Author/s:
Gou, M;Hu, HW;Zhang, YJ;Wang, JT;Hayden, H;Tang, YQ;He, JZ

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Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils

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Corresponding Author: Dr. Hangwei Hu,

Corresponding Author's Institution:

First Author: Min Gou, PhD

Order of Authors: Min Gou, PhD; Hangwei Hu; Yu-Jing Zhang, BSc; Jun-Tao Wang, PhD; Helen Hayden; Yue-Qin Tang, PhD; Ji-Zheng He, PhD

Abstract: Composting has been suggested as a potential strategy to eliminate antibiotic residues and pathogens in livestock manure before its application as an organic fertilizer in agro-ecosystems. However, the impacts of composting on antibiotic resistance genes (ARGs) in livestock manure and their temporal succession following the application of compost to land are not well understood. We examined how aerobic composting affected the resistome profiles of cattle manure, and by constructing laboratory microcosms we compared the effects of manure and compost application to agricultural soils on the temporal succession of a wide spectrum of ARGs. The high-throughput quantitative PCR array detected a total of 144 ARGs across all the soil, manure and compost samples, with Macrolide-Lincosamide-Streptogramin B, aminoglycoside, multidrug, tetracycline, and β-lactam resistance as the most dominant types. Composting significantly reduced the diversity and relative abundance of ARGs and mobile genetic elements (MGEs) in the cattle manure. In the 120-day microcosm incubation, the diversity and abundance of ARGs in manure-treated soils were significantly higher than those in compost-treated soils at the beginning of the experiment. The level of antibiotic resistance rapidly declined over time in all manure- and compost-treated soils, coupled with similar temporal patterns of manure- and compost-derived bacterial communities as revealed by SourceTracker analysis. The network analysis revealed more intensive interactions/associations among ARGs and MGEs in manure-treated soils than in compost-treated soils, suggesting that mobility potential of ARGs was lower in soils amended with compost. Our results provide evidence that aerobic composting of cattle manure may be an effective approach to mitigate the risk of antibiotic resistance propagation associated with land application of organic wastes.

Response to Reviewers: Responses to Editor and Reviewers’ comments on STOTEN-D-17-05504

Dear Prof Gan

Title page

Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils

Min Gou[1,2], Hang-Wei Hu[1,]*, Yu-Jing Zhang[1], Jun-Tao Wang[3], Helen Hayden[4], Yue-Qin Tang[2], Ji-Zheng He[1,3,]*

[1]*Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia*

[2]*College of Architecture and Environment, Sichuan University, Chengdu, Sichuan 610065, China*

[3]*State Key Laboratory of Urban and Regional Ecology, Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*

[4]*Department of Economic Development, Jobs, Transport and Resources, AgriBio, 5 Ring Rd, Bundoora, Victoria 3083, Australia*

*Author for correspondence:

Ji-Zheng He, Email: jizheng.he@unimelb.edu.au; or Hang-Wei Hu, Email: hang-wei.hu@unimelb.edu.au;

Abstract

Composting has been suggested as a potential strategy to eliminate antibiotic residues and pathogens in livestock manure before its application as an organic fertilizer in agro-ecosystems. However, the impacts of composting on antibiotic resistance genes (ARGs) in livestock manure and their temporal succession following the application of compost to land are not well understood. We examined how aerobic composting affected the resistome profiles of cattle manure, and by constructing laboratory microcosms we compared the effects of manure and compost application to agricultural soils on the temporal succession of a wide spectrum of ARGs. The high-throughput quantitative PCR array detected a total of 144 ARGs across all the soil, manure and compost samples, with Macrolide-Lincosamide-Streptogramin B, aminoglycoside, multidrug, tetracycline, and β-lactam resistance as the most dominant types. Composting significantly reduced the diversity and relative abundance of ARGs and mobile genetic elements (MGEs) in the cattle manure. In the 120-day microcosm incubation, the diversity and abundance of ARGs in manure-treated soils were significantly higher than those in compost-treated soils at the beginning of the experiment. The level of antibiotic resistance rapidly declined over time in all manure- and compost-treated soils, coupled with similar temporal patterns of manure- and compost-derived bacterial communities as revealed by SourceTracker analysis. The network analysis revealed more intensive interactions/associations among ARGs and MGEs in manure-treated soils than in compost-treated soils, suggesting that mobility potential of ARGs was lower in soils amended with compost. Our results provide evidence that aerobic composting of cattle manure may be an effective approach to mitigate the risk of antibiotic resistance propagation associated with land application of organic wastes.

Keywords

Antibiotic resistance genes; manure; aerobic composting; human health; resistome

1 Introduction

The increasing emergence and dissemination of antibiotic resistance genes (ARGs) in environmental settings represents a major threat to clinical infection treatment and human health (Berendonk et al., 2015). The use or abuse of antibiotics for disease control or growth promotion in livestock husbandry, which comprises more than 70% of global consumption of antibiotics, has been widely reported to select and enrich for antibiotic resistant bacteria in the gastrointestinal tracts of animals (Jechalke et al., 2014). Therefore, animal manure is recognized as a rich reservoir of antibiotic residues, antibiotic-resistance bacteria and ARGs (Zhu et al., 2013). Additionally, many studies have demonstrated that land application of animal manure without proper treatment can substantially increase the diversity and abundance of ARGs in agricultural soils (Heuer et al., 2011; Zhu et al., 2013; Hu et al., 2016a). Manure-derived bacteria and pathogens can persist at high abundance in soils with a survival time of several weeks to several months depending on the sources of manure and the history of antibiotic use (Heuer et al., 2008; Chee-Sanford et al., 2009; Leclercq et al., 2016). These ARGs may migrate into the food chain via transmission to the phyllosphere, endosphere and rhizosphere of plants grown in manured soils (Marti et al., 2013; Chen et al., 2017; Zhu et al., 2017); or may disseminate into a broader range of soil bacterial communities via horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs) such as integrons, transposons or plasmids (Jechalke et al., 2013; Kopmann et al., 2013; Blair et al., 2014; Gillings et al., 2015). Manure application may also enhance the abundance of resident soil ARGs through the facilitation of nutrients enabling the growth of their bacterial hosts (Udikovic-Kolic et al., 2014; Hu et al., 2016a). The emerging antibiotic resistance in agro-ecosystems due to land application of manure is causing global concern, and thus there is an imperative need to develop effective agronomic management options to mitigate the dissemination of antibiotic resistance in the environment (Pruden et al., 2013

There are generally three methods of application for livestock manure: direct land). application, anaerobic digestion for methane production, and aerobic composting. Manure composting is recognized as a common and effective way of reducing chemical and biological hazards in organic wastes prior to land application (Bernal et al., 2009; Xie et al., 2016). Previous studies have demonstrated that composting was effective for the degradation of antibiotic residues in beef cattle manure (Sura et al., 2015), however an intensive composting process only slightly reduced the degradation period for three antibiotics in horse and dairy cattle manure (Storteboom et al., 2007). Chlortetracycline, monensin and tylosin concentrations were reduced by 54-99%, but sulfamethazine concentrations were not reduced after 35 days of composting (Dolliver et al., 2008). Most pathogenic organisms and ARGs-bearing bacteria are rapidly killed by the high temperature from self-heating during thermophilic composting (Wang et al., 2017a), however, specific types of ARGs and MGEs may persist in the compost (Peng et al., 2015; Xie et al., 2016) and even significantly increase in abundance after traditional waste composting by aerobic digestion (Zhu et al., 2013; Su et al., 2015) and vermicomposting for swine manure (Wang et al., 2017b). The total abundance of tetracycline resistance genes was not significantly reduced by manure composting (Peng et al., 2015), though most of the sulfadiazine resistance genes became undetectable after the composting process (Selvam et al., 2012). Several field studies have also reported that some ARGs and MGEs increased in abundance when composted manure was applied to soils compared to inorganic fertilizer-treated or untreated soils (Zhu et al., 2013; Lin et al., 2016). Despite these limited reports on the efficacy of composting to reduce several types of well-studied ARGs, our understanding of the effect of composting on a wide range of ARGs in animal manure remains limited, and the fate of ARGs after land application of manure and the corresponding compost was rarely compared.

