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Effect of antibiotic use and composting on antibiotic resistance gene abundance and resistome risks of soils receiving manure-derived amendments

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ABSTRACT

Manure-derived amendments are commonly applied to soil, raising questions about whether antibiotic use in livestock could influence the soil resistome (collective antibiotic resistance genes (ARGs)) and ultimately contribute to the spread of antibiotic resistance to humans during food production. Here, we examined the metagenomes of soils amended with raw or composted manure generated from dairy cows administered pirlimycin and cephapirin (antibiotic) or no antibiotics (control) relative to unamended soils. Initial amendment (Day 1) with manure or compost significantly increased the diversity (richness) of ARGs in soils ($p < 0.01$) and resulted in distinct abundances of individual ARG types. Notably, initial amendment with antibiotic-manure significantly increased the total ARG relative abundances (per 16S rRNA gene) in the soils (2.21 × unamended soils, $p < 0.001$). After incubating 120 days, to simulate a wait period before crop harvest, 282 ARGs reduced 4.33-fold (median) up to 307-fold while 210 ARGs increased 2.89-fold (median) up to 76-fold in the antibiotic-manure-amended soils, resulting in reduced total ARG relative abundances equivalent to those of the unamended soils. We further assembled the metagenomic data and calculated resistome risk scores, which was recently defined as a relative index comparing co-occurrence of sequences corresponding to ARGs, mobile genetic elements, and putative pathogens on the same scaffold. Initial amendment of manure significantly increased the soil resistome risk scores, especially when generated by cows administered antibiotics, while composting reduced the effects and resulted in soil resistomes more similar to the background. The risk scores of manure-amended soils reduced to levels comparable to the unamended soils after 120 days. Overall, this study provides an integrated, high-resolution examination of the effects of prior antibiotic use, composting, and a 120-day wait period on soil resistomes following manure-derived amendment, demonstrating that all three management practices have measurable effects and should be taken into consideration in the development of policy and practice for mitigating the spread of antibiotic resistance.

1. Introduction

There is increasing evidence that antibiotic use in livestock production can contribute to the emergence and spread of antibiotic resistant bacteria and ultimately undermine efficacy of their application to treat and prevent bacterial infections in humans (Chang et al., 2015; Landers et al., 2012). In the United States, domestic sale and distribution of antibiotics approved for use in food-producing animals increased by 24% from 2009 through 2015 (United States Food and Drug Administration, 2015b). Antibiotics administered to animals can result in selection of antibiotic resistant bacteria and enrichment of antibiotic resistance genes (ARGs) in the resulting manure (Carlet, 2012; Shterzer and Mizrahi, 2015; Zhang et al., 2013). Antibiotic residues excreted by animals, including metabolites, can further exert selection pressure on bacteria in manure-impacted environments (Heuer et al., 2008). Among the various environmental compartments, manure is often directly

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applied to soil (Boschiroli et al., 2016; Heuer et al., 2008; Xie et al., 2018), as this practice is used worldwide to provide nutrients to crops and improve soil quality (Binh et al., 2008). However, application of manure can also result in the infusion of antibiotics, antibiotic resistant bacteria, and ARGs into the soil.

Horizontal gene transfer of ARGs is a fundamental driver of the spread of antibiotic resistance to pathogens (von Wintersdorff et al., 2016) and has been identified as a key factor needed to advance human health risk assessment models of environmental sources of antibiotic resistance (Ashbolt et al., 2013). Horizontal gene transfer has been documented to occur in livestock gastrointestinal tracts (Shterzer and Mizrahi, 2015) and can be enhanced by the presence of antibiotics (Ubeda et al., 2005). Mobile genetic elements (MGEs), such as plasmids, transposons, and bacteriophage elements, facilitate horizontal transfer of ARGs generally and to pathogens specifically (Jasni et al., 2010). Notably, manure introduced into soils is not only enriched in antibiotics, resistant bacteria, and ARGs, but also pathogens and MGEs (Chee-Sanford et al., 2009). Manure-borne antibiotic resistant bacteria can persist in soil via adaptation, particularly in the presence of antibiotics (Baquero et al., 1998; Biswas et al., 2018; Sharma and Reynnells, 2016). Horizontal gene transfer can further occur between indigenous manure-borne and soil bacteria following manure application (Xie et al., 2018). In addition, manure provides nutrients, which can further enhance the proliferation of bacteria (Heuer and Smalla, 2007; Udikovic-Kolic et al., 2014). ARGs can amplify during bacterial replication and be passed on to the next generation through vertical gene transfer (Martinez et al., 2007). Overall, emergence, survival, and proliferation of resistant bacteria (including non-pathogens) carrying MGEs can present risk of dissemination of ARGs within the diverse pool of soil bacteria and ultimately to human pathogens.

It is generally known that resistant pathogens and associated ARGs from manure and soils can be carried over onto produce during crop production (Tien et al., 2017). However, both the U.S. Food and Drug Administration's Produce Safety Rule in the Food Safety and Modernization Act (FSMA) and the U.S. Department of Agriculture's National Organic Program standards solely focus on reduction of pathogens and prevention of foodborne illness and do not address antibiotic resistance (United States Food and Drug Administration, 2015a). The importance of environmental dimensions in the spread of antibiotic resistance and rise in resistant human pathogens is gaining increasing attention (Bengtsson-Palme et al., 2018; Martinez and Baquero, 2014; Westphal-Settele et al., 2018) and has important implications for agroecosystems and food production. Specifically, ARGs are now being tracked as culture-independent contaminants and indicators of antibiotic resistance potential (Martinez, 2008; Pruden et al., 2006). To minimize the potential for spreading antibiotic resistance and reduce associated human health risk, additional manure management guidelines are worthy of consideration. In particular, such management guidelines should be considerate of microbial ecological factors, such as potential for manure-associated ARGs and MGEs to mobilize from environmental reservoirs to human pathogens.

Previous studies employed high-throughput quantitative PCR arrays to examine a wide spectrum of ARGs in manure-amended soil (Gou et al., 2018; Muurinen et al., 2017; Zhang et al., 2017; Zhu et al., 2013), while others have focused specifically on the fate of a few select groups of ARGs, such as those conferring resistance to tetracyclines or sulfonamides (Heuer and Smalla, 2007; Peng et al., 2015; Tang et al., 2015). Only a few studies have recently employed shotgun metagenomic sequencing to obtain a deeper profile of the ARGs present in soils (Fang et al., 2015; Pitta et al., 2016), but generally these have focused on effects of one or a few factors related to animal production and manure management. Such studies have also been limited to field-scale, where numerous uncontrolled and unaccounted factors are typically at play, making it difficult to draw firm conclusions and extrapolate results to typical manure land application practices. For example, Zhu et al. (2013) found significant enrichment of ARGs in soils near swine feedlots receiving manure or compost relative to pristine soils, but the amendment application rates and intervals between application and sampling were unknown. Although Gou et al. (2018) found reduced ARG levels in soils amended with composted cattle manure without antibiotics relative to raw manure, it is unclear if the same difference would be observed if the manure with antibiotics were composted. In addition, many studies employ manure spiked with antibiotics, often at higher than realistic levels, rather than manure from animals treated with antibiotics according to standard practice. Integrated experiments examining multiple upstream factors in animal production and soil amendment can provide insight into the relative contributions of various manure mitigation strategies, while metagenomics provides the opportunity for high-resolution assessment of the effects of various factors on the resulting resistomes.

This study employed controlled, replicated microcosms for an integrated examination of the effect of manure collected during antibiotic administration and composting on the resistomes (i.e., total ARGs) of soils receiving manure-derived amendments. Microcosms were set up with three distinct soil textures amended at typical agronomic rates with raw manure or composts derived from dairy cattle undergoing standard antibiotic treatment or control cows that had not received antibiotics the previous lactation cycle, with unamended background soils as controls. Microcosms were sacrificed and sampled one day and 120 days after amendment to simulate a harvest wait period. Shotgun metagenomic sequencing was used in this study to comprehensively compare resistomes across conditions, and MetaCompare (Oh et al., 2018) was used to assess effects on resistome risk as an integrative indicator of potential for ARGs to mobilize to human pathogens.

2. Materials and methods

2.1. Manure and compost

Details of manure and compost collection were described previously (Ray et al., 2017). Briefly, three cows were treated therapeutically with pirlimycin (intramammary dose typical for clinical mastitis; two doses of 50 mg each, 24 h apart), and three other cows received cephapirin (intramammary dry cow therapy; single dose of 300 mg into each of four quarters). Manure collected from the two treated groups were mixed thoroughly to generate raw manure containing the two antibiotics (antibiotic-manure). Manure generated from cows that had not been administered antibiotics during their previous lactation cycle was also collected (control-manure). More specifically, the control cows were administered cephapirin 10–12 months prior to this study, while none of the control cows had a history of pirlimycin treatment. A proportion of the raw manure was composted for 42 days in small-scale tumblers (wet mass: 20–22 kg) using static techniques, as recommended by the FDA (United States Food and Drug Administration, 2014). The compost resulting from control-manure was regarded as control-compost, while the compost resulting from antibiotic-manure was regarded as antibiotic-compost. Antibiotic-manure, control-manure, antibiotic-compost, and control-compost were stored for four to six months at 4 °C prior to amending the soils. The physical and chemical properties of the manure and compost are summarized in Supplementary Table S1.

