Author/s:
Zhang, YJ;Hu, HW;Gou, M;Wang, JT;Chen, D;He, JZ

Title:
Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics

Date:
2017-12-01

Citation:
Zhang, Y. J., Hu, H. W., Gou, M., Wang, J. T., Chen, D. & He, J. Z. (2017). Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environmental Pollution, 231 (Pt 2), pp.1621-1632. https://doi.org/10.1016/j.envpol.2017.09.074.

Persistent Link:
https://hdl.handle.net/11343/208831

Title page

Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics

Yu-Jing Zhang[1], Hang-Wei Hu[1,*], Min Gou[1], Jun-Tao Wang[2], Deli Chen[1], Ji-Zheng He[1, 2*]

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

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

*Author for correspondence: Ji-Zheng He, E-mail: jizheng.he@unimelb.edu.au or Hang-Wei Hu, E-mail: hang-wei.hu@unimelb.edu.au

Running head: Effects of different animal manures on soil ARGs

Abstract

Land application of animal manure is a common agricultural practice potentially leading to dispersal and propagation of antibiotic resistance genes (ARGs) in environmental settings. However, the fate of resistome in agro-ecosystems over time following application of different manure sources has never been compared systematically. Here, soil microcosm incubation was conducted to compare effects of poultry, cattle and swine manures spiked with or without the antibiotic tylosin on the temporal changes of soil ARGs. The high-throughput quantitative PCR detected a total of 185 unique ARGs, with Macrolide-Lincosamide-Streptogramin B resistance as the most frequently encountered ARG type. The diversity and abundance of ARGs significantly increased following application of manure and tylosin, with more pronounced effects observed in the swine and poultry manure treatments than in the cattle manure treatment. The level of antibiotic resistance gradually decreased over time in all manured soils but was still significantly higher in the soils treated with swine and poultry manures than in the untreated soils after 130 days' incubation. Tylosin-amended soils consistently showed higher abundances of ARGs than soils treated with manure only, suggesting a strong selection pressure of antibiotic-spiked manure on soil ARGs. The relative abundance of ARGs had significantly positive correlations with integrase and transposase genes, indicative of horizontal transfer potential of ARGs in manure and tylosin treated soils. Our findings provide evidence that application of swine and poultry manures can enrich more soil ARGs than cattle manure, which necessitates the appropriate treatment of raw animal manures prior to land application to minimise the spread of environmental ARGs.

One-sentence summary: Swine and poultry manures are more enriched in ARGs than cattle manure, and can exert stronger selection pressure on soil resistome in soil microcosm incubations.

Keywords: animal manure; tylosin; antibiotic resistance genes; mobile genetic elements; public health

1 Introduction

The increasing prevalence of antibiotic resistance genes (ARGs) in environmental settings and their potential acquisition by human pathogens have become a global public health concern in the 21st century (Rossolini et al., 2014; WHO, 2014). Antibiotic-resistant infections are now responsible for more than half a million human deaths globally each year (Dominey-Howes and Labbate, 2014), while over 10 million deaths in 2050 is predicted if the antibiotic resistance issue is not tackled from now onwards. The over-prescription and abuse of antibiotics in clinical environments, and especially in livestock production, is considered the major cause leading to the global spread of antibiotic resistant bacteria (Sarmah et al., 2006). Environmental ARGs are subjected to potential transmission to human pathogens and commensals via the route of horizontal gene transfer (HGT; conjugation, transduction and transformation) mediated by mobile genetic elements (e.g., plasmids, integrons, and transposons) (Thomas and Nielsen, 2005; Heuer et al., 2011). Therefore, ARGs have been recognized as a novel type of environmental contaminant (Pruden et al., 2006) attracting

emerging efforts to understand the behaviors and mobility of ARGs in diverse environments and their links with clinically-relevant pathogens (Forsberg et al., 2012).