The aim of this study was to explore the fate of a wide spectrum of ARGs during aerobic composting of cattle manure, and to compare the effects of manure and compost application on the abundance, diversity and dynamics of these ARGs and manure/compost-associated bacteria over 4 months in laboratory microcosms incubated with agricultural soil. We used a high-throughput quantitative PCR (HT-qPCR) technique to profile 285 ARGs conferring potential resistance to eight major categories of antibiotics and 10 MGEs as indicators for the HGT potential of ARGs, and used Illumina Miseq sequencing of 16S rRNA gene amplicons to track the dynamics of bacterial community members. We hypothesized that: (i) aerobic composting can substantially reduce the levels of ARGs in cattle manure; (ii) compost application has less impacts on the ARG profiles in agricultural soils compared with manure application, and the compost-/manure-derived ARGs will diminish over time during the soil microcosm incubation; and (iii) dynamics of ARGs are associated with changes in bacterial communities, which have been recognized as an important determinant of ARG contents (Forsberg et al., 2014).

2 Materials and methods

2.1 Sampling of soil, manure, and compost

Soil samples used in this study were taken from an arable field (a vegetable farm planted with celery) at Clyde (38°07′S, 145°19′E), Victoria, Australia in 2016. The average annual precipitation at this site is 819 mm, and the average annual temperature is 19.4°C. The soil is classified as a Loamy Sand, and has a pH (H_2O) of 7.19. Total carbon and total nitrogen are 3.75% and 0.43%, respectively, as determined using a LECO macro-CN analyzer (LECO, St Joseph, MI, USA). Prior to the collection of soil samples only inorganic fertilizers had been applied to the field for more than two years. Surface soils (0-15 cm) were collected from the field, sieved through a 2.0 mm mesh, and transported to the laboratory on ice. Cattle manure and a compost derived from the same source of cattle manure were collected from a large-scale beef cattle feedlot in Charlton (36°23′S, 143°21′E), Victoria, Australia. The raw cattle manure was collected from the cattle feedlot pens within seven days of defecation, while the compost was collected from the top, middle, and bottom of composting stockpiles and thoroughly mixed. The basic properties of manure, compost and soil samples are shown in Table S1.

Aerobic composting was undertaken using low windrows constructed by forming cattle manure harvested from pens and sawdust added as a bulking agent into a long pile with a triangular cross-section, 1.5-2 m high and 3-4 m wide at the base. The composting process consisted of an active stage and a curing stage. The whole process lasting for several months with a thermophilic (active) phase (50–60°C) of several weeks provided there is sufficient nitrogen. The temperature and moisture content within the piles was monitored weekly. The compost piles were turned only after a minimum three consecutive days at high temperature (>55°C). The active phase was considered complete when the temperature within the piles was no longer able to reach >55°C after turning. After completion of the active phase, the compost was formed into a stockpile where it cured for several weeks. Curing is important since immature compost may contain high levels of organic acids, a high carbon: nitrogen ratio and other properties that can be detrimental to crops.

2.2 Soil microcosms and DNA extraction

Five sets of treatments with three replicates were established in 500 ml vials containing 20 g of soil (oven dry-weight equivalent): untreated soil samples (CK, control), low-level manure application (LM, 40 mg manure g^{-1} soil), high-level manure (HM, 80 mg manure g^{-1} soil), low-level compost (LC, 40 mg compost g^{-1} soil), and high-level compost (HC, 80 mg compost g^{-1} soil). The compost and manure applied to the vials as organic fertilizers were based upon oven dry-weight, and corresponded to amounts of 30 or 60 m^3 manure/compost ha^{-1} (~30 or 60 tons manure/compost ha^{-1}) in agronomic practices. Soil moisture contents were maintained at 60% of the water filled pore space through weekly replenishment of water. Aerobic conditions were maintained by opening the microcosms for 10 min every three days throughout the incubation to refresh the air. Soil microcosms were incubated at 25°C in the dark and destructively sampled at days 1, 15, 32, 60, 90, and 120. Genomic DNA was extracted from 0.25 g of soil, manure or compost samples using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and concentration of the extracted DNA were assessed using the NanoDrop ND2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA concentrations varied between 10-20 ng μl^{-1} for all the samples, and the A260/A280 ratios were greater than 1.8 for all DNA extracts.

2.3 HT-qPCR analysis of ARGs and MGEs

Quantification of ARGs and MGEs was conducted on the Wafergen Smart-Chip Real-Time System (Fremont, CA, USA) using thermal cycling conditions and reaction systems as described previously (Su et al., 2015; Hu et al., 2016b). The HT-qPCR array contained a total of 296 validated primer sets (Looft et al., 2012; Johnson et al., 2016), including 285 primer sets targeting eight major classes of antibiotic resistance, 10 primer sets targeting MGEs, and one 16S rRNA gene (Table S2). One negative control with no DNA template added was included in each HT-qPCR run to eliminate false positive detections. The criteria used for a positive detection of the ARGs were: (i) a threshold cycle value (C_T) of 31 was used as the detection limit to differentiate between positive amplification and primer-dimers (Su et al., 2015; Hu et al., 2017); (ii) all three technical replicates of each sample were above the detection limit; and (iii) amplicons with multiple melting curves were removed from the analysis. The $2^{-\Delta CT}$ method where $\Delta C_T = (C_{T\ \text{detected ARGs}} - C_{T\ \text{16S rRNA gene}})$ was used to calculate the relative abundances of ARGs and MGEs normalized to the 16S rRNA gene according to a comparative C_T method (Schmittgen and Livak, 2008).

2.4 Miseq sequencing of 16S rRNA gene

The V3-V4 regions of the bacterial 16S rRNA gene were amplified using the primer pair Bakt_341F/Bakt_805R (Herlemann et al., 2011) with Illumina adapter overhang sequences attached. The reaction was carried out in 25 μl mixtures containing 1.5 U of MyTaq DNA polymerase (Bioline), 0.5 μl of each primer (10 μM), 5 μl of reaction buffer, and 2 μl of template DNA. The resultant PCR products were purified using Agencourt

AMPure XP beads (Beckman Coulter, Beverly, MA, USA), linked with dual indices and Illumina sequencing adapters using the Nextera XT index kit (Illumina Inc., San Diego, CA, USA), and purified again using Agencourt AMPure XP beads. The final library made by mixing all PCR products in equimolar ratios was quantified using the JetSeq library quantification Lo-ROX Kit (Bioline), and sequenced on an Illumina MiSeq sequencer.

2.5 Processing of 16S rRNA gene amplicon sequences

Raw sequences obtained from the paired-end sequencing were quality filtered to remove low-quality reads and ambiguous nucleotides, assembled using the Fast Length Adjustment of Short Reads (FLASH) (Magoè and Salzberg, 2011), de-multiplexed, and assigned to individual samples using Quantitative Insights Into Microbial Ecology (QIIME) analysis (Caporaso *et al.*, 2010). Assembled sequences were classified into operational taxonomic units (OTUs) at the 97% sequence similarity using a chimera filtering pipeline UPARSE (Edgar, 2013). The same number of sequences (11,862 sequences per sample) was randomly selected from each sample to compensate for variability in sequencing depth before downstream analyses. Taxonomic identity was assigned to OTUs using Greengenes as the reference database (DeSantis *et al.*, 2006). The temporal changes of the microbial communities were investigated using non-metric multidimensional scaling (NMDS) ordination based on the weighted UniFrac distances (Lozupone and Knight, 2005) and Bray-Curtis distances computed between all samples.

2.6 Manure and compost-associated community analyses

We used SourceTracker to calculate the contribution of soil, compost and manure microbial communities within each microcosm following the procedures as described previously (Knights et al., 2011; Leclercq et al., 2016). SourceTracker is a Bayesian approach to estimate the most probable proportion of user-defined “source” microbial communities in a given “sink” community. In this study, the raw cattle manure, compost and untreated soil samples at day 0 were defined as manure, compost and soil “sources”, respectively, and manure- and compost-treated soil samples at all sampling points as “sinks”. OTUs present in only one sample were removed prior to the analysis, and the algorithm was run with default parameters. We identified bacteria taxa that were enriched in manure-/compost- treated soils compared to the untreated soils on day 0 by performing Mann-Whitney tests at a significance level of 0.05.