2.2. Soil microcosms

The detailed procedure of soil microcosm set up was described previously (Chen et al., 2018). In summary, top soils (0–5 cm) were collected from three farms in Virginia. The three soils were classified according to their texture content (sand: silt: clay) as a loamy sand (S1), a silt loam (S2), and a silty clay loam (S3). Soil information, physical and chemical properties, and soil heavy metal concentrations are summarized in Supplementary Table S2. The soil microcosms consisted of triplicate glass jars containing soils amended with manure or compost at a typical agronomic nitrogen application rate for Virginia

(Evanylo, 2009). The amendments were thoroughly mixed with the soils by hand shaking and stirring. Microcosms were incubated under aerobic conditions, in the dark, at 20 °C, and with soil moisture maintained at 50% field capacity by watering one time weekly with sterile water. Microcosms were sacrificed for soil collection on Day 1 and Day 120 after set up, freeze dried, and stored at −20 °C for DNA extraction.

2.3. DNA extraction, sequencing, and bioinformatic analysis

DNA extraction, sequencing, and bioinformatic analysis are described in detail in the Supplementary information, S1. Briefly, DNA was extracted from 0.5 g of soil using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) followed by clean-up using a OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA). Shotgun metagenomic sequencing, including Truseq library construction, was carried out by the Biocomplexity Institute of Virginia Tech, Blacksburg, VA, using an Illumina HiSeq 2500 in high output mode with a paired-end 2 × 100 read length protocol. DNA sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive (accession number PRJNA489261, Table S9).

Sequencing data were uploaded to MetaStorm (Arango-Argoty et al., 2016), an online platform where ARG-like fragments were searched against publicly-available databases. The Comprehensive Antibiotic Resistance Database (CARD v1.0.6) was used for the ARG annotation (McArthur et al., 2013), with cutoff criteria of E-value < 1e-10, identity > 60%, and minimum alignment length of 25 amino acids. Relative ARG abundances (i.e., per 16S rRNA gene) were determined from shotgun metagenomic data in MetaStorm by normalizing the ARG reads to 16S rRNA gene reads, accounting for relative read length, as described by (Li et al., 2015). The Greengenes database was used for bacteria taxonomy annotation (DeSantis et al., 2006).

ARGs were categorized according to the antibiotic class to which they confer resistance. ARGs conferring resistance to macrolide, lincosamide, and streptogramin ARGs are typically categorized together (i.e. MLS), as the three antibiotics have similar antibacterial spectra and mechanism (Ungureanu, 2010) and the mechanisms of resistance are similar (i.e., modification by methylase(s) reduces the binding of all three classes of antibiotics) (Roberts et al., 1999). If an ARG was found to confer resistance to more than one antibiotic class, it was categorized as “multidrug”. The ARG profiles were compared using Bray-Curtis based non-metric multidimensional scaling (NMDS) plots in R with the vegan package. Heatmaps were used to display the abundances of individual ARGs in R with the superheat package.

2.4. Resistome risk score determination

Resistome risks scores of the soil samples were calculated and ranked using MetaCompare (Oh et al., 2018) and described in detail in the Supplementary information S2. This computational pipeline estimates the potential for ARGs to be disseminated into human pathogens by identifying gene fragments annotated as ARGs, MGEs, and human pathogens co-occurring on assembled scaffolds derived from the metagenomics reads. Specifically, the IDBA-UD assembler and the Prodigal gene-prediction tool were applied to pinpoint alignment regions for BLAST searches against CARD (McArthur et al., 2013), ACLAME (Leplae et al., 2004), and PATRIC (Wattam et al., 2014) databases for ARGs, MGEs, and human pathogen-like sequences, respectively. Based on these annotations, three types of assembled scaffolds were tabulated: 1) scaffolds annotated as containing ARGs, 2) scaffolds annotated as containing ARGs and MGEs, and 3) scaffolds annotated as containing ARGs, MGEs, and pathogen-like sequences. The number of scaffolds of each type was normalized by the total number of scaffolds, resulting in 3-dimensional coordinates ($Q_{(ARG)}$, $Q_{(ARG,\ MGE)}$, $Q_{(ARG,\ MGE,\ PATH)}$). Each sample was projected into 3-dimensional hazard space and the environmental resistome risk score was obtained by calculating the distance between each sample point s and the theoretical point h representing the greatest theoretical resistome risk in the hazard space:

$$\text{resistome risk score } (s) = \frac{1}{(2 + \log_{10} dist(s,h))^2}$$

where $dist(s,h)$ is the Euclidean distance between s and h.

2.5. Statistical analysis

A companion study of the fate of antibiotics in the soil microcosms indicated that there was no significant effect of soil types on the dissipation patterns or rates (Chen et al., 2018). Therefore, soil type was considered as a secondary factor in this study and the three soils were categorized by the amendment condition for various statistical comparison using JMP®, Version 13.0 (SAS Institute Inc., Cary, NC), for significance tests. This approach also provided a robust comparison of the main affects across three distinct soils. Effect of amendments was evaluated by comparing soils receiving manure-derived amendments to unamended soils. Effect of antibiotic administration was evaluated by comparing soils receiving antibiotic amendments to soils receiving control amendments. Effect of composting was evaluated by comparing soils receiving compost to soils receiving manure. Effect of time was evaluated by examining the fate of individual ARGs in the antibiotic-manure-amended soils, where significant reduction of total ARG relative abundances was observed. For ARG richness, total ARG relative abundance, and resistome risk score, significance tests were carried out at the 95% confidence level. For ARGs categorized by antibiotic class to which they confer resistance and individual ARG types, fold differences in abundances were determined using geometric means of the pairs and statistical significance was declared for p-values falling below the threshold defined by controlling false discovery rate (FDR) at level 0.05 (Benjamini and Hochberg, 1995) and at least a two-fold difference.

3. Results

3.1. Effect of amendments on diversity (richness) and relative abundances (per 16S rRNA gene) of total ARGs detected in the soils

A total of 665 unique ARG types, i.e., assigned a unique antibiotic resistance ontology number by CARD, conferring resistance across 21 classes of antibiotics were annotated across all soil samples. In subsequent sections, diversity of ARGs is examined in terms of total number of unique ARG types encountered in the amendment and soil reservoirs. Diversity is of interest as a measure of the available pool of antibiotic resistance traits that could be subject to selection pressure or horizontal gene transfer. Relative ARG abundance accounts for minor variation in DNA recovery during extraction and serves as an indicator of the proportion of bacteria carrying ARGs and thus potential selective pressures at play affecting this proportion.

One day after soil amendment and incubation, the diversity of ARGs in manure- and compost- amended soils were significantly higher than in unamended soils (Fig. 1a). The diversity of ARGs ranged from 224 to 347 and 147 to 172 in the amended and unamended soils, respectively (Fig. 1a, Table S3). However, a higher diversity did not necessarily indicate a higher total ARG relative abundance. Relative abundance of total ARGs was only significantly higher than the unamended soils after one day of incubation in the antibiotic-manure condition ($p < 0.001$, Fig. 1b). Specifically, relative abundance in the antibiotic-manure-amended soils was calculated to be 2.21 × higher than in the unamended soils. When the soils were amended with control-compost, antibiotic-compost, or control-manure, the relative abundances of total ARGs remained at a level comparable to the unamended soils ($p > 0.05$, Fig. 1b).

