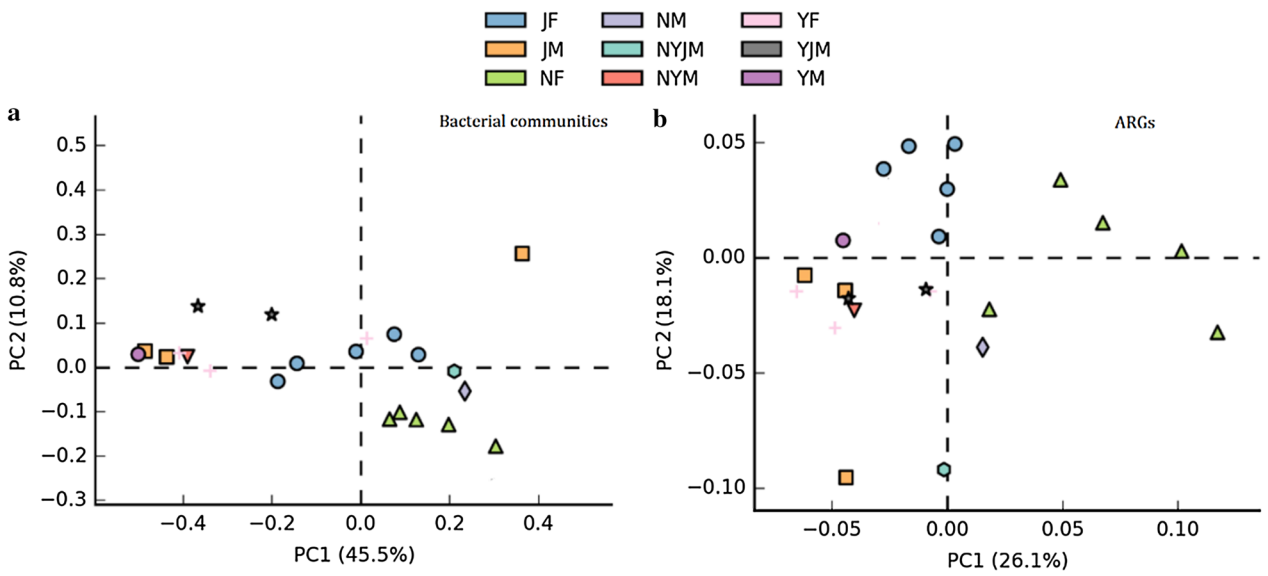

Fig. 4 Principal component analysis (PCA) showing the bacterial communities

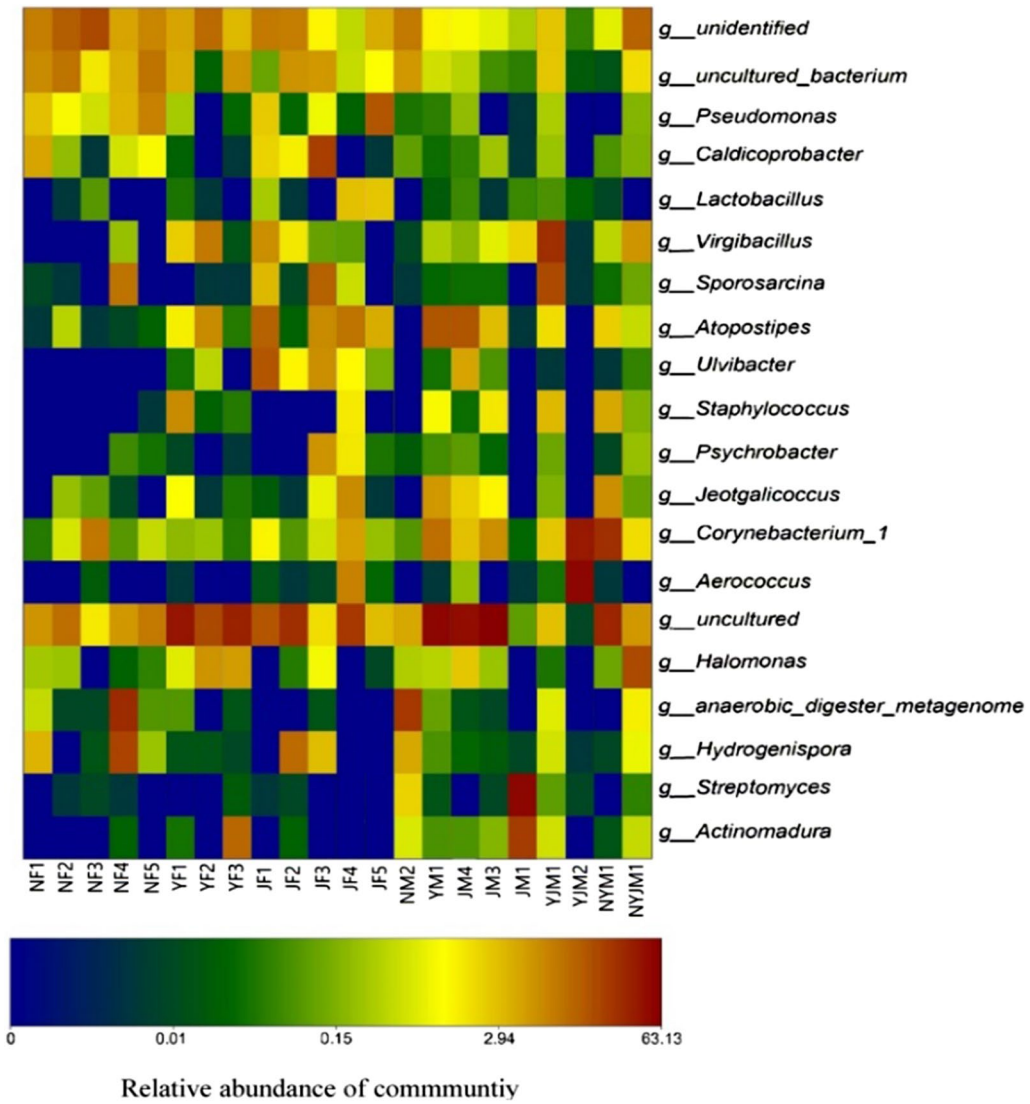

Fig. 5 Heatmap of the relative abundance of the top 20 genera in animal manure and organic fertilizer

to organic fertilizer [30, 51]. Network analysis was conducted to determine the correlation between ARGs and the top bacterial genera, which may allow the potential host bacteria for ARGs to be determined. As illustrated in Fig. 6, there were significant correlations between ARGs and the potential host bacteria in the animal manure and their derived organic fertilizer samples ($p < 0.05$ and $R > 0.60$). In addition to the host bacteria obtained above, the genus *Atopostipes* belonging to the phylum *Firmicutes* were found to contain potential host bacteria for *erm*A, *erm*B, *erm*X, *tet*K, and *tet*M-01.

Pathogens could not be completely removed after composting animal manure to organic fertilizer, causing partial proliferation. Significant correlations between pathogens and ARGs were observed, suggesting that the pathogens might become important hosts of ARGs [49]. Therefore, once antibiotic-resistant pathogens are ubiquitous in organic fertilizer, they are bound to pose a threat to the health of farmland soil and crops.

Conclusions

This study investigated the composition of ARGs and bacterial community structure between different animal manures and their derived organic fertilizers. The diversity and abundance of most ARGs significantly decreased from animal manure to organic fertilizer. Microorganisms in the prepared organic fertilizer may mainly be inherited from animal manure. The results