2.7 Network analysis and visualization

The co-occurrence/interaction patterns among the detected ARGs and MGEs as well as between ARGs and microbial taxa were explored in network analysis using the CoNet Cytoscape plug-in (Faust et al., 2012) as described previously (Li et al., 2015; Hu et al., 2017). Briefly, the pairwise correlation scores were calculated using two dissimilarity methods (Bray-Curtis and Kullback-Leibler) and two correlation methods (Spearman and Pearson), and the *P*-values (<0.05) were merged using the Brown method. The Benjamini-Hochberg multiple test was performed to control the expected number of false positives among all significant relationships. The chance of falsely rejecting the null hypothesis was 0.05. The resultant pairwise correlations were visualized in Cytoscape plug-in Network Analyzer (Assenov et al., 2008), and network topology was explored using the Frucherman Reingold algorithm in Gephi (Bastian *et al.* 2009). Only correlations with a correlation coefficient (ρ-value) above 0.6 and a significance level (*P*-value) below 0.05 were displayed in the networks (Junker and Schreiber, 2008).

2.8 Statistical analysis

One-way analysis of variance (ANOVA) was performed to test the difference in the diversity and relative abundance of ARGs and MGEs across different treatments in SPSS 20 (IBM, Armonk, NY, USA). The data were normalized using log-transformation when necessary prior to statistical analysis. Spearman's rank correlation was performed to test the relationships between the relative abundance of ARGs and MGEs across all the samples. A heat map visualization of the relative abundances of individual ARG subtypes was generated using the "gplots" package in the R platform (version 3.3.1) (R Development Core Team, 2008). The changes of ARG compositions across different treatments were visualized by NMDS ordinations based on the Bray-Curtis dissimilarity distances using the "vegan" package with 999 permutations in R. A Mantel test was performed to analyse the relationships between ARGs and bacterial communities based on Bray-Curtis dissimilarity matrices using "vegan" in R. The threshold for significance was $P < 0.05$.

3 Results

3.1 Diversity and relative abundance of ARGs and MGEs in manure, compost and soil

The HT-qPCR detected a total of 144 unique ARGs and 10 MGEs across all the soil, manure and compost samples. The detected ARGs can encode potential resistance to eight major classes of antibiotics, with MLSB, aminoglycoside, multidrug, tetracycline, and β-lactam resistance as the most frequently encountered types (Fig. 1a). These ARGs represented all three resistance mechanisms: antibiotic deactivation (43%), efflux pump (34%) and cellular protection (20%) (Fig. S1). Aerobic composting significantly reduced the average number of ARGs detected from 103.3 in raw manure to 71.3 in mature compost, which was comparable to 70.0 in untreated agricultural soils (Fig. 1b). The relative abundance of ARGs in manure was approximately five times higher than that in compost, but the compost samples still harboured a significantly higher abundance of ARGs than the untreated soils (Fig. 1c). A similar pattern was found for MGEs, with an average number of 8.7 and 6.3 MGEs detected in manure and compost, respectively, while the untreated soil harboured averagely 4.0 MGEs (Fig. 1d). The relative abundance of MGEs in manure was significantly higher than that in the compost and untreated soils (Fig. 1e).

3.2 Temporal changes of ARGs and MGEs following application of manure/compost to an agricultural soil

Laboratory soil microcosms were destructively sampled at six time points (days 1, 15, 32, 60, 90, and 120) to explore the time-course succession of ARGs and MGEs in agricultural soils treated with different levels of manure or compost (Fig. 2). The numbers of ARGs detected in manure-treated soils were significantly higher than those in compost-treated soils at the beginning of the experiment, and they all gradually declined over time to reach the same background level as in the untreated soils by the end of the incubation (day 120) (Fig. 2a). The application of manure significantly increased the relative abundance of ARGs in both HM and LM treatments at day 1, which rapidly decreased to the background levels by day 60, while compost application only slightly increased the relative abundance of ARGs and also returned to the background levels by day 32 (Fig. 2c). The diversity and relative abundance of ARGs in untreated soils remained largely unchanged throughout the course of the experiment (Figs. 2a and 2c). A similar temporal pattern was found for the diversity and relative abundance of MGEs across different treatments in the microcosm incubation (Figs. 2b and 2d).

Spearman’s correlation analysis revealed that the relative abundance of total ARGs had significantly positive relationships with that of total MGEs ($P < 0.001$), total integrase genes ($P < 0.001$) and total transposase genes ($P < 0.001$) (Fig. 3). The relative abundance of MGEs, integrase and transposase genes was also significantly and positively correlated with those of specific categories of ARGs, except the β-lactam and vancomycin genes (Fig. 3).

3.3 Temporal changes of individual subtypes of ARGs and MGEs

We further explored the temporal succession of the relative abundance of specific subtypes of ARGs and MGEs during the soil microcosm incubation (Fig. 4). The profiles of ARGs in compost tended to be very different to those of untreated soils, with soil samples more enriched in β-lactam, tetracycline, vancomycin, and multidrug resistance while compost samples were more enriched in aminoglycoside and sulphonamide resistance. The untreated soil samples and the compost-amended soil samples exhibited very similar ARGs and MGEs patterns, which remained largely stable during the incubation. Compared with untreated soil and compost samples, manure samples were distinctly more enriched in almost all the major categories of ARGs and MGEs. Manure amendment resulted in obvious increases in the relative abundances of a majority of ARGs and MGEs at days 1 and 15, which then rapidly decreased over time and tended to approach the background levels in untreated soils by the end of the incubation. These results were further supported by the NMDS ordination based on the Bray-Curtis dissimilarity matrices (Fig. 5) which revealed that: (i) compost and manure samples were clearly separated from all other samples, and also clustered separately from each other; (ii) the untreated soil samples and the compost-treated soil samples at all sampling points clustered together with the manure-treated soils collected after day 15; (iii) no clear differences were observed in soils treated with low and high levels of manure or compost.

3.4 Dynamics of bacterial communities in soil microcosms

We performed Miseq sequencing of 16S rRNA gene amplicons to investigate the dynamics of manure- and compost-derived bacteria in an agricultural soil and the effects of manure and compost application on the soil bacterial community compositions in soil microcosms. A total of 3,670,764 high-quality sequences were obtained from all samples. The alpha-diversity measures in manure and compost samples including observed species (343 and 361, respectively) and phylogenetic diversity metrics (PD whole tree; 55.4 and 58.8, respectively) were significantly lower than those in untreated soil samples (2046 for observed species and 74.6 for PD whole tree). Rarefaction curves of OTUs at 11,862 reads per sample revealed that the alpha diversity based on PD whole tree analysis approached a plateau at this sequencing depth (Fig. S2).

The manure and compost samples were dominated by the phyla Proteobacteria (43.3% vs. 43.9%), Bacteroidetes (27.6% vs. 37.7%), Actinobacteria (16.2% vs. 14.1%) and Firmicutes (12.0% vs. 3.0%), while the initial bacterial communities in untreated soil samples (at day 1) were dominated by Proteobacteria (30.1%), Actinobacteria (22.1%), Acidobacteria (10.7%), Chloroflexi (9.7%) and Firmicutes (7.2%) (Fig. 6a). The bacterial community compositions in manure- and compost-treated soils at day 1 were similar to those in manure and compost samples respectively, and gradually shifted over time during the incubation to become more similar to those of the untreated soil samples than to manure/compost samples after day 15. The NMDS ordination of bacterial communities based on the weighted UniFrac and Bray-Curtis distances between samples revealed that, similar to the ARG patterns, manure and compost samples together with manure-treated soil samples at days 1 and 15 were separate from all other samples (Figs. S3 and S4). Mantel test analysis demonstrated that bacterial community compositions were significantly correlated with ARG profiles on the basis of Bray-Curtis dissimilarity metrics ($R = 0.634$, $P < 0.001$). Furthermore, the network analysis revealed strong and significant co-occurrence patterns between ARGs and microbial taxa (Fig. S5). The bacterial phyla FCPU426, Bacteroidetes, Acidobacteria, Gemmatimonadetes, Parvarchaeota, and Chloroflexi were the most prevalent predicted taxa that were intensively correlated with multiple ARGs conferring resistance to different classes of antibiotics.