After 120 days of incubation, soils receiving the manures still had significantly higher diversity of ARGs compared to the unamended soils ($p < 0.05$, Fig. 1a). However, the relative ARG abundances in the manure-amended soils were reduced to levels equivalent to the

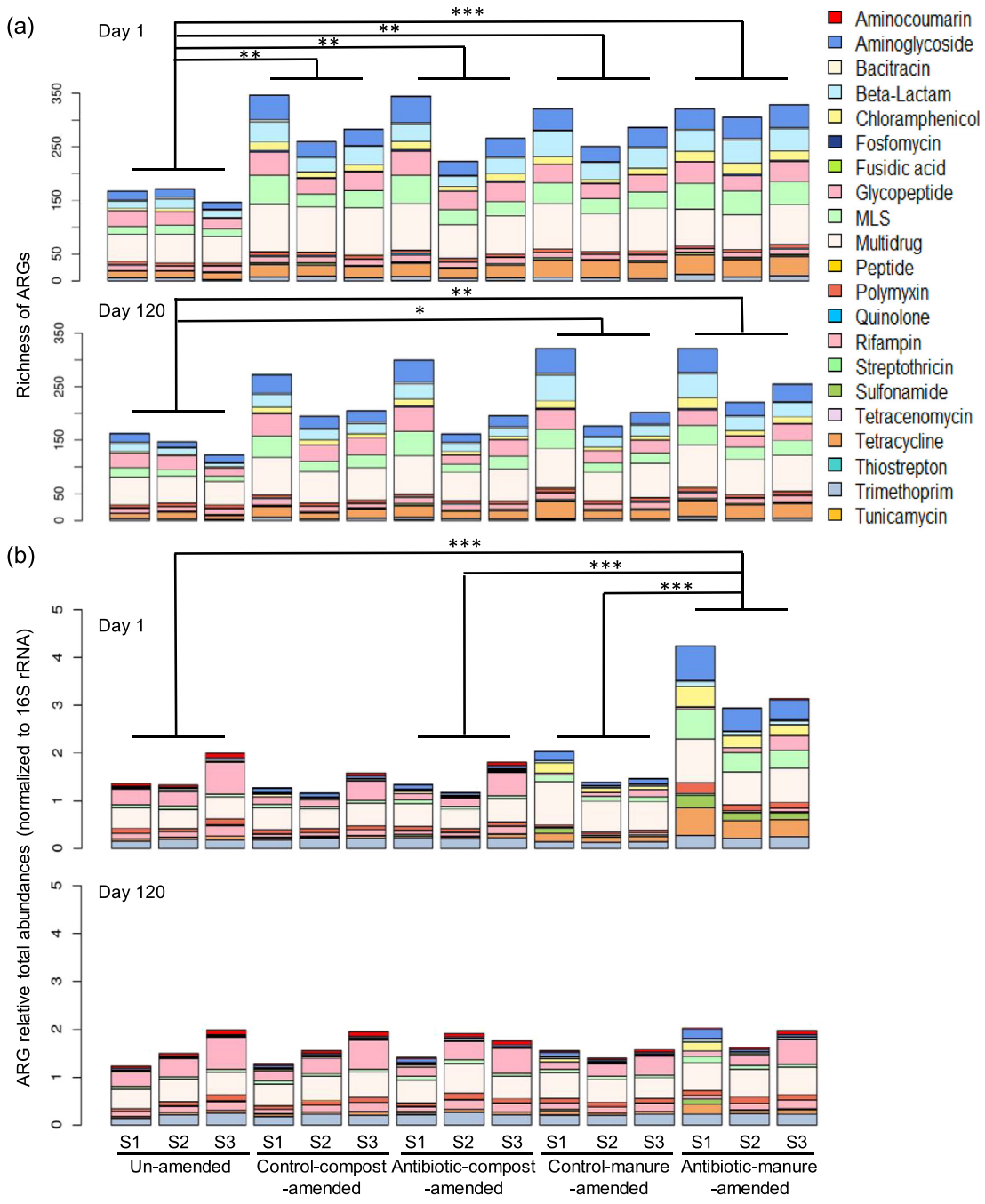

Fig. 1. Comparison of ARG profiles, categorized by class of resistance, detected by shotgun metagenomic sequencing of soils on Day 1 and Day 120 after amendment, reported as: (a) richness (i.e., total number of individual ARG types detected within each class) and (b) relative abundances (normalized to 16S rRNA). Asterisks indicate significance by multiple pairwise comparisons of total richness and relative abundances (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, Table S8). S1, S2, and S3 represent loamy sand, silt loam, and silty clay loam soils, respectively. ARGs were annotated via comparison to the Comprehensive Antibiotic Resistance Database v1.0.6 with cutoff criteria of E-value $< 1e-10$, identity $> 60\%$, and minimum alignment length is 25 amino acids.

unamended soils ($p > 0.05$, Fig. 1b).

3.2. Effect of amendments on ARG profiles in soils

Comparing ARG profiles indicated that all soils receiving manure or compost were significantly different from the unamended soils on Day 1 (Fig. 2). Notably, on the NMDS plots, the distances from the unamended soils to the manure-amended soils were greater than to the compost-amended soils, illustrating a stronger effect of manure than compost amendment. Further, ARG profiles were distinct between antibiotic-manure-amended and control-manure-amended soils, indicating an effect on antibiotic administration as well (Fig. 2). In addition, the ARG profiles were significantly different between the control-compost-amended and control-manure-amended soils, and also between antibiotic-compost-amended and antibiotic-manure-amended soils. After 120 days, the ARG profiles were not significantly different among the soils (Fig. 2).

On Day 1, relative to the unamended soils, soils receiving manure or compost contained higher diversity of ARGs conferring resistance to multidrug, aminoglycoside, beta-lactam, chloramphenicol, MLS, and tetracycline classes of antibiotics. After 120 days, the diversity of ARGs belonging to these classes were still higher in soils receiving manure or

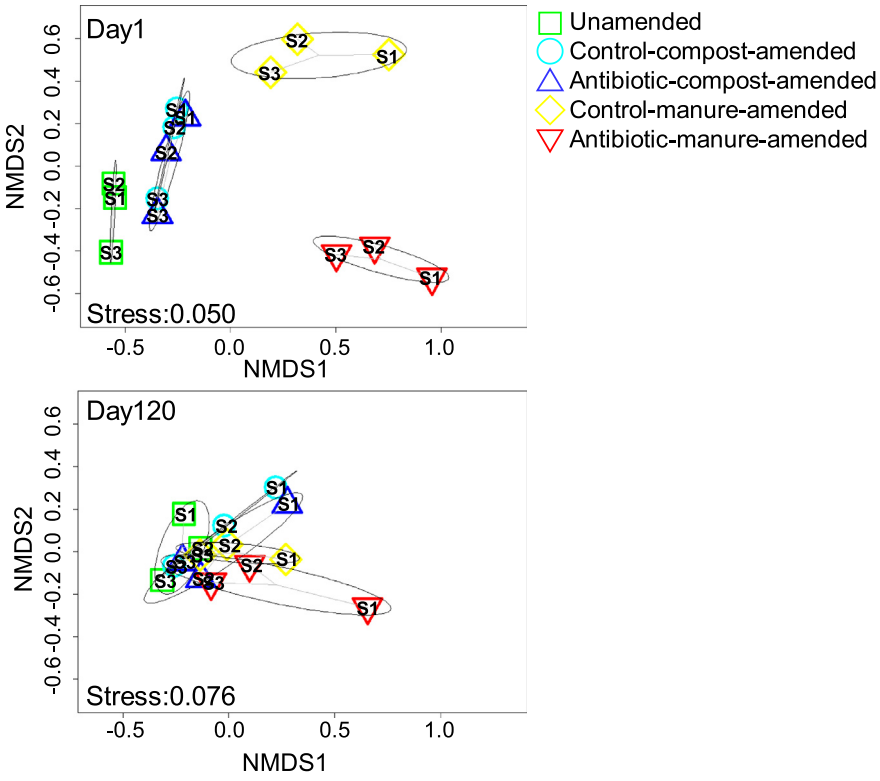

Fig. 2. Nonmetric multidimensional scaling (NMDS) comparing ARG profiles among individual soil microcosm samples. Samples are categorized according to the amendment types (shown in different colors). Gray lines indicate the centroids of samples for each amendment type. Gray circles around the centroids indicate the 95% confidence range for standard error of the centroids. Samples are not significantly different if these confidence ranges overlap. S1, S2, and S3 represent loamy sand soil, silt loam soil, and silty clay loam soil, respectively.

compost (Fig. 1a, Table S3).

Following amendment of any of the four manure or compost types, the relative abundances of ARGs conferring resistance to chloramphenicol, fosfomycin, and sulfonamide antibiotics were significantly higher relative to the unamended soils (Fig. 1b, Table S4). Additionally, the relative abundances of ARGs conferring resistance to MLS and multidrug antibiotics were significantly higher relative to the unamended control following amendment of control-manure or antibiotic-manure (Fig. 1b, Table S4). Additionally, the relative abundances of beta-lactam, polymyxin, streptothricin, and trimethoprim classes of ARGs were also significantly higher in the antibiotic-manure-amended soils. Notably, the relative abundance of aminocoumarin, glycopeptide, rifampin, and tetracenomycin classes actually reduced initially in manure-amended-soils below that of the unamended soil.

After 120 days, the relative abundance of chloramphenicol and sulfonamide classes of ARGs remained significantly higher in all of the amended soils relative to unamended soils. Additionally, the relative abundances of ARG conferring resistance to aminoglycoside, beta-lactam, and tetracycline resistance classes of antibiotics remained significantly higher in antibiotic-manure-amended soils relative to the unamended soils. All other classes of ARGs were statistically equivalent in relative abundance between the amended and unamended conditions after 120 days (Table S4).