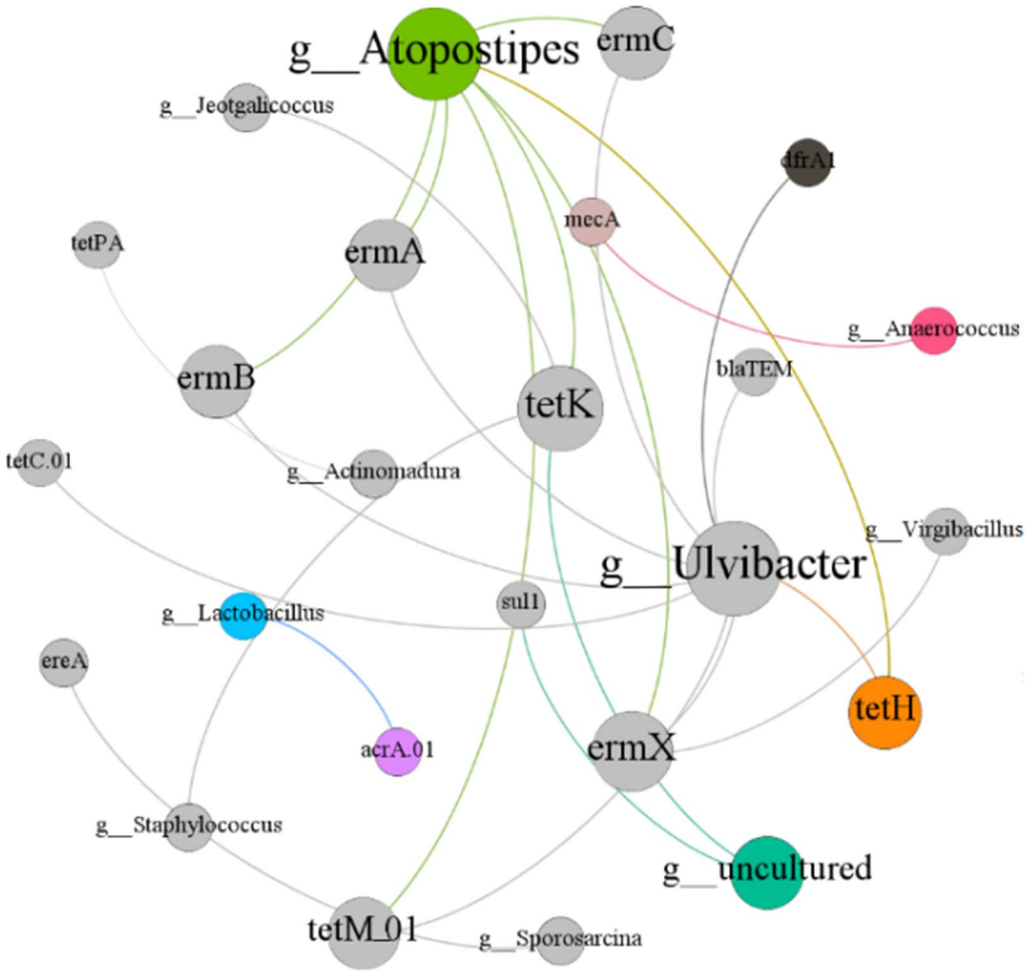

Fig. 6 Network analysis of ARGs and potential host bacteria (top 20 genera) ($p < 0.05$, $R > 0.60$)

also showed that the pathogens in the prepared organic fertilizer would significantly reduce, but would still cause partial pathogen proliferation. It is urgent and necessary to explore optimal fermentation processes to improve the removal efficiency of ARGs and pathogens in animal manure.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00381-y.

Additional file 1: Table S1. The 52 genes primers.

Abbreviations

ARGs: Antibiotic resistance genes; NMDS: Nonmetric multidimensional scaling; SRGs: Sulfonamide resistance genes; TRGs: Tetracycline resistance genes; FRGs: Fluoroquinolone resistance genes; MLSB: Macrolide–lincosamide–streptomycin B; OTUs: Operational classification units; PCA: Principal component analysis; RDA: Redundancy analysis.

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Authors' contributions

YX, HL and RS were involved in the experiments and manuscript writing, and JL, BL, FY and XZ were responsible for the data analysis. JX contributed to the study design and manuscript correction. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and analysed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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