3.5 Fate of manure- and compost-derived bacterial communities in soil microcosms

SourceTracker analysis was further performed to explore the fate of manure- and compost-derived bacteria in soil microcosms. According to this analysis, manure addition accounted for 52.9-68.6% and 73.2-76.8% of the bacterial communities in the LM and HM treatments at day 1, respectively, while compost addition was responsible for 30.0-43.5% and 42.9-60.1% of the bacterial communities in the LC and HC treatments at day 1, respectively (Fig. 6b). The remaining proportions were ascribed to “soil source” and “unknown source”. The unknown source likely represented a subset of the soil resident bacterial communities that were not sampled in the untreated soils at day 1. The proportion of compost-derived bacteria decreased continually to zero after 15 and 32 days in the LC and HC treatments respectively. The proportion of manure-derived bacteria also rapidly decreased within 32 days, but still consisted of 12.1-16.7% of the bacterial communities in the LM and HM treatments at the end of the incubation.

3.6 Co-occurrence patterns of ARGs and MGEs

The co-occurrence patterns among ARGs and MGEs subtypes in untreated, manure-treated, and compost-treated soils were further visualized as association networks (Fig. 7). Strong co-occurring correlations were observed in the ARGs-MGEs networks, which consisted of 37 nodes and 32 edges in untreated soils (Fig. 7a), compared to 27 nodes and 35 edges in compost-treated soils (Fig. 7b) and 58 nodes and 346 edges in manure-treated soils (Fig. 7c). The entire ARGs-MGEs networks across the three treatments can be clearly separated into six major modules. Interestingly, in the network of compost-treated soils (Fig. 7b), the MGEs *tnpA-02* and *intl1* genes in Module II, *intl* gene in Module III, and *tnpA-05* gene in Module IV had most intensive connections with ARGs, which can potentially confer resistance to multiple classes of antibiotics. In the network of manure-treated soils (Fig. 7c), the MGEs *tnpA-03* and *Tp614* genes in Module I and *tnpA-02* and *intl1* genes in Module IV also correlated with a variety of ARG subtypes. By contrast, not a single MGE was found in the network of untreated soils, though there were intensive associations among the detected ARGs from different categories (Fig. 7a).

4 Discussion

4.1 Composting reduced the diversity and abundance of ARGs in cattle manure

Composting can reduce the abundance of antibiotic residues and pathogenic organisms, but there is limited information on the effects of composting on the diversity and composition of ARG profiles in manure. Recently, metagenomics and metatranscriptomic analyses revealed that composting markedly reduced the aggregated expression level of the manure resistome, but a varied transcriptional response of different ARGs was observed (Wang et al., 2017a). For example, composting can significantly reduce the expression of tetracycline resistance genes but had no effect on the expression of sulfonamide and fluoroquinolone resistance genes (Wang et al., 2017a). Similarly, another study reported that vermicomposting of swine manure using housefly larvae significantly reduced the abundance of 94 out of 158 ARGs, but significantly enriched 23 ARGs by 3.9-fold (Wang et al., 2017b). By using the HT-qPCR array, a key finding of this study is that aerobic composting under field conditions can significantly reduce the diversity and relative abundance of all major classes of ARGs in the cattle manure tested, and we did not observe a significant increase in abundance of any ARGs after composting (Figs. 1 and 4). In agreement with previous screenings of ARGs in Chinese swine farms (Zhu et al., 2013), commercial composts (Xie et al., 2016), and antibiotic-free cattle manure (Hu et al., 2016a), the genes conferring potential resistance to Macrolide-Lincosamide-Streptogramin B, aminoglycoside, multidrug, tetracycline, and β-lactam were the major ARG types detected in our tested manure and compost samples. Our findings contribute to the growing body of evidence that livestock manure is a significant reservoir of ARGs, and highlight that aerobic composting may be an effective approach to reduce the level of antibiotic resistance in the environment. Given that organic farming is becoming increasingly popular all over the world, effective composting is required to eliminate ARGs in all types of livestock manures to reduce the risk of the environmental ARG dissemination before manures are applied to the field as organic fertilizers.

The significant reduction in ARGs in composted manure might be attributed to dilution by the addition of sawdust and/or the decrease of ARG-harbouring bacteria during the thermophilic composting process, which is known to kill the majority of bacteria in animal manure (Bernal et al., 2009). The majority of bacterial communities in animal manure are thought to be coliforms adapted to the anaerobic conditions, which cannot tolerate high temperatures or adapt to the composting conditions (Leclercq et al., 2016). In this study three phyla Firmicutes, Bacteroidetes and Chloroflexi known to be important components of the gut microbiome of cattle (Shoaie et al., 2013) were found to be significantly reduced in abundance after composting compared to manure samples (Fig. 6a). These bacteria phyla have been recognized through metagenomics analyses of dairy cow manure to be important hosts carrying diverse ARGs (Wichmann et al., 2014). The reduction in manure-derived bacterial communities that probably carry ARGs may explain the observed decline of a wide variety of ARGs during the composting process. Although the number of detected ARGs in compost is similar to that of untreated agricultural soils, the abundance of ARGs in compost is still significantly higher than in untreated soils (Fig. 1). The presence of persistent ARGs and even some attenuated ARGs (e.g. *sul2* gene belonging to the sulfonamide resistance) in compost poses a potential threat to the environment through the dispersal of ARGs when compost is applied to agro-ecosystems. Therefore, we argue that further improvement is required in aerobic composting to eliminate the level of antibiotic resistance prior to the application of compost to land, and that further studies are required to get a comprehensive understanding of the effects of composting on the ARG profiles in other types of animal manures (e.g. poultry and swine manures).

4.2 Composting reduced the risk potential of ARG dissemination in agricultural soils

Land application of untreated animal manure, as a common agronomic practice, has been reported to introduce the inflow of a large amount of manure-derived microbiome including antibiotic resistant bacteria and potential human pathogens into the soil environment (Chee-Sanford et al., 2009; Heuer et al., 2011; Hu et al., 2016a). Functional metagenomic analysis revealed that manure-derived ARGs can account for up to 70% of the total soil ARGs following manure application (Su et al., 2014). Although composting has been suggested as a potential strategy to reduce the risk of ARG dissemination associated with organic fertilizer application, few studies have systematically compared the temporal patterns of soil ARGs following application of manure and the corresponding compost. In this study, we found that composting had significantly reduced the diversity and abundance of ARGs in cattle manure, however the ARG abundance in compost was still significantly higher than that of untreated soils (Fig. 1). Therefore, it is not surprising to observe that the abundance of soil ARGs was dramatically increased at the beginning of the experiment upon the application of manure and compost as a number of manure-/compost-derived ARGs were introduced into the soil (Fig. 2). Compost application had less impacts on soil ARG patterns compared to manure application, but still significantly increased soil ARG diversity and abundances, in particular, the abundances of aminoglycoside and sulfonamide resistance genes (Fig. 4). Therefore, cattle manure and compost might manipulate the soil resistome in varying magnitudes with compost application possibly having minor consequences for soil and public health.

Despite the different ARG profiles in soils treated with manure and compost, the diversity and abundance of ARGs in all treated soils gradually decreased over time (Fig. 2). Notably, the abundance of ARGs in manure-amended soils dramatically declined to the background levels of ARGs in untreated soils within 60 days, whereas ARGs in compost-treated soils approached the background levels within 32 days. A similar temporal pattern could be observed for the abundance of MGEs in both manure- and compost-treated soils. Therefore, these ARGs during the early period of application might have the highest potential to be captured by human pathogens and pose a potential threat to human health if vegetables are harvested during this period. Our findings have implications for agronomic practice from the perspective of minimizing the dispersal risk of antibiotic resistance: raw cattle manure might not be applied to the field two months before the vegetable harvest, but compost may be used one month before the harvest. It is highly recommended that raw cattle manure needs to be properly treated by composting procedures to reduce the levels of ARGs before land application.