3.3. Effect of amendments on relative abundances of individual ARGs in soils

The relative abundance of individual ARGs was examined to identify which were the main drivers of the distinct profiles observed among the soils on Day 1 (Fig. 3 and Fig. S3). Among the 665 ARGs, the initial abundances of 9 ARGs were significantly higher in all soils one day after receiving manure-derived amendments relative to the unamended soil, including some ARGs that have been widely associated with agriculture, such as *sul*1, *sul*2, and *tet*W (Ben et al., 2017; He et al., 2014). qPCR further confirmed that addition of manure specifically enriched *sul*1 and *tet*W in the amended soils compared to the unamended soils, which was consistent with the quantitative comparisons of these ARGs discerned via metagenomics analysis (Fig. S2).

On Day 1, relative to the unamended soils, 96, 64, 21, and 24 individual ARGs were significantly higher in soils receiving antibiotic-manure-amendment, control-manure-amendment, antibiotic-compost-amendment, and control-compost-amendment, respectively (Fig. 3 and Fig. S3, Table S5). Notably, 9 and 24 ARGs were actually significantly lower on Day 1 after amending soils with antibiotic-manure or control-manure, respectively, while no ARGs were significantly lower in the compost-amended soils (Fig. 3 and Fig. S3, Table S5).

3.4. Effect of 120-day wait period after amendment of manure collected during antibiotic administration

On Day 1, among the four amendments, addition of antibiotic-manure resulted in the highest relative abundance of total ARGs and was the only amendment to result in significantly higher relative abundance of total ARGs in soils compared to the unamended soil. Specific antibiotic classes that were greater in ARG relative abundance in the antibiotic-manure-amended soils were: aminoglycoside (4.9-fold), beta-lactam (2.4-fold), chloramphenicol (2.7-fold), MLS (4.0-fold), polymyxin (3.5-fold), sulfonamide (3.6-fold), and tetracycline (3.5-fold). Generally, the 120-day incubation period aided in attenuating ARGs in the antibiotic-manure condition. Specifically, a total of 282 ARGs decreased by 4.33 fold (median), but 210 ARGs increased by 2.89-fold (median) by Day 120 (Fig. 4a). Among these, decreases of 39 ARGs and increases of 9 ARGs were statistically significant (FDR p values < 0.05), with a maximum increase and maximum decrease of 76-fold and 307-fold, respectively (Fig. 4a). Attenuation of ARGs during soil incubation was gene specific. More individual ARGs decreased than increased, together with greater degrees of ARG fold decrease than ARG fold increase, resulting in an overall reduction relative abundance of total ARGs in the antibiotic-manure-amended soils over the 120-day incubation period.

Fig. 4b shows the fate of 42 clinically-important ARGs, defined for the purpose of this study as ARGs conferring resistance to last-resort

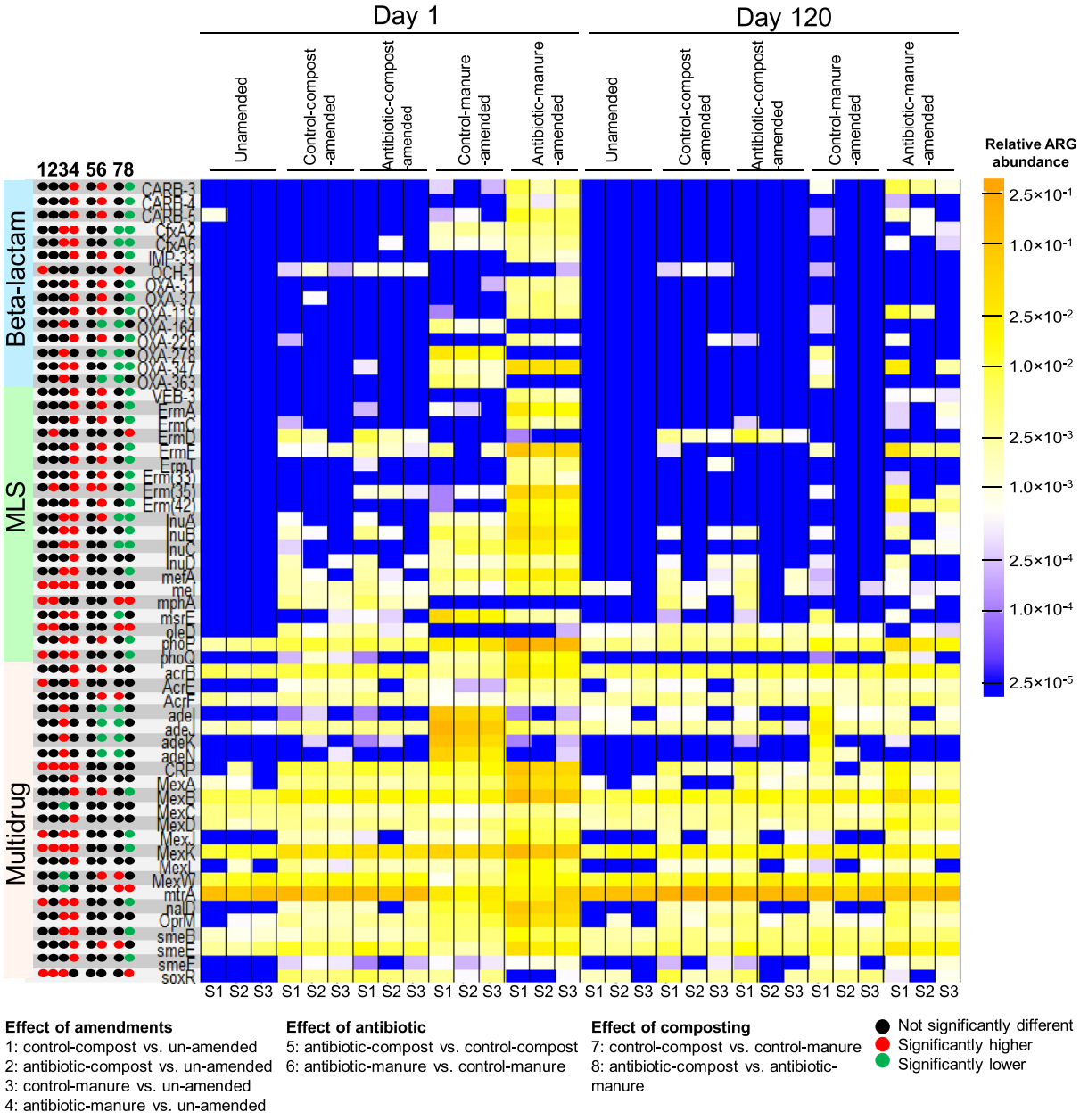

Fig. 3. Relative ARG abundances (normalized to 16S rRNA genes) for beta-lactam-, MSL-, and multidrug- (including beta-lactam and/or MSL resistance and other resistance classes) classes of ARGs detected in the soils. S1, S2, and S3 represent loamy sand, silt loam, and silty clay loam soils, respectively. Significance testing was conducted accounting for false discovery rate (FDR) (FDR p-value < 0.05 and a at least two-fold change considered as significant). A red, green, or black circle indicates a "significantly higher", "significantly lower", or "not significantly different" for the eight indicated pairwise comparisons on Day 1, respectively. Effect of manure-derived amendments is evaluated by comparing to the un-amended soils (1 to 4). Effect of collecting feedstock manure during antibiotic administration is evaluated by comparing soils receiving "antibiotic" amendments to soils receiving "control" amendments (5 and 6). Effect of composting is evaluated by comparing soils receiving "compost" versus "manure" (7 and 8). All comparisons were carried out for Day 1 samples, ARGs bearing statistical significant differences for at least one of the comparisons are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

antibiotics, such as third and fourth generation cephalosporins (beta-lactam antibiotics), colistin (peptide antibiotics), and vancomycin (glycopeptide antibiotics) (see Table S6 for full list applied in this study). Notably, clinically-important ARGs were significantly higher in the manure-amended condition on Day 1 compared to other soils, but some decreased and others increased following 120-day incubation.

In particular, shifts in beta-lactam-resistance genes were noted to be generally dependent on the specific beta-lactamase family to which they belonged. For example, most of the ARGs in the CARB and OXA family decreased, while most of the ARGs in the CTX-M and TEM family increased after 120 days (Fig. 4b). In addition, the abundances of *van*A (vancomycin) and *mcr*-1 (colistin) increased by 8.5 fold and 1.6 fold, respectively, after 120 days in antibiotic-manure-amended soils (Fig. 4b).