The global consumption of antibiotics substantially increased from 50 billion to 70 billion standard units between 2000-2010, among which 70-80% are applied to livestock industry, especially in poultry, swine, and cattle husbandry (Gelband et al., 2015). Tetracyclines, macrolides, and sulfonamides are three most commonly used antibiotics in livestock, and are also widely used in human medicines for treatment of infections (Jechalke et al., 2014b; Gelband et al., 2015). Along with the increasing consumption of antibiotics, it was found that veterinary antibiotics are poorly digested in the guts of animals, and a significant amount (30-90%) of them are released into the environment through animal urination and defecation (Zhang and Zhang, 2011). Use of veterinary antibiotics posed a strong pressure on the gut microbiomes by selecting antibiotic resistant bacteria through mutation or acquisition of ARGs (Tenover, 2006). An emerging body of studies have reported that there is a positive correlation between dosage of veterinary antibiotics and the occurrence of ARGs in manure and agricultural soils (Bibbal et al., 2007; Byrne-Bailey et al., 2009; Heuer and Smalla, 2007; Hölzel et al., 2010). Therefore, it is imperative to understand the impact of antibiotic usage on ARGs in veterinary settings and their fate after release into the environment.

The abuse of antibiotics in livestock production and the prevalence of ARGs in animal gastrointestinal tract have triggered global concerns that land application of animal manures may lead to the dissemination of ARGs in agro-ecosystems (Udikoviv-Kolic et al., 2014; Hu et al., 2016a). Apart from being recognized as a rich reservoir of ARGs phylogenetically

close to potential human pathogens (Heuer et al., 2011; Forsberg et al., 2012), manure can also provide nutrients for favoring the occurrence of HGT (Smalla et al., 2000). These manure-derived ARGs are in high risk of spreading into the food chain (Marti et al., 2013; Zhu et al., 2017), and may pose a potential threat to public health when vegetables grown in manured soils are consumed by humans. Recent studies have started to shed light on the transmission of manure-derived ARGs in agricultural soils following land application of animal manures (Jechalke et al., 2013, 2014a; Garder et al., 2014; Peng et al., 2015; Hu et al., 2016a; Luby et al., 2016). Most of these studies, however, focused on only single type of manure or a limited number of well-documented ARG types (Heuer and Smalla, 2007). To our knowledge, no studies have attempted to systematically compare the impacts of different sources of animal manures, in the presence or absence of antibiotics, on the temporal patterns of a broad spectrum of ARGs. An improved understanding of this knowledge is critical to prediction of ARG behaviours in soil environments, and development of appropriate manure treatment approaches to minimise the spread of environmental ARGs.

Therefore, the objective of this study is to compare the effects of amendment of three animal manure sources (swine, cattle, and poultry) on the temporal succession of a diverse array of ARGs in soil microcosms incubated with agricultural soils. Tylosin, as a macrolide antibiotic which is used in livestock industries for growth promotion and therapy (Sarmah et al., 2006; Apley et al., 2012), was spiked into manure to test their synergistic selection effects on antibiotic resistance. The high-throughput quantitative PCR array was performed to target 285 ARGs which confer resistance to all major classes of antibiotics; and 10 mobile genetic elements (MGEs) as a proxy for HGT potential of ARGs. We hypothesized that: (i)

amendment of different animal manure sources would differ in their impacts on the temporal succession of soil ARGs owing to their different intrisic ARG profiles and selection pressure; (ii) Tylosin-spiked manure would further enhance the selection of soil ARGs and their HGT potential.

2 Materials and Methods

2.1 Soil and manure sampling

The soil used in this study was taken from a vegetable farm at Clyde (38°07′S, 145°19′E), Victoria, Australia. The soil in this site has a pH value of 7.2 and is classified as loamy sand. Total carbon and total nitrogen are 3.75% and 0.43%, respectively. Cattle manure and swine manure were collected from the Dookie Farm (36°25′S, 145°42′E), University of Melbourne, and poultry manure was taken from a chicken farm in Mornington, Melbourne. Manure samples were collected within three days after excretion without composting, and had no known history of antibiotic treatment. Soil and manure samples were kept on ice during transportation and stored at 4°C before analysis. The antibiotic tylosin used in the experiment was purchased from Sigma-Aldrich company (St Louis, MO, USA).