The time-course reduction of ARGs following manure application may be explained by the gradual out-competition of manure-derived bacteria by the soil indigenous microbiomes, and the different conditions between animal gut and soil environments (Chee-Sanford et al., 2009; Hu et al., 2016a). Consistent with the temporal patterns of ARGs and MGEs, the SourceTracker analysis revealed that manure- and compost-derived bacteria gradually diminish over time (Fig. 6). Compost-derived bacteria cannot survive in the soil environment and reduced to zero within 32 days of incubation, and therefore would not contribute to ARG persistence in soils (Fig. 6). By comparison, manure-derived bacteria rapidly decreased within 60 days of incubation, but a minor proportion of them persisted until the end of the incubation, suggesting that manure-derived ARGs might still persist in the soil (Fig. 6). The composition of ARGs was significantly correlated to those of bacteria as revealed by Mantel tests and network analysis (Fig. S5). These findings led to the conclusion that bacterial communities are important predictors of the dynamics of the ARG contents of manure- and compost-treated soils. Previous studies have reported that microbial phylogenetic and taxonomic structure is an important determinant of ARG composition (Forsberg *et al.*, 2014; Su *et al.*, 2015; Hu *et al.*, 2016b; Wang et al., 2017), because microbes are hosts to many ARGs. For example, Actinobacteria are a well-known group of bacteria producing antibiotics, and Proteobacteria and Actinobacteria are among the most prevalent predicted hosts of multi-resistant ARGs in metagenomics studies of soils (Forsberg *et al.*, 2014). Members of the gut-associated genus *Clostridium*, and environmental species of the genera *Acinetobacter* and *Pseudomonas* are responsible for the persistence of ARGs in manure-treated soils (Leclercq et al., 2016). The strong impact of microbial community structure on ARG contents might be also attributed to a low rate of HGT which has not decoupled ARG contents and bacterial phylogeny in manure- and compost-treated soils during the incubation.

4.3 The HGT potential of ARGs in manure- and compost-treated soils

The enhanced diversity and abundance of ARGs and MGEs in both manure- and compost-treated soils suggested that manure/compost application might increase the likelihood of these ARGs being transferred to other environmental bacteria and pathogens. The class 1 integron-integrase genes and the transposon-transposase genes were detected in all the manure- and compost-treated soils. Class 1 integrons are widely distributed in over 70% of Gram-negative bacteria of clinical importance (Stalder *et al.*, 2012), and have been repeatedly reported to be an important proxy for acquisition and dissemination of multiple ARGs in circular cassettes in the environment (Gillings *et al*., 2015). Integrons have a site recombination system which could capture and express gene cassettes, and they are often located on plasmids and transposons which further facilitate their HGT potential (Heuer et al., 2012). We found that the relative abundances of MGEs were positively correlated with those of total ARGs and most individual classes of ARGs (Fig. 3). In the network analysis, we found that the MGEs had intensive connections with ARG subtypes, and co-occurred with multiple ARGs conferring resistance to all major classes of antibiotics (Fig. 7). Some of these

ARG subtypes have been reported to be located within integron gene cassettes, such as the *aac* and *aad* families of ARGs (Zhu *et al.*, 2017). The co-occurrence of ARGs and MGEs suggest that these ARGs might be genetically linked together in integrons, transposons and microbial genomes conferring resistance to multiple antibiotics, and likely subject to a synchronous dispersal non-pathogens, pathogens, and even distantly related organisms through HGT.

We also found that there were significantly less interactions/associations in the network of compost-treated soils than that of manure-treated soils (Fig. 7), suggesting that composting might negatively influence the co-occurrence patterns of ARGs and MGEs, and therefore there might be a lower frequency of HGT for ARGs in compost-treated soils. Despite the genetic potential of HGT, the actual frequencies of HGT for environmental ARGs are also influenced by a range of abiotic (e.g. soil pH, soil types, water content, and temperature) and biotic factors (e.g. commensal, antagonistic and mutualistic relationships between soil organisms) in the environment (Aminov, 2011). In fact, it is assumed that the rate of HGT is very low in soils, where ARGs content and phylogenetic compositions are strongly associated (Forsberg *et al.*, 2014) suggesting that HGT might not be frequent enough to effectively decouple resistomes from phylogeny.

5 Conclusions

In summary, by combining HT-qPCR arrays with a composting trial in the field and laboratory soil microcosm incubations, we provide evidence that (i) aerobic composting can significantly reduce the diversity and abundance of the resistome in the tested cattle manure; (ii) compared with manure application, compost amendment may have less impacts on the dynamics of ARGs in soils, suggesting that composting may reduce the risk of ARG dispersal to plants grown in the field; and (iii) the co-occurrence pattern of ARGs and MGEs was negatively affected by composting, indicative of lower HGT potential of ARGs in compost-treated soils than in manure-treated soils. Our findings have important implications for understanding the impacts of agronomic practices on the propagation of ARGs in environmental systems, and necessitate future studies to (i) examine the effects of composting on the resistome profiles in diverse types of animal manures and (ii) how to improve composting procedures to further minimise the level of antibiotic resistance in manure and the subsequent dissemination of environmental ARGs into the food chain.

Acknowledgement

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Figure legends

Fig. 1 Comparison of the major classes of ARGs and MGEs among the manure, compost and untreated soil samples (a). The number of unique ARGs (b) and MGEs (d) and the relative abundances of ARGs (c) and MGEs (e) in the manure, compost and untreated soil samples. Error bars indicate standard errors (n = 3). Different letters above the bars indicate a significant difference for ARGs and MGEs. (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance genes)

Fig. 2 Dynamics of the number of unique ARGs (a) and MGEs (b) and the relative abundances of ARGs (c) and MGEs (d) over the time-course of the microcosm experiment. Error bars indicate standard errors (n = 3).

Fig. 3 Correlation matrices of the relative abundances of ARGs and MGEs across all the treatments as revealed by Spearman's rank correlation analysis. The pie plots represent correlation coefficients and only statistically-significant relationships ($P < 0.05$) are shown. (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance genes)

Fig. 4 The heat map showing the dynamics of ARGs and MGEs across the treatments over time-course of the microcosm experiment. Each row represents the results from a specific type of ARG/MGE primer set, and three columns at the sampling time point represent three replicates. The plotted values are the natural logarithm transformed proportion of each ARG/MGE to the bacterial 16S rRNA gene abundance. (Abbreviations: MLSB, Macrolide-Lincosamide-Streptogramin B resistance genes)

Fig. 5 The non-metric multidimensional scaling (NMDS) ordination plot showing the Bray-Curtis dissimilarity matrices between all the samples based on the relative abundance of ARGs/MGEs over the time course of the soil microcosms. The 2D stress value of 0.11, indicating that the ordination can well represent the data.

Fig. 6 Dynamics of the bacterial community compositions at the phylum level over the time-course of the microcosm experiment (a). Relative contribution of the manure- and compost-derived, soil and unknown sources of bacterial communities over the time course of the soil microcosms estimated using SourceTracker analysis (b).

Fig. 7 The network analysis showing the co-occurrence patterns among the detected ARG and MGE subtypes in the untreated soils (a), compost-treated soils (b), and manure-treated soils (c). The nodes with different colours represent different modularity classes, and the edges correspond to strong ($\rho > 0.6$) and significant ($P < 0.05$) correlations between nodes. The size of each node is proportional to the number of significant correlations between nodes.