3.5. Soil resistome risk

MetaCompare was applied to assembled metagenomic data as a means to compare the effects of the amendments on the relative resistome risks represented by the soils. The total number of assembled scaffolds obtained among the soil metagenomes ranged from 6182 to 164,902 (Table S7). Among these, scaffolds with ARG annotations were identified, ranging from 4 to 827 and accounting for up to 0.5% of the total assembled scaffolds (Table S7). Among these, 177 scaffolds were

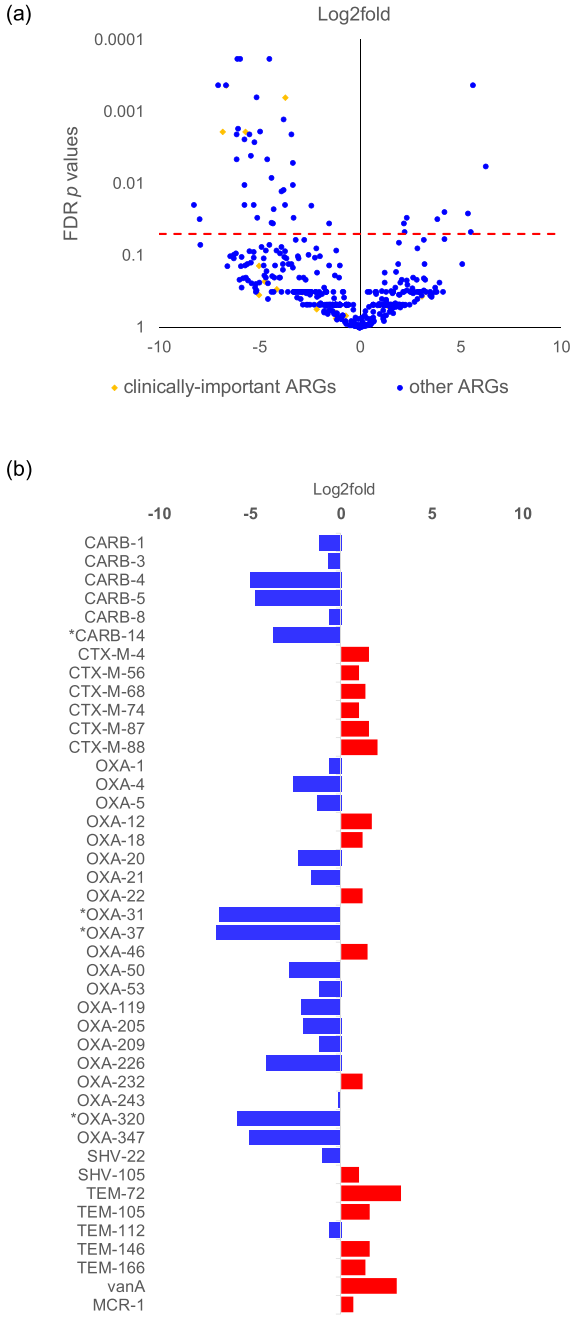

Fig. 4. Fate of ARGs in the antibiotic-manure-amended soils after 120-day incubation. (a) fold changes and FDR *p* values of 210 ARGs that increased and 282 ARGs that decreased; (b) fold change of 42 clinically important ARGs (ARGs conferring resistance to last resort of antibiotics). Asterisks indicate significance with FDR p value < 0.05. Fold change of ARGs in soils from Day 1 to Day 120 was log 2 transformed.

annotated as containing ARGs and MGEs and 163 as containing an ARG, MGEs, and human pathogen-like sequences (Table S7). According to the framework proposed by Martinez et al. (2015) and elaborated upon by Oh et al. (2018), the 163 scaffolds annotated as containing all three of these elements are considered to represent the greatest relative resistome risk. Notably, none of the scaffolds containing all three elements were assembled from the unamended soil, indicating that such scaffolds likely mostly originated from the manure-derived amendments.

On Day 1, manure amendment resulted in significantly higher resistome risk scores relative to the unamended soil, while compost had

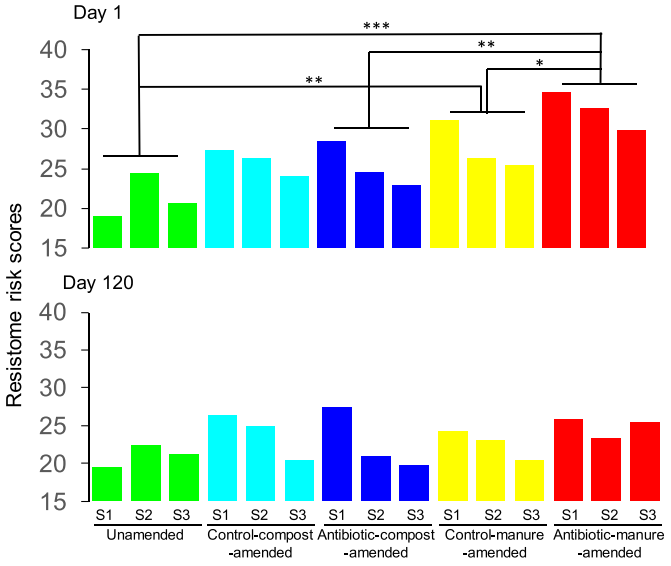

Fig. 5. Soil resistome risk scores (Asterisks indicate significance by multiple pairwise comparisons of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, Table S8).

no significant effect on soil resistome risks (Fig. 5). Further, resistome risk scores of antibiotic-manure-amended soils were significantly greater than those for the control-manure-amended and antibiotic-compost-amended soils (Fig. 5), indicating that antibiotic administration and composting had measurable effects on soil resistome risk. After 120 days, the environmental resistome risk scores of all amended soils were equivalent to that of the unamended soils (Fig. 5).

4. Discussion

4.1. Effect of applying manure collected during antibiotic administration

Here, we examined ARGs in soils receiving amendments derived from manure of dairy cows treated with cephapirin, a beta-lactam antibiotic, and pirlimycin, a lincosamide antibiotic, classes that are categorized by the World Health Organization as highly important in human medicine (WHO, 2016). Both antibiotics are commonly used for the treatment of bovine mastitis in North America, while pirlimycin is especially used prophylactically to prevent metritis (Roy and Keefe, 2012). We observed that soils amended with manure collected during antibiotic administration were significantly elevated in relative abundance of total ARGs compared to control-manure-amended soils (Fig. 1). Elevated relative ARG abundance in the antibiotic conditions suggests that selective pressures could have been at play in enriching ARG content in the bacterial community, which is consistent with selection by antibiotics and metabolites in the gastrointestinal tract and in the resulting manures. The antibiotic-manure contained 117 and 8.37 $\mu g\ kg^{-1}$ of pirlimycin and lincosamide, respectively (Table S1). As expected, these antibiotics were never detected in the control manure.

Amendment of soil with manure collected during antibiotic administration resulted in significantly elevated abundance of 36 ARGs (Fig. 3 and Fig. S3). Among them, 23 confer resistance to beta-lactam and/or MLS and increased by 6.9 to 701-fold (Fig. 3, Table S5). The elevated relative abundances ARGs conferring resistance to beta-lactam and MLS-classes of antibiotics is a strong indicator of direct selection pressure exerted by the classes of antibiotics administered. It is also possible that antibiotics or residuals could stimulate proliferation of ARGs by horizontal gene transfer (Hastings et al., 2004; Lopatkin et al., 2016). In mouse models, intramammary injection of tetracycline significantly increased the level of tetracycline ARGs in the mouse gut microbiota, from below detection limit before injection to over 10^5 copies/g after injection (Zhang et al., 2013). The enrichment of

ARGs is also consistent with a previous study where the injection with ceftiofur (a third generation cephalosporin) significantly doubled the abundance of beta-lactam ARGs in the feces relative to un-treated cows (Chambers et al., 2015). Selection and horizontal gene transfer could have also been at play during storage of amendments at cold temperatures prior to this study. One study found that the ARG levels in manure increased by five-fold compared to fresh manure after winter storage, suggesting that residual antibiotics continue to exert selective pressure in the manure (Ruuskanen et al., 2016). Similarly, the relative abundances of *sul*1 and *intI*1 in sludge stored at 4 °C for 2 months significantly increased by 0.5–3 log units, suggesting that the cold induced ARG increases due to horizontal gene transfer or preferential survival of resistant bacteria (Miller et al., 2014). A previous study showed that, after the drugs were administered to dairy cows by intramammary injection, the concentrations of pirlimycin and cephapirin increased up to 2.12 $\mu g\,kg^{-1}$ and 287 $\mu g\,kg^{-1}$ in the feces, respectively, and up to 480 $\mu g\,L^{-1}$ and 232 $\mu g\,L^{-1}$ in the urine, respectively (Ray et al., 2014a, 2014b). The present study suggests that the excreted antibiotic or their metabolites may have continued to exert selective pressure favoring resistant bacteria or promote horizontal gene transfer in the manure as it was stored prior to amendment and possibly during the first day of incubation in the soil. It is also important to recognize that, once ARGs are enriched or fixed in bacterial populations, they do not necessarily easily lose them in the absence of selection pressure (Bjorkman et al., 2000).