2.2 Soil microcosm incubations

Three sets of soil microcosm incubation experiments were established depending on the amendment of different animal manure sources, and each set included two treatments (Fig. S1). In the first treatment, manure was mixed thoroughly with sieved soils (<2 mm) to reach a final concentration of 80 mg g^{-1} soil, which is corresponded to a typical agricultural amount of 60 m^3 manure per hectare. In the second treatment, tylosin in aqueous solutions was spiked to the manure and the tylosin-spiked manure was mixed with soil to reach a concentration of

0.1 mg tylosin g^{-1} soil and 60 m^3 manure per hectare. The untreated soil sample with same water content was used as the control treatment. Soil microcosms were established in 250 ml vials with 20 g of soil or manured soil, loosely covered, and incubated in the dark at 25°C. The aerobic condition in the microcosms was maintained by opening the vials for air refreshing every three days. Soil moisture contents were maintained at 60% of the water filled pore space by adding sterilized water regularly. Soil microcosms were destructively sampled at eight time points on days 1, 7, 20, 35, 50, 70, 100, and 130 after manure application.

2.3 Soil physicochemical analysis and genomic DNA extraction

Soil total nitrogen and total carbon were measured using the classic Dumas method of combustion on the isotope-ratio mass spectrometry (Sercon Hydra, Crewe, United Kingdom). Soil pH were measured with a soil to water ratio of 1: 5 using the Orion Star A211 pH Meter (Thermo Scientific Inc., Melbourne, Australia). Soil water content was measured by oven-drying soils samples at 105°C for 24 h. DNA was extracted from soil and manure samples using the MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed using the NanoDrop ND2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.4 High-throughput quantitative PCR

High-throughput qPCR (HT-qPCR) was performed to determine the diversity and abundance of ARGs using the Wafergen SmartChip Real-time PCR system (Fremont, CA, USA). The Wafergen system is a high-throughput qPCR platform with the capability to run

5184 reactions with each volume of 100 nl using the SensiMix SYBR No-ROX reagent (Bioline, London, UK). Primers, DNA and reagents were dispensed into the 5184-nanowell SmartChip using a Multisample NanoDispenser (Fremont). The HT-qPCR array contained a total of 296 primer sets (Su *et al.*, 2015; Hu et al., 2016b), including 285 primer sets targeting eight major classes of ARGs, 10 primer sets targeting MGEs, and one 16S rRNA gene as the internal control (Table S1). The specificity of all primers was verified through amplicon sequencing, BLAST search and amplification efficiency check (Looft *et al.*, 2012; Johnson *et al.*, 2016). The SmartChip was loaded into the Wafergen SmartChip Real-Time PCR Cycler using the thermal-cycling conditions as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30s and 60°C for 30s. Only well data with the amplification efficiencies within 1.7-2.3 were remained. A threshold cycle (C_T) value of 31 was used as the detection limit (Su et al., 2015). A comparative C_T method, also referred to as the $\Delta\Delta C_T$ method of relative profiling, was performed to assess the temporal changes of ARGs across all the samples (Schmittgen and Livak, 2008).

2.5 Quantitative PCR analysis of the bacterial 16S rRNA gene

The absolute abundance of 16S rRNA gene was determined on a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Herculers, USA) using the primer set BACT1369F/PROK1492R (Suzuki et al., 2000). Each 10 µl reaction system included 5 µl SensiMix SYBR No-ROX reagent (Bioline), 0.4 µl of each primer (10 µM), 2 µl DNA template, and 2.2 µl nuclease-free PCR-grade water. Thermal-cycling conditions were as follows: an initial enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s and a final annealing and extension at 60°C for 1 min. A

plasmid containing correct inserts of the bacterial 16S rRNA gene fragment was used as standard curves in tenfold serial dilutions.

2.6 Co-occurrence analysis and network generation

The CoNet Cytoscape plug-in method was used to visualize the co-occurrence patterns of ARGs and MGEs in the network interface (Soffer et al., 2015). ARGs with a minimum occurrence of 3 across all soil samples were regarded as poorly represented ARG subtypes and discarded to reduce artificial correlation bias. The *P*-values were calculated based on two dissimilarity methods (Bray-Curtis and Kullback-Leibler) and two correlation methods (Spearman and Pearson) to control the potential of obtaining false-positive results. The significant pairwise correlations (*P*-value < 0.05) between the ARG and MGE subtypes were utilized to form their co-occurrence networks. The resultant co-occurrence network was visualized using the Frucherman Reingold algorithm on the interactive platform of Gephi (Bastian et al., 2009). A correlation with a correlation coefficient (ρ-value) > 0.8 and a significant level (*P*-value) < 0.05 were considered statistically robust (Junker and Schreiber, 2008).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyse the effects of manure application and tylosin addition on the diversity, relative and absolute abundances of ARGs across different treatments. Pearson's correlation test was performed to test the correlations between the relative abundance of ARGs and MGEs. Non-metric multidimensional scaling (NMDS) analysis based on the relative abundance of ARGs was

191 performed to visualize the Bray-Curtis dissimilarity matrices. The heatmap showing the
192 overall HT-qPCR array data of ARGs with log-transformed relative abundances was
193 generated with the "ggplot2" package in R platform. The Venn diagrams showing the unique
194 and shared ARG subtypes across different treatments and manure sources were performed
195 with the "gplots" package in R platform.