Figure 1

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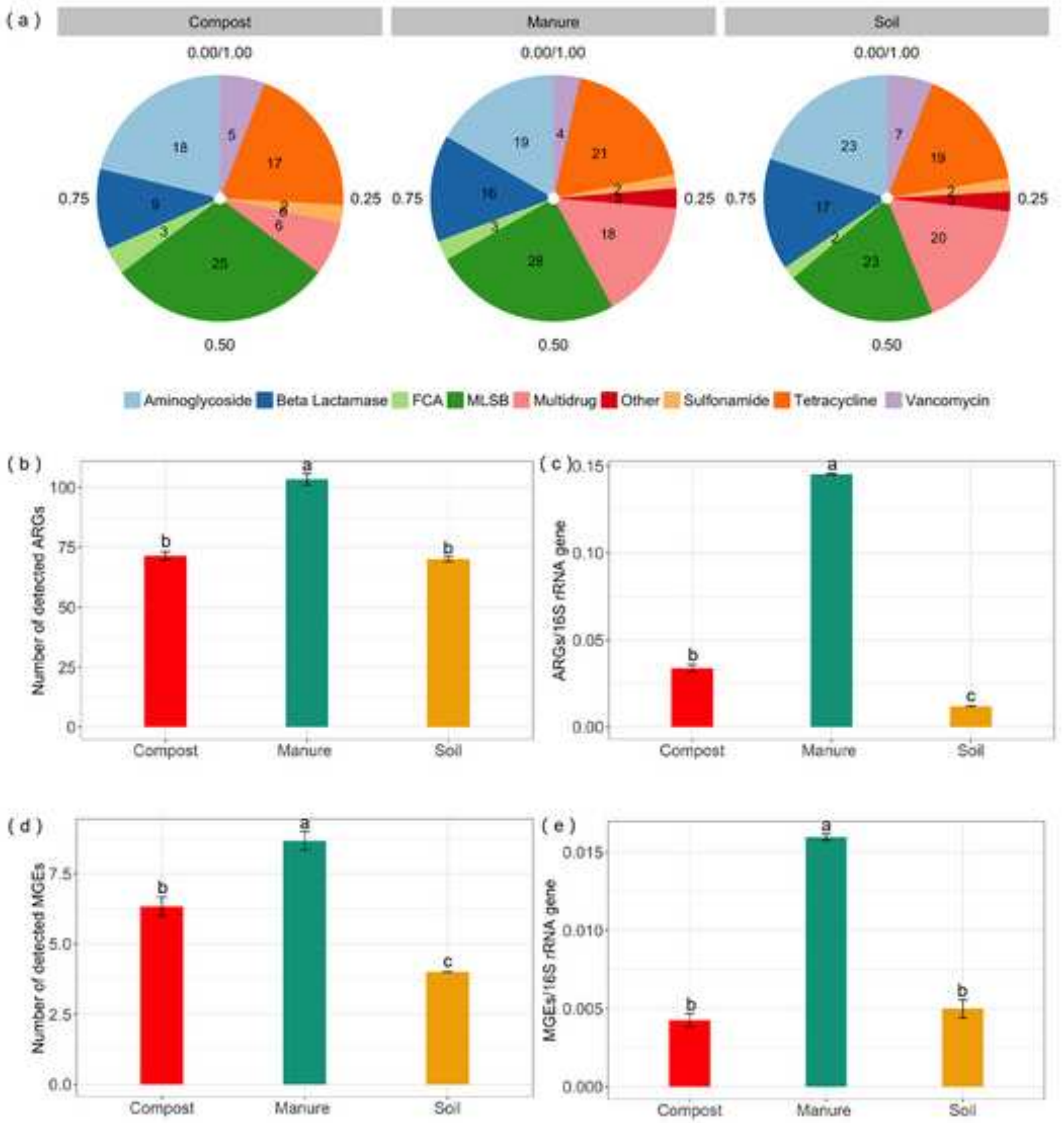

Figure 2

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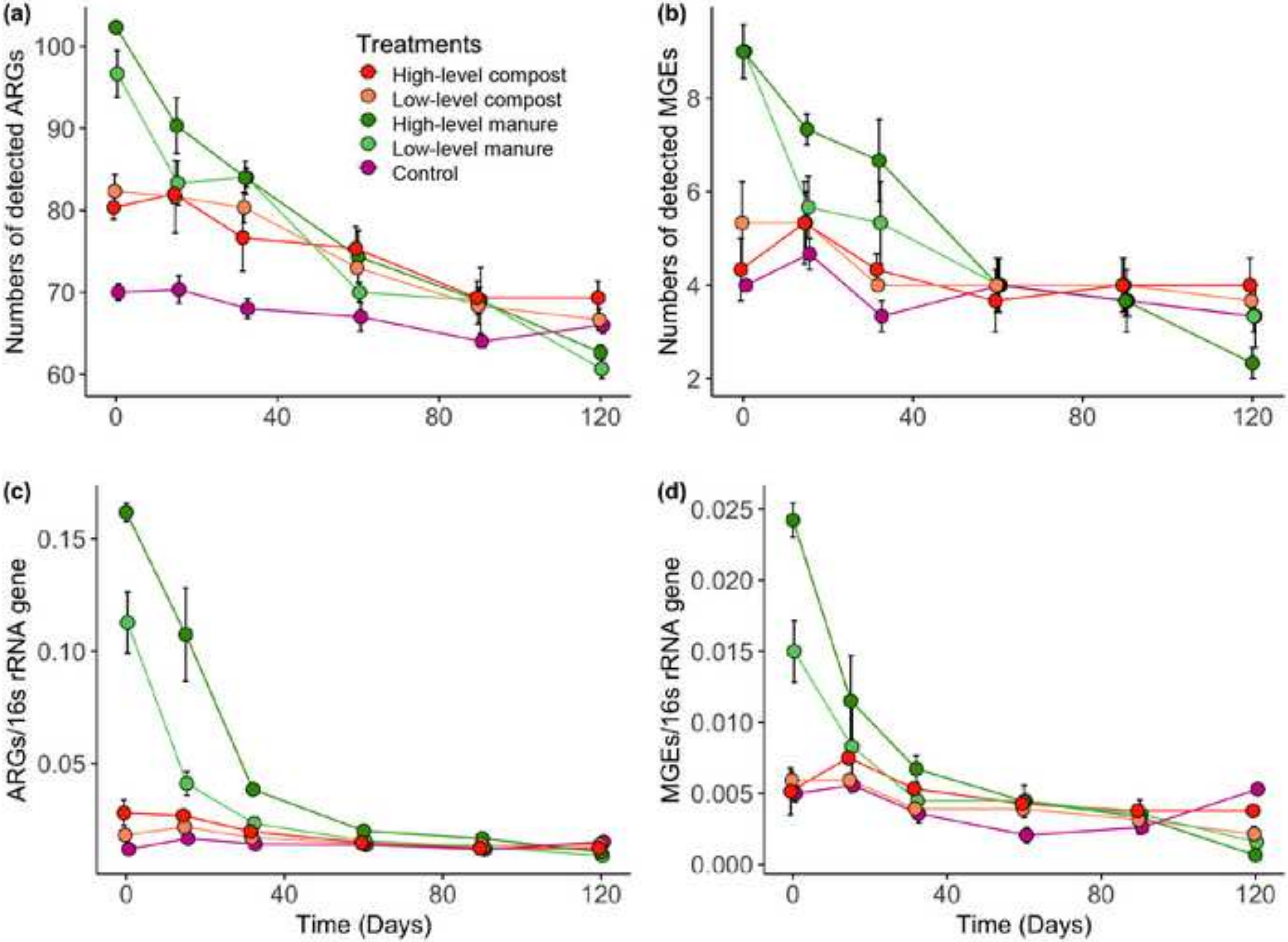