Using manure collected during antibiotic administration was also associated with an increase in 13 ARGs conferring resistance to antibiotic classes other than beta-lactam or MLS (Fig. S3, Table S5). These results further indicated potential co-selective effects of cephapirin and pirlimycin on ARGs belonging to several other classes. The relative abundance of these 13 ARGs were positively correlated with ARGs conferring resistance the beta-lactam, MLS and multidrug classes of antibiotics (Table S10), suggesting potential co-selective effects on a wide spectrum of ARGs. Analysis of assembled scaffolds also supports this conclusion, where multiple coding regions of ARGs conferring resistance to different antibiotic classes were found to be located on the same scaffolds (Fig. S1). For instance, a scaffold assembled from Day 1 antibiotic-manure-amended soils contained open-reading frames of ARGs belonging to four different classes, including CARB-3 (beta-lactam), *aad*A3 (aminoglycoside), *qac*H (quinolone), and *sul*1 (sulfonamide) (Fig. S1). Such co-occurrence of ARGs on the same DNA strand would indicate that the proliferation of beta-lactam-resistant bacteria can also result in the amplification of sulfonamide-, quinolone-, and aminoglycoside-ARGs.

Applying antibiotic-manure to soils resulted in a significant increase of resistome risk scores relative to the control-manure (Fig. 5). This further suggests that limiting antibiotic use could be a viable means of reducing potential for ARGs to proliferate and disseminate via horizontal gene transfer to pathogens. However, it is important to recognize that the effects of antibiotic withdrawal may not be immediate, as time may be required for ARGs to attenuate in the microbial community and some ARGs may become fixed in the population because they do not incur a fitness cost (Schulz zur Wiesch et al., 2010). Besides, it is important to note that the injection of pirlimycin and cephapirin, as applied in this study, was at a typical dose for therapeutic and prophylactic purposes in dairy cattle, not routine subtherapeutic application to promote weight-gain as is more commonly done for other types of livestock. Subtherapeutic antibiotic use was banned in the EU in 2006 and most recently in the US in 2017 in accordance with the Veterinarian's Feed Directive. This study indicates that even antibiotic dosages intended specifically to treat and prevent illnesses could still stimulate the spread of antibiotic resistance. Maintaining good animal health and hygiene through alternative means is important to reduce the need for antibiotics. Further, diverting and separately treating manure containing antibiotics when livestock are known to be undergoing antibiotic therapy is advisable.

4.2. Effect of composting manure prior to amending soil

Composting is a common manure management practice for pathogen reduction (Mc Carthy et al., 2011), and previous research has also shown its effectiveness for reduction of antibiotics (Dolliver et al., 2008; Ray et al., 2017; Shan et al., 2018). However, prior research has been unclear with respect to whether composting provides benefits for ARG reduction. For instance, composting of horse manure resulted in a decrease by up to 100-fold of tetracycline-resistance genes (Storteboom et al., 2007). By contrast, composting of swine manure increased tetracycline- and sulfonamide- resistance genes by 10- to 100-fold (Wang et al., 2015). A recent study employing high-throughput qPCR provided insight into the responses of a broad range of ARGs and indicated that the tendency of an ARG to increase or decrease during composting is highly gene-specific (Wang et al., 2017; Xu et al., 2018). Still, questions remain with respect to the behavior of other neighboring genes, MGEs, and host bacteria, especially after amending to soil. Further, manure is commonly applied to soil without composting, and thus direct comparison of compost versus manure amendments is needed. In fact, sometimes nitrogen loss during composting can be undesirable (Hoornweg et al., 2000), and thus higher rates of compost application are needed to achieve target agronomic nitrogen application rates. For example, in this study, it was necessary to add twice the mass of compost than manure to achieve the same nitrogen application rate. Thus, the dilution factor by the soil matrix was lower for compost than for the manure and there may have been more differences than observed here when comparing ARG profiles had they been added at the same mass.

Even in the case of control-manure, addition of compost rather than corresponding manure resulted in distinct soil ARG profiles, with less separation relative to the unamended soils (Fig. 2). The abundances of 33 ARGs were significantly lower, by 10- to 1789- fold, in the control-compost- relative to the control-manure amended soils (Fig. 3 and S3, Table S5). Although composting did not significantly reduce total ARG relative abundances in the soil, relative to the control-manure (Fig. 1), it still had measureable impacts in terms of reducing several individual ARG types. The reduction in ARGs during composting may be attributed to the removal of ARG-harboring microorganisms during the thermophilic stage, as well as the aerobic conditions, which can result in the reduction of enteric bacteria carrying ARGs. Furthermore, extracellular ARGs can be biodegraded along with other organic material in the compost (Gou et al., 2018). Still, it is important to recognize that the shifts in microbial community composition that take place during composting could also increase some ARGs. Notably, there were 23 ARGs that were significantly higher in the compost-amended versus manure-amended soils, by 3.0- to 174- fold. In addition to enrichment of certain ARGs during composting, higher application rate of compost to soils could play a role.

Strikingly, composting resulted in much lower relative abundance of total ARGs in the soil when applied to antibiotic-manure (Fig. 1). Specific antibiotic classes that were lower in ARG relative abundance in the antibiotic-compost-amended soils were: aminoglycoside (8.1-fold), beta-lactam (4.6-fold), chloramphenicol (9.8-fold), MLS (7.0-fold), sulfonamide, (8.9-fold), and tetracycline (11.6-fold). A total of 66 individual ARG types were significantly lower in the compost condition, while only 9 ARGs were lower in the antibiotic-manure condition (Fig. 3 and Fig. S3). Specifically, 31 ARGs conferring resistance to beta-lactam and/or MLS were significantly lower in the compost condition, by 3.3 to 868-fold (Table S5), which is consistent with the removal of 99% of the initial concentrations of pirlimycin and cephapirin during composting (Ray et al., 2017). This suggests that there could be benefits of composting reducing antibiotic selective pressure once the amendment is added to the soil. Antibiotic-manure-amended soils harbored significantly higher initial abundances of ARGs and distinct ARG profiles relative to the control-manure-amended soils. After composting, such differences were no longer significant. These results clearly

indicated that composting helped to reduce the effect of prior antibiotic use and provide benefits in controlling the release of elevated ARGs to the soil environment.

4.3. Simulating a 120-day harvest wait period

After manure application, imposing a wait period prior to crop harvest can reduce manure-borne pathogens in soil, as their survival can be suppressed by indigenous soil microorganisms due to factors such as predation, substrate competition, and antagonism (van Elsas et al., 2011). In addition, antibiotic dissipation can take its course over the weeks and months following amendment (Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005; Pan and Chu, 2016). Our previous study showed a rapid removal of pirlimycin in the antibiotic-manure-amended soils, resulting in undetectable concentrations after 120 days (Chen et al., 2018). Based on available methods, we concluded that the 120 day concentrations were too low to impose risk of selection pressure by these antibiotics (Chen et al., 2018). This assessment is consistent with our current finding that even in the worst case scenario (amending soil with antibiotic-manure), there was no difference in terms of total ARG relative abundance, ARG profiles, or environmental resistome risk scores relative to the unamended soils after 120-day incubation. However, it should also be noted that the abundance of certain individual ARGs, including several clinically-important ARGs, increased during soil incubation in the antibiotic-manure-amended soils (Fig. 4b, c). In addition, the ARG diversity in the manure-amended soils was still significantly higher than in the unamended soils (Fig. 1a), indicating that the attenuation of unique ARGs is not complete within 120-day incubation. In terms of mechanisms at play during the 120-day wait period, the antibiotic and antibiotic residues in the antibiotic-manure amended soils may exert selection pressure on bacteria carrying ARGs, since the manure also contained the highest levels of antibiotics. Also, the organisms carrying these ARGs may have happened to have a competitive advantage in using the manure as a carbon source. Thirdly, there could have been horizontal transfer of these ARGs to other hosts. Although the 120-day incubation period was selected to simulate a harvest wait period, the basic findings indicate that wait periods could be beneficial for other scenarios as well, such as sowing or grazing.