196 **3 Results**

197 **3.1 Diversity of ARGs and MGEs in different treatments**

198 The HT-qPCR array targeting 285 ARGs and 10 MGEs was performed to compare the
199 effects of three types of animal manure with or without tylosin addition on the diversity of
200 ARGs and MGEs in an agricultural soil. The ARGs detected in these samples encompass
201 three major resistance mechanisms: antibiotic deactivation, efflux pump, and cellular
202 protection, with antibiotic deactivation (39% of total ARGs) as the most common resistance
203 mechanism (Fig. S2). The detected ARGs can potentially confer resistance to eight major
204 classes of antibiotics, of which MLSB (20%) and aminoglycoside (19%) were the two most
205 frequently detected ARG types (Fig. 1a). Other frequently detected ARGs included multidrug
206 (18%), tetracycline (17%) and β-lactamase (13%) (Fig. 1a).

207 Untreated soil samples harbored the lowest ARG diversity (68.8 ± 2.7 on average)
208 among all the treatments (Fig. 1b). The average numbers of ARGs detected in poultry manure
209 (109.7 ± 5.9) and swine manure (109.0 ± 4.2) were significant higher than that in cattle
210 manure (82.0 ± 5.0) ($P < 0.05$). Among all the manure-treated samples without tylosin, the
211 number of detected ARGs ranged from 58 to 133 in individual samples and a significantly

higher ARG diversity was observed in poultry and swine manure-treated samples compared with cattle manure-treated samples (Fig. 1b, $P < 0.05$). Tylosin-spiked cattle manure also harbored the lowest diversty of ARGs among all manure-treated samples spiked with tylosin (Fig. 1b). The average numbers of detected MGEs were similar among all the samples but slightly higher in the manure samples (Fig. 1b).

Venn diagrams showed the number of unique and shared ARGs among different manure and soil samples (Figs. 1c and 1d). A total of 86 ARGs were shared among the three types of animal manures, and 22, 19 and 3 unique ARGs were observed in swine, poultry, and cattle manures, respectively (Fig. 1c). Untreated soil samples shared 129 ARGs with manure-treated samples, and amendment of poultry, cattle, and swine manures could introduce 32, 28, and 28 unique ARGs, respectively, into soils (Fig. 1d).

3.2 Abundance of ARGs and MGEs in different treatments

The comparative C_T method was used to calculate the relative abundances of ARGs and MGEs by normalizing to 16S rRNA gene in the same HT-qPCR array (Schmittgen and Livak, 2008). The relative and absolute abundances of ARGs in cattle manure samples were significantly lower compared with those in swine and poultry manure samples, with swine manure harbouring the highest abundance of ARGs (Figs. 2a and 2c). When animal manure was applied to the soil, the relative and absolute abundances of ARGs in soil samples treated with cattle and swine manure were significantly lower than those in poultry manure-treated samples (Figs. 2b and 2d). In manure-treated samples spiked with tylosin, the ARG abundances were consistently higher than those in samples without tylosin addition (Figs. 2b

and 2d). The pattern of MGE abundances was quite similar to that of ARG abundances, with the lowest abundances found in cattle manure and cattle manure-treated soil samples (Fig. 2).