Figure 3

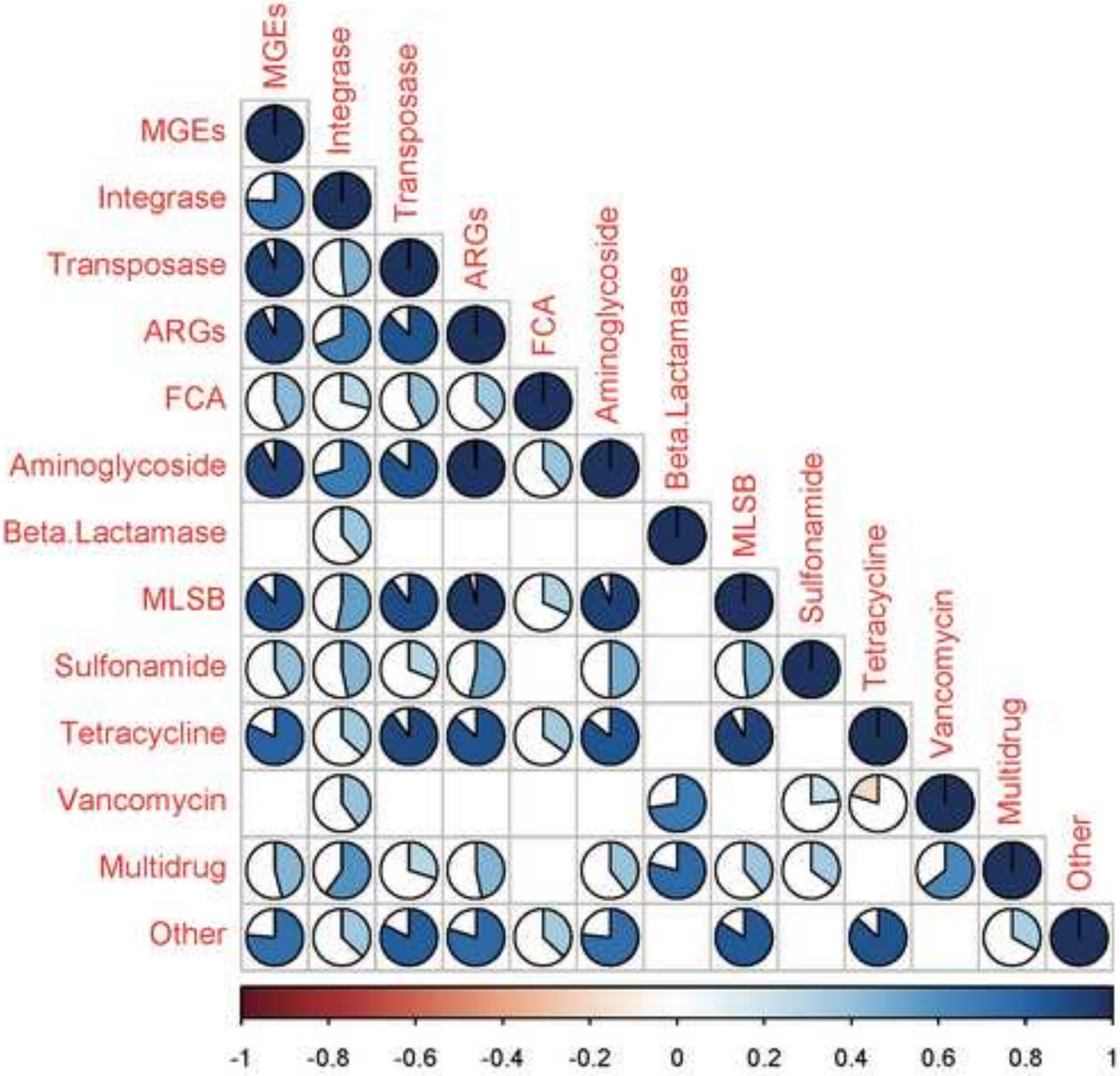
MGEs
Integrase
Transposase
ARGs
FCA
Aminoglycoside
Beta.Lactamase
MLSB
Sulfonamide
Tetracycline
Vancomycin
Multidrug
Other
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 4


MGEs
Aminoglycoside
β- lactamase
MLSB
Sulfonamide
Tetracycline
Vancomycin
Multidrug
Other
Relative Abundance
-10
-8
-6
-4
-2
0
Day 1
Day 15
Day 32
Day 60
Day 90
Day 120
CK
Day 1
Day 15
Day 32
Day 60
Day 90
Day 120
LC
Day 1
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Day 32
Day 60
Day 90
Day 120
HC
Compost
Day 1
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LM
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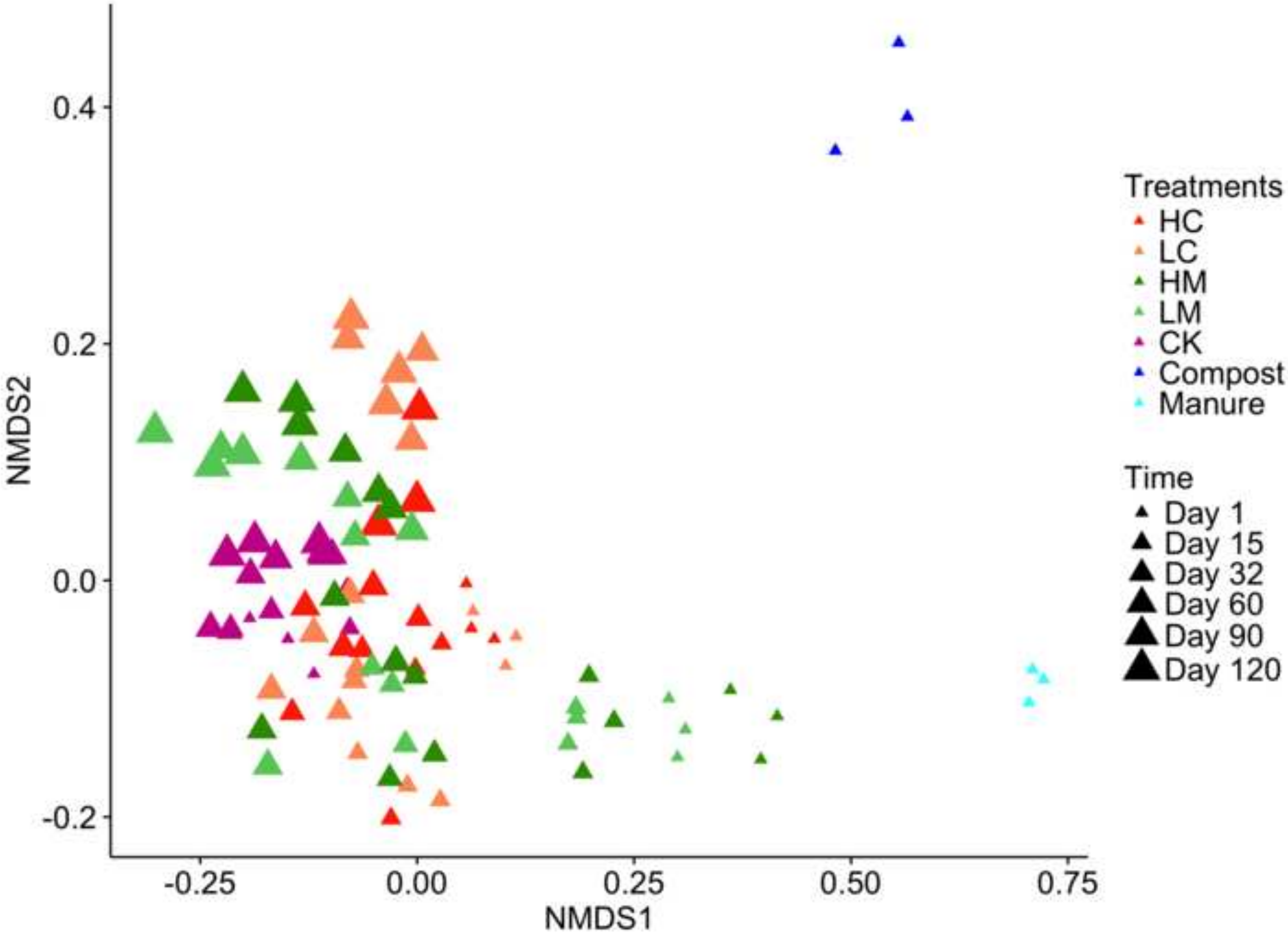
Manure

Figure 5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Treatments
HC
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Compost
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Time
Day 1
Day 15
Day 32
Day 60
Day 90
Day 120