4.4. Limitations and suggested further studies

Shotgun metagenomic sequencing provides a powerful tool for accessing the full range of ARGs and other genes relevant to antibiotic resistance in complex environmental samples, which is impossible with culture-based or PCR amplification-based techniques. While interpretation of shotgun metagenomics sequencing data presents several opportunities for developing various metrics for assessing antibiotic resistance; including total ARGs, ARGs corresponding to antibiotic classes of interest, diversity of ARGs, and resistome risk, is important to bear in mind various limitations of metagenomics in interpreting the results. Firstly, as the ARGs identified represent “potential” genotypic resistance, as it could be possible that the genes were incomplete or non-functional in the bacterial host. Further, the linkages between ARGs, MGEs, and pathogen gene markers were predicted based on assembly, which incurs considerable uncertainty for metagenomic versus whole genome sequence data. Systematic assembly of genomes can be problematic in terms of generating chimeric artifacts and at present there is no standard means to assess the confidence in assembled data. In the present study, we employed one of the most advanced assembly algorithms available to date, IDBA-UD assembler, to obtain the highest quality assemblies possible (Supplementary information, S2). Finally, one potential improvement to MetaCompare in the future would be to also take into account the scaffold length, for which there is commonly a wide distribution in assembled data. If the distribution of scaffold lengths varies significantly across samples, this could potentially bias the resistome risk scores given that longer scaffolds have a greater chance of be annotated with ARGs, MGEs and/or pathogens.

Limitations in experimental design are also important to consider. While the results here were obtained using microcosms and a closed system, it would be valuable validate the effect of the factors studied here at field-scale. Additional factors that could be at play in the field include crop type, rainfall, sunlight/photodegradation, and soil history. In particular, transfer of soil microbes or other influences of amendments on edible crops is of particular interest as a potential human exposure route. At present, only limited studies have examined the effect of soil amendments on markers of antibiotic resistance on crops (Fogler et al., 2019; Marti et al., 2013).

5. Conclusion

This study provided valuable insight into the potential benefits of multiple on-farm practices for reducing the potential for antibiotic resistance to spread through a highly integrated, replicated experimental design and employing shotgun metagenomics for a comprehensive assessment of the effects on the soil resistome. Collecting manure during antibiotic administration, specifically cephapirin and pirlimycin, significantly elevated abundance of a wide spectrum of ARG types in corresponding manure-amended soils, especially beta-lactam and MLS classes, as well as soil resistome risk scores. Applying compost resulted in a reduced abundance of total ARGs in the antibiotic-manure, but some ARGs were still elevated compared to unamended soil. Total ARGs generally diminished over the 120-day incubation period, corresponding with the reduction of soil resistome risk scores, indicating that time restrictions between amendment application and sowing or harvest might present a buffer to ARG dissemination. However, some ARGs were still elevated at 120 days, suggesting that further management efforts, such as application of adsorbing materials to soils (Jiao et al., 2018), could be beneficial for achieving conditions indistinguishable from unamended soils.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.04.043.

References

Arango-Argoty, G., Singh, G., Heath, L.S., Pruden, A., Xiao, W., Zhang, L., 2016. MetaStorm: a public resource for customizable metagenomics annotation. PLoS One 11, e0162442.

Ashbolt, N.J., Amezquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G., McEwen, S.A., Ryan, J.J., Schonfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001.

Baquero, F., Negri, M.C., Morosini, M.I., Blazquez, J., 1998. Antibiotic-selective environments. Clin. Infect. Dis. 27 (Suppl. 1), S5–11.

Ben, W., Wang, J., Pan, X., Qiang, Z., 2017. Dissemination of antibiotic resistance genes and their potential removal by on-farm treatment processes in nine swine feedlots in

Shandong Province, China. Chemosphere 167, 262–268.
Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B Met. 57, 289–300.
Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol. Rev. 66, 25–37.
Biswas, S., Niu, M., Pandey, P., Appuhamy, J., Leytem, A.B., Kebreab, E., Dungan, R.S., 2018. Effect of dairy manure storage conditions on the survival of E. coli O157:H7 and Listeria. J. Environ. Qual. 47, 185–189.
Bjorkman, J., Nagaev, I., Berg, O.G., Hughes, D., Andersson, D.I., 2000. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science 287, 1479–1482.
Boschiroli, M.L., Falkinham, J., Favre-Bonte, S., Nazaret, S., Piveteau, P., Sadowsky, M.J., Byappanahalli, M., Delaquis, P., Hartmann, A., 2016. Natural soil reservoirs for human pathogenic and fecal indicator bacteria. In: Manual of Environmental Microbiology, Fourth edition. (3.3.2-1-3. 3.2-12).
Carlet, J., 2012. The gut is the epicentre of antibiotic resistance. Antimicrob. Resist. Infect. Control 1.
Carlson, J.C., Mabury, S.A., 2006. Dissipation kinetics and mobility of chlortetracycline, tylosin, and monensin in an agricultural soil in Northumberland County, Ontario, Canada. Environ. Toxicol. Chem. 25 (1), 10.
Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A., Strickland, M., Knowlton, K., 2015. Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. PLoS One 10, e0133764.
Chang, Q.Z., Wang, W.K., Regev-Yochay, G., Lipsitch, M., Hanage, W.P., 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol. Appl. 8, 240–247.
Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. 38, 1086–1108.
Chen, C., Ray, P., Knowlton, K.F., Pruden, A., Xia, K., 2018. Effect of composting and soil type on dissipation of veterinary antibiotics in land-applied manures. Chemosphere 196, 270–279.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072.
Dolliver, H., Gupta, S., Noll, S., 2008. Antibiotic degradation during manure composting. J. Environ. Qual. 37, 1245–1253.
Evanylo, G.K., 2009. Agricultural land application of biosolids in Virginia: managing biosolids for agricultural use. In: Virginia Cooperative Extension.
Fang, H., Wang, H., Cai, L., Yu, Y., 2015. Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. Environ. Sci. Technol. 49, 1095–1104.
Fogler, K., Guron, G.K., Wind, L.L., Keenum, I.M., Hession, W.C., Krometis, L.A., Strawn, L.K., Ponder, M.A., Pruden, A., 2019. Microbiota and antibiotic resistome of lettuce leaves and radishes grown in soils receiving manure-based amendments derived from antibiotic-treated cows. Front. Sustain. Food Syst. 3.
Gou, M., Hu, H.W., Zhang, Y.J., Wang, J.T., Hayden, H., Tang, Y.Q., He, J.Z., 2018. Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. Sci. Total Environ. 612, 1300–1310.
Halling-Sorensen, B., Jacobsen, A.M., Jensen, J., Sengelov, G., Vaclavik, E., Ingerslev, F., 2005. Dissipation and effects of chlortetracycline and tylosin in two agricultural soils: a field-scale study in southern Denmark. Environ. Toxicol. Chem. 24, 802–810.
Hastings, P.J., Rosenberg, S.M., Slack, A., 2004. Antibiotic-induced lateral transfer of antibiotic resistance. Trends Microbiol. 12, 401–404.
He, L.Y., Liu, Y.S., Su, H.C., Zhao, J.L., Liu, S.S., Chen, J., Liu, W.R., Ying, G.G., 2014. Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. Environ. Sci. Technol. 48, 13120–13129.
Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657–666.
Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., 2008. Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol. Biochem. 40, 1892–1900.
Hoornweg, D.; Thomas, L.; Otten, L. Composting and Its Applicability in Developing Countries (English). Urban Waste Management Working Paper Series; No 8 Washington, DC: The World Bank http://documentsworldbankorg/curated/en/483421468740129529/Composting-and-its-applicability-in-developing-countries 2000
Jasni, A.S., Mullany, P., Hussain, H., Roberts, A.P., 2010. Demonstration of conjugative transposon (Tn5397)-mediated horizontal gene transfer between Clostridium difficile and Enterococcus faecalis. Antimicrob Agents Ch 54, 4924–4926.
Jiao, W., Du, R., Ye, M., Sun, M., Feng, Y., Wan, J., Zhao, Y., Zhang, Z., Huang, D., Du, D., Jiang, X., 2018. 'Agricultural waste to treasure' - biochar and eggshell to impede soil antibiotics/antibiotic resistant bacteria (genes) from accumulating in Solanum tuberosum L. Environ. Pollut. 242, 2088–2095.
Landers, T.F., Cohen, B., Wittum, T.E., Larson, E.L., 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep. 127, 4–22.
Leplae, R., Hebrant, A., Wodak, S.J., Toussaint, A., 2004. ACLAME: a CLAssification of Mobile genetic Elements. Nucleic Acids Res. 32, D45–D49.
Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 9, 2490–2502.
Lopatkin, A.J., Huang, S., Smith, R.P., Srimani, J.K., Sysoeva, T.A., Bewick, S., Karig, D.K., You, L., 2016. Antibiotics as a selective driver for conjugation dynamics. Nat. Microbiol. 1, 16044.
Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709.
Martinez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365–367.
Martinez, J.L., Baquero, F., 2014. Emergence and spread of antibiotic resistance: setting a parameter space. Ups. J. Med. Sci. 119, 68–77.
Martinez, J.L., Baquero, F., Andersson, D.I., 2007. Predicting antibiotic resistance. Nat. Rev. Microbiol. 5, 958–965.
Martinez, J.L., Coque, T.M., Baquero, F., 2015. Prioritizing risks of antibiotic resistance genes in all metagenomes. Nat. Rev. Microbiol. 13, 396.
Mc Carthy, G., Lawlor, P.G., Coffey, L., Nolan, T., Gutierrez, M., Gardiner, G.E., 2011. An assessment of pathogen removal during composting of the separated solid fraction of pig manure. Bioresour. Technol. 102, 9059–9067.
McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., Canova, M.J., Pascale, G.D., Ejim, L., Kalan, L., King, A.M., Koteva, K., Morar, M., Mulvey, M.R., O'Brien, J.S., Pawlowski, A.C., Laura, J.V.P., Spanogiannopoulos, P., Sutherland, A.D., Tang, I., Taylor, P.L., Thaker, M., Wang, W., Yan, M., Yu, T., Wright, G.D., 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Ch 57, 3348–3357.
Miller, J.H., Novak, J.T., Knocke, W.R., Pruden, A., 2014. Elevation of antibiotic resistance genes at cold temperatures: implications for winter storage of sludge and biosolids. Lett. Appl. Microbiol. 59, 587–593.
Muurinen, J., Stedtfeld, R., Karkman, A., Parnanen, K., Tiedje, J., Virta, M., 2017. Influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use. Environ. Sci. Technol. 51, 5989–5999.
Oh, M., Pruden, A., Chen, C., Heath, L.S., Xia, K., Zhang, L., 2018. Meta Compare: A computational pipeline for prioritizing environmental resistome risk. FEMS Microbiol. Ecol. 94.
Pan, M., Chu, L.M., 2016. Adsorption and degradation of five selected antibiotics in agricultural soil. Sci. Total Environ. 545-546, 48–56.
Peng, S., Wang, Y., Zhou, B., Lin, X., 2015. Long-term application of fresh and composted manure increase tetracycline resistance in the arable soil of eastern China. Sci. Total Environ. 506-507, 279–286.
Pitta, D.W., Dou, Z.X., Kumar, S., Indugu, N., Toth, J.D., Vecchiarelli, B., Bhukya, B., 2016. Metagenomic evidence of the prevalence and distribution patterns of antimicrobial resistance genes in dairy agroecosystems. Foodborne Pathog. Dis. 13, 296–302.
Pruden, A., Pei, R., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450.
Ray, P., Knowlton, K.F., Shang, C., Xia, K., 2014a. Development and validation of a UPLC-MS/MS method to monitor cephapirin excretion in dairy cows following intramammary infusion. PLoS One 9, e112343.
Ray, P., Knowlton, K.F., Shang, C., Xia, K., 2014b. Method development and validation: solid phase extraction-ultra performance liquid chromatography-tandem mass spectrometry quantification of pirlimycin in bovine feces and urine. J. AOAC Int. 97, 1730–1736.
Ray, P., Chen, C., Knowlton, K.F., Pruden, A., Xia, K., 2017. Fate and effect of antibiotics in beef and dairy manure during static and turned composting. J. Environ. Qual. 46, 45–54.
Roberts, M.C., Sutcliffe, J., Courvalin, P., Jensen, L.B., Rood, J., Seppala, H., 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. 43, 2823–2830.
Roy, J.P., Keefe, G., 2012. Systematic review: what is the best antibiotic treatment for Staphylococcus aureus intramammary infection of lactating cows in North America? Vet. Clin. North Am. Food Anim. Pract. 28, 39.
Ruuskanen, M., Muurinen, J., Meierjohan, A., Parnanen, K., Tamminen, M., Lyra, C., Kronberg, L., Virta, M., 2016. Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment. J. Environ. Qual. 45, 488–493.
Schulz zur Wiesch, P., Engelstadter, J., Bonhoeffer, S., 2010. Compensation of fitness costs and reversibility of antibiotic resistance mutations. Antimicrob. Agents Chemother. 54, 2085–2095.
Shan, S.D., Wang, H., Fang, C.R., Chu, Y.X., Jiang, L.H., 2018. Effects of adding biochar on tetracycline removal during anaerobic composting of swine manure. Chem. Ecol. 34, 86–97.
Sharma, M., Reynnells, R., 2016. Importance of soil amendments: survival of bacterial pathogens in manure and compost used as organic fertilizers. Microbiol. Spectr. 4.
Shterzer, N., Mizrahi, I., 2015. The animal gut as a melting pot for horizontal gene transfer. Can. J. Microbiol. 61, 603–605.
Storteboom, H.N., Kim, S.C., Doesken, K.C., Carlson, K.H., Davis, J.G., Pruden, A., 2007. Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. J. Environ. Qual. 36, 1695–1703.
Tang, X.J., Lou, C.L., Wang, S.X., Lu, Y.H., Liu, M., Hashmi, M.Z., Liang, X.Q., Li, Z.P., Liao, Y.L., Qin, W.J., Fan, F., Xu, J.M., Brookes, P.C., 2015. Effects of long-term manure applications on the occurrence of antibiotics and antibiotic resistance genes (ARGs) in paddy soils: evidence from four field experiments in south of China. Soil