3.3 Temporal changes of ARGs and MGEs in manure-treated soils

Soil microcosms were destructively sampled at eight time points (days 1, 7, 20, 35, 50, 70, 100, and 130) during the incubation to explore the temporal changes of ARGs and MGEs in soils treated with three sources of animal manures with or without tylosin (Fig. 3). The numbers of detected ARGs in manure-treated soils gradually decresed over time during the incubation, regardless of whether or not tylosin was added (Fig. 3a). Interestingly, the ARG numbers in soils treated with cattle manure approached the background levels of ARGs in untreated soils at the end of incubation (day 130), but swine and poultry manure-amended soils had a significantly higher level of ARGs than untreated soils at day 130 (Fig. 3a). The relative abundances of ARGs in soils treated with poultry and cattle manures declined over time but were still higher than that in untreated soils, with a more pronounced selection effect observed in tylosin-amended samples (Fig. 3b). The tylosin-amended manure application showed consistently higher relative abundances of ARGs compared with their corresponding no-tylosin treatments (Fig. 3b). The ARG abundances in swine manure-treated samples declined sharply to background levels in untreated soils within 20 days of incubation, and then remained largely unchanged till day 130 (Fig. 3b).

The number and relative abundances of MGEs exhibited highly similar temporal patterns to ARGs across different treatments (Figs. 3c and 3d). Pearson's correlation analysis

revealed that the relative abundance of total ARGs was significantly correlated with that of total transposase genes ($P < 0.001$) and total integrase genes ($P < 0.001$) (Fig. 4). The abundances of the eight major classes of ARGs were also significantly and positively correlated with total transposase genes and total integrase genes, except MLSB and vancomycin resistance genes (Table 1).

3.4 Temporal changes of individual subtypes of ARGs

The distribution and temporal changes of individual ARG subtypes across different treatments were assessed at a higher resolution level in heatmap based on the log-transformed relative abundance of each ARG subtypes (Fig. 5). The untreated soil samples showed the lowest detection frequencies of ARGs, which remained largely stable during the incubation. In general, manure application resulted in obvious increases in the relative abundances of a majority of ARG subtypes, and the effect was more pronounced in manure spiked with tylosin treatments. Poultry and swine manures had much stronger selection pressure on enhancing the ARGs abundance than cattle manure. The NMDS ordination based on the Bray-Curtis dissimilarity matrices revealed that soil samples treated with different animal manures clustered separately, and samples treated with both manure and tylosin tended to separate from those treated with manure only (Fig. 6).

These ARGs showed different temporal patterns during the incubation (Fig. 6): (i) Some genes conferring resistance to aminoglycoside (*aacC4, aadA-01, aadA-02, aadA-1-02, aadA2-01, aadA2-02*), FCA, MLSB (*ermY, lnuA-01*), multidrug (*oprD*) and tetracycline (*tetD-02*), which were present in untreated soil samples, became remarkably more abundant

in soils treated with manure and tylosin, especially in the poultry and swine manure treatments; (ii) Some genes conferring resistance to multidrug (*yceL/mdtH-03*, *yidY/mdtL-01*, *yidY/mdtL-02*) and tetracycline (*tetA-02*, *tetB-01*, *tetB-02*, *tetC-01*), which were absent in untreated soil samples, appeared in manure and tylosin treated samples, and persisted until the end of the incubation; (iii) Several multidrug resistance genes (*qacH-01*, *qacH-02*, *rarD-02*, *tolC-01*, *tolC-02* and *tolC-03*) and vancomycin resistance genes (*vanRA-02*, *vanRB*) were abundant in three types of manures, however, decreased over time in all the manured soils and became undetected at the end of the incubation; (iv) Most β-lactam resistant genes kept relatively unchanged in soils amended with manure in the presence or absence of tylosin.

3.5 Co-occurrence patterns among ARGs and MGEs

The network analysis was performed to explore the co-occurrence patterns of ARGs and MGEs based on strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations. The resultant network was composed of 73 nodes (ARG subtypes) and 209 edges (pairwise correlations) (Fig. 7a), and could be clearly separated into six modules (Fig. 7b). Each module consisted of different types of ARGs, except module IV which included exclusively five tetracycline resistance genes. The most densely connected node in each module was defined as the 'hub', for example, the '*tolC-03*' (multidrug resistance) and '*aadA2-01*' (aminoglycoside resistance) were the hubs for modules I and II, respectively (Fig. 7b). The module III had three equivalent hub genes *vgb-01*, *acrA-05* and *yidY/mdtL-01*. It has been suggested that hubs could be regarded as indicator ARGs to represent the quantity of other co-occurring ARGs in the same module (Li et al., 2015). Interestingly, the *intI1* gene (belonging to MGEs) had

296 intensive connections with multiple ARGs which can potentially confer resistance to multiple
297 classes of antibiotics (Fig. 7a).