Figure 6

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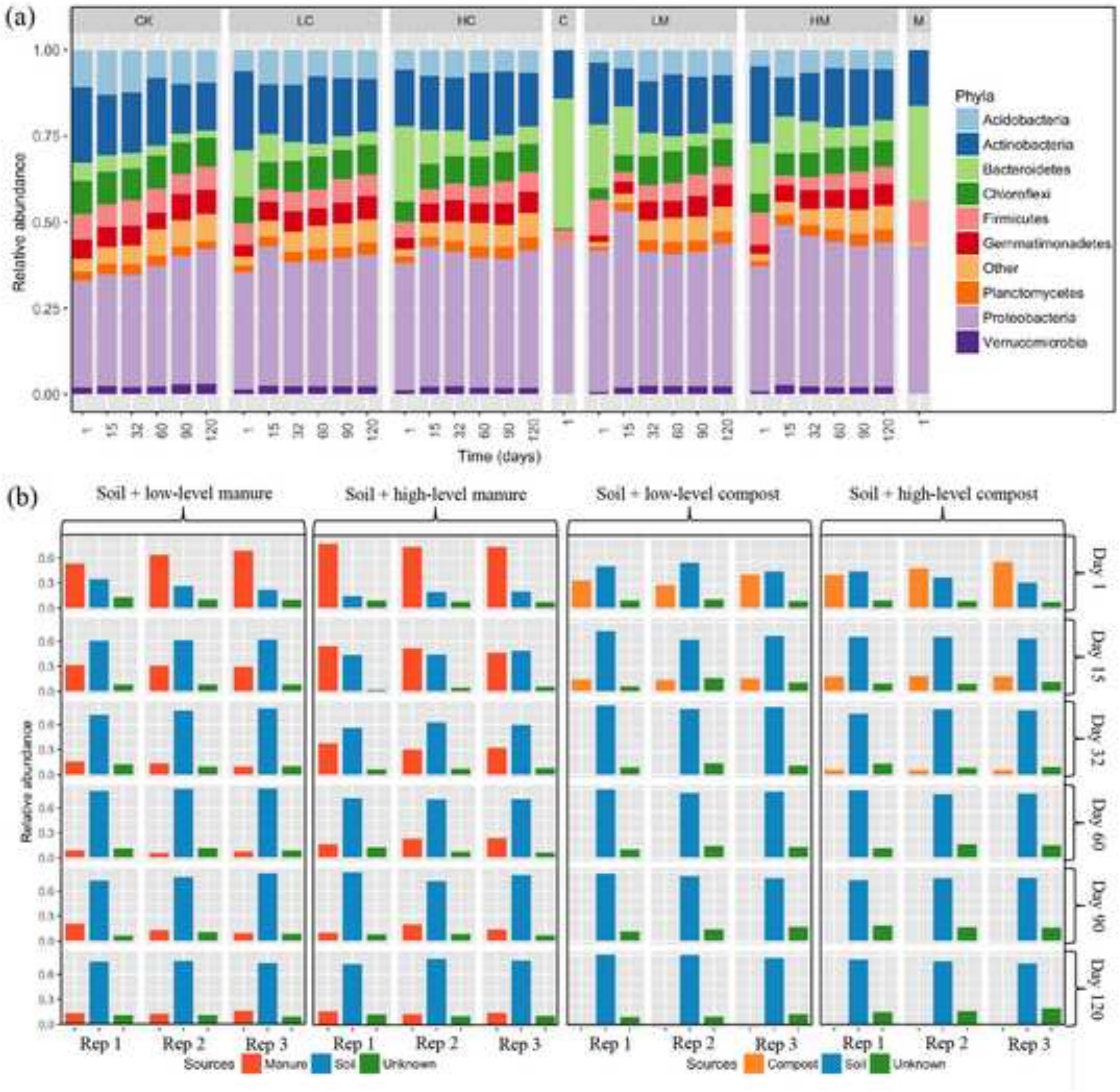
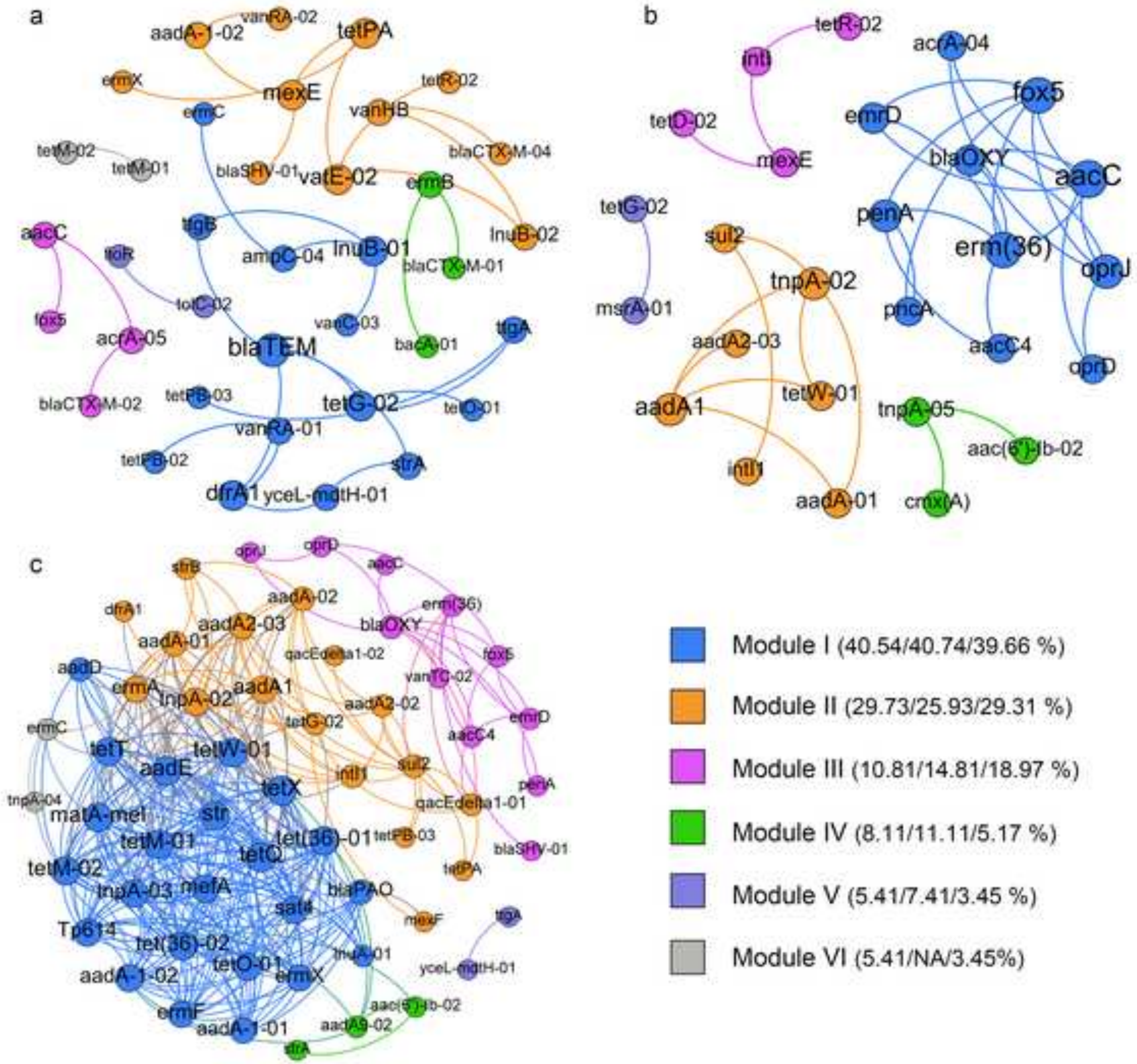


Figure 7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aadA-1-02
vanRA-02
tetPA
ermX
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tetM-02
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vatE-02
blaCTX-M-04
ermB
aacC
tetB
lnuB-02
tolR
ampC-04
lnuB-01
blaCTX-M-01
fox5
tolC-02
vanC-03
acrA-05
blaTEM
bacA-01
tetA
blaCTX-M-02
tetPB-03
tetG-02
tetO-01
vanRA-01
tetPB-02
strA
dfrA1
yceL-mdtH-01
b
tetR-02
intI
acrA-04
fox5
tetO-02
emrD
mexE
blaOXY
aacC
tetG-02
penA
sul2
erm(36)
oprJ
tnpA-02
msrA-01
pncA
aadA2-03
aacC4
oprD
aadA1
tetW-01
tnpA-05
aac(6')-Ib-02
intI1
aadA-01
cmx(A)
c
oprJ
oprD
strB
aacC
aadA-02
dfrA1
erm(36)
aadA2-03
blaOXY
aadA-01
qacEdelta1-02
fox5
aadD
ermA
tnpA-02
aadA1
vanTC-02
aadA2-02
emrD
ermC
tetG-02
aacC4
tetT
tetW-01
aadE
intI1
sul2
penA
tetX
tnpA-04
qacEdelta1-01
matA-mel
str
tetM-01
tet(36)-01
tetPB-03
blaSHV-01
tetM-02
tetQ
tetPA
tnpA-03
mefA
blaPAO
sat4
mexF
tetA
Tp614
lnuA-01
tet(36)-02
yceL-mdtH-01
tetO-01
aadA-1-02
ermX
aac(6')-Ib-02
ermF
aadA9-02
aadA-1-01
strA
Module I (40.54/40.74/39.66 %)
Module II (29.73/25.93/29.31 %)
Module III (10.81/14.81/18.97 %)
Module IV (8.11/11.11/5.17 %)
Module V (5.41/7.41/3.45 %)
Module VI (5.41/NA/3.45%)