Biol. Biochem. 90, 179–187.
Tien, Y.C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017. Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. Sci. Total Environ. 581–582, 32–39.
Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R.P., Penades, J.R., 2005. Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. Mol. Microbiol. 56, 836–844.
Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. U. S. A. 111, 15202–15207.
Ungureanu, V., 2010. Macrolides, lincosamides, streptogramines (MLS): mechanisms of action and resistance. Bacteriol. Virusol. Parazitol. Epidemiol. 55, 131–138.
United States Food and Drug Administration. Food safety modernization act facts: biological soil amendments: subpart F, 2014. Accessed online at: http://wwwfdagov/downloads/Food/GuidanceRegulation/FSMA/UCM359281pdf 2014;
United States Food and Drug Administration, 2015a. In: Federal Register (Ed.), Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Final Rule. United States Department of Health and Human Services.
United States Food and Drug Administration, 2015b. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. https://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM534243.pdf.
van Elsas, J.D., Semenov, A.V., Costa, R., Trevors, J.T., 2011. Survival of Escherichia coli in the environment: fundamental and public health aspects. ISME J. 5, 173–183.
von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7.
Wang, J., Ben, W., Zhang, Y., Yang, M., Qiang, Z., 2015. Effects of thermophilic composting on oxytetracycline, sulfamethazine, and their corresponding resistance genes in swine manure. Environ. Sci.: Processes Impacts 17, 1654–1660.
Wang, H., Sangwan, N., Li, H.Y., Su, J.Q., Oyang, W.Y., Zhang, Z.J., Gilbert, J.A., Zhu, Y.G., Ping, F., Zhang, H.L., 2017. The antibiotic resistome of swine manure is significantly altered by association with the Musca domestica larvae gut microbiome. ISME J. 11, 100–111.
Wattam, A.R., Abraham, D., Dalay, O., Disz, T.L., Driscoll, T., Gabbard, J.L., Gillespie, J.J., Gough, R., Hix, D., Kenyon, R., Machi, D., Mao, C., Nordberg, E.K., Olson, R., Overbeek, R., Pusch, G.D., Shukla, M., Schulman, J., Stevens, R.L., Sullivan, D.E., Vonstein, V., Warren, A., Will, R., Wilson, M.J., Yoo, H.S., Zhang, C., Zhang, Y., Sobral, B.W., 2014. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res. 42, D581–D591.
Westphal-Settele, K., Konradi, S., Balzer, F., Schonfeld, J., Schmithausen, R., 2018. The environment as a reservoir for antimicrobial resistance: a growing problem for public health? Bundesgesundheitsbla 61, 533–542.
WHO, 2016. Critically important antimicrobials for human medicine. 5th revision. Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. Accessed online at. http://appswhoint/iris/bitstream/handle/10665/255027/9789241512220-engpdf?sequence=1.
Xie, W.Y., Shen, Q., Zhao, F.J., 2018. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur. J. Soil Sci. 69, 181–195.
Xu, S., Amarakoon, I.D., Zaheer, R., Smith, A., Sura, S., Wang, G., Reuter, T., Zvomuya, F., Cessna, A.J., Larney, F.J., McAllister, T.A., 2018. Dissipation of antimicrobial resistance genes in compost originating from cattle manure after direct oral administration or post-excretion fortification of antimicrobials. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 53, 373–384.
Zhang, L., Huang, Y., Zhou, Y., Buckley, T., Wang, H.H., 2013. Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. Antimicrob. Agents Chemother. 57, 3659–3666.
Zhang, Y.J., Hu, H.W., Gou, M., Wang, J.T., Chen, D.L., He, J.Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environ. Pollut. 231, 1621–1632.
Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. U. S. A. 110, 3435–3440.