298 **4. Discussion**

299 **4.1 Poultry and swine manures have stronger selection pressure on soil resistome than**
300 **cattle manure**

301 Animal manure has been long regarded as an important reservoir of ARGs, no matter
302 whether or not the animals have been treated with antibiotics (Heuer et al., 2011; Hu et al.,
303 2016a). Land application of animal manure, as a common agricultural practice, may
304 introduce the inflow of a large amount of fecal microbiome including antibiotic resistant
305 bacteria and potential human pathogens into the soil environment (Chee-Sanford et al., 2009;
306 Heuer et al., 2011; Wang et al., 2015). Functional metagenomic analysis revealed that
307 manure-derived ARGs can account for up to 70% of the total ARGs in soils following
308 manure application (Su et al., 2014). Swine, cattle, and poultry manures are three major types
309 of widely-used organic fertilizers worldwide (Wichmann et al., 2014), but no studies have
310 systematically compared their effects on the temporal patterns of soil ARGs. In this study, we
311 found that swine manure harboured the highest diversity and abundance of ARGs, followed
312 by poultry manure, while the diversity and abundance of ARGs in cattle manure were
313 significantly lower than the other two manure types. This result supported previous findings
314 that animal manure is a “hotspot” for environmental contamination with ARGs (Chee-
315 Sanford et al., 2001; Sengeløv et al., 2003; Heuer and Smalla, 2007) even from antibiotic-free
316 animals (Jackson et al., 2004; Looft et al., 2012; Udikoviv-Kolic et al., 2014; Hu et al.,

317 2016a). Our findings imply that attentions should be particularly paid to swine and poultry
318 manures which need to be properly treated, i.e. by composting, to reduce the levels of ARGs
319 before land application.

320 Although the untreated agricultural soil examined in this study had a diverse array of
321 ARGs (Fig. 1b), its ARG abundances were significantly lower than all three types of animal
322 manures. Therefore, manure application can dramatically increase the abundance of soil
323 ARGs (Figs. 2b and 2d), and can also introduce a number of unique manure-derived ARGs
324 into soil (Fig. 2). For example, swine manure is highly enriched in tetracycline, sulfonamide,
325 MLSB, and β-lactam resistance genes, while poultry manure is enriched in multidrug, and
326 aminoglycoside resistance genes, and thus application of these two manures substantially
327 enhanced abundances of these genes, resulting in contrasting profiles of ARGs across the
328 different manure treatments (Fig. 2). Cattle manure application had relatively slight impacts
329 on soil ARG diversity compared to poultry and swine manures (Fig. 1), but still significantly
330 increased soil ARG abundances, in particular, the abundances of aminoglycoside and
331 multidrug resistance genes (Fig. 2). Therefore, different sources of animal manures could
332 manipulate soil resistome in varying magnitudes, and different soil ARG profiles in manured
333 soils would differ in their consequences for soil and public health. The differences in ARG
334 profiles in the three manure sources could be attributed to the dietary, antibiotic use history,
335 and indigenous gut resistome of the animals.

336 Despite the different ARG profiles in soils treated with different animal manure
337 sources, the diversity and abundance of ARGs in all manured soils gradually decreased over
338 time (Fig. 3). Notably, the abundance of ARGs in swine manure-amended soils dramatically

339 declined to the background levels of ARGs in untreated soils within 20 days, whereas ARGs
340 in poultry and cattle manure-treated soils persisted in the entire course of incubation and were
341 still more abundant than background levels at day 130. This finding has implications for
342 agricultural management practice from the perspective of minimizing antibiotic resistance:
343 raw swine manure can be applied to the field one month before the vegetable harvest, but raw
344 poultry and cattle manures should be used at least three ~ four months before harvest, with
345 poultry manure having the highest risk of ARGs spreading. The time-course reduction of
346 ARGs following manure application may be explained by the gradual out-competition of
347 manure-derived bacteria by the soil indigenous microbiomes, and the different conditions
348 between animal gut and soil environments (Chee-Sanford et al., 2009; Hu et al., 2016a). A
349 number of ARGs, such as *aacC4* and *aadA* genes (aminoglycoside resistance); *ermY* and
350 *lnuA-01* genes (MLSB resistance); *oprD* gene (multidrug resistance); and *tetD-02* gene
351 (tetracycline resistance), persisted until the end of the incubation, particularly in the poultry
352 and swine manure treatments (Fig. 5). Therefore, these ARGs might have the highest
353 potential to be captured by human pathogens and pose a threat to human health.

354 **4.2 Antibiotic tylosin amendment imposed an additional selection pressure on ARGs**

355 The use of human-made antibiotics in livestock production and human medicines was
356 considered as the major reason for the exponentially increasing ARGs in environmental
357 samples since 1940 (Knapp et al., 2010). Many previous studies have investigated the effects
358 of manure collected from antibiotic-treated animals on the composition and mobility potential
359 of ARGs in natural settings (Jechalke et al., 2014a; Jechalke et al., 2013; Luby et al., 2016).
360 No studies have, however, provided comprehensive insights into the impacts of the antibiotic

tylosin on the temporal patterns of a wide spectrum of soil ARGs. Tylosin belongs to macrolide subclass in the MLSB class, which was the most frequently detected antibiotic resistance type in this experiment (Fig. 1a). Although it is supposed that tylosin has a short half-life of a couple of days in soils (Kolz et al., 2005), its impacts would last for a long period after its decomposition, as demonstrated by the persistence of ARGs for many years in the absence of the corresponding antibiotic (Johnsen et al., 2009). Therefore, it is not surprising to observe increased abundances of MLSB resistance genes in tylosin-spiked poultry and swine manure-treated soils until the end of the incubation (Fig. 2), indicating the direct selection effect of tylosin on the MLSB resistance type.

Erythromycin ribosome methylation (*erm*) genes encoding tylosin resistance by reducing the ability of tylosin from binding to the 50S ribosomal subunit (Leclercq and Courvalin, 1991; Weisblum, 1998), and they are detected in bacteria isolates of human origin which may constitute a health risk for human (Rollins et al., 1985). Three main *erm* genes have been described in *staphylococci*: *erm*(A) gene is located on transposon Tn*554*, *erm*(B) gene on transposon Tn*551* and *erm*(C) gene on a plasmid (Saribas et al., 2006), suggesting the HGT potential of the *erm* genes. We detected 11 *erm* genes in this study, and these genes were obviously more abundant in the tylosin-amended treatments, especially in the poultry manure and swine manure treatments (Fig. 5). In the swine manure treatments, the relative abundances of *erm* genes were significantly higher in the tylosin-amended treatments compared with the tylosin-absent treatments (Fig. S3). Therefore, the presence of tylosin in animal manures can impose strong selection pressure on soil microbiome, and increase the propagation of tylosin-resistant bacteria.

Beyond the selection effect of tylosin on MLSB resistance genes, it is interesting to find that ARGs encoding resistance to almost all the major classes of ARGs were clearly enriched in tylosin-amended treatments compared with manured soils without tylosin (Fig. 2). This no-targeted selection phenomenon might be explained by the co-selection mechanisms (Hu et al., 2016b) in which genes encoding resistance to different antibiotics may reside in the same MGEs (plasmid, integrin, or transposon) or single genes can encode resistance to various classes of ARGs. As shown in the co-occurrence network (Fig. 7), genes conferring resistance to different categories of antibiotics were shared in the same module, suggesting that these ARGs might be carried by the same bacterial cells or MGEs, and they can change and transfer together under the selection pressure imposed by tylosin. Therefore, our findings caution that the addition of tylosin can select for a broad range of ARGs apart from MLSB resistance, and elongate the lifespan of ARGs throughout the whole incubation period (in poultry and swine manured soils).

4.3 The HGT potential of ARGs in soils treated with manure and tylosin

The HGT of ARGs among environmental bacteria of different taxa is an important pathway for resistance dissemination and the subsequent acquisition of resistance by human pathogens and commensals (Heuer et al., 2011; Forsberg et al., 2012). Previous studies have demonstrated that manure addition may promote the HGT potential of soil ARGs, because manure generally contains high loads of broad-host-range plasmids, which are important vectors of ARGs (Heuer et al., 2011). Antibiotics could stimulate the HGT of ARGs as well mostly via accelerating conjugation (Whittle et al., 2002; Ohlsen et al., 2003). Under the selection pressure of antibiotic, fecal microbes that cannot persist in the soil environment may

transfer ARGs to resident soil bacteria via HGT mediated by MGEs (Karami et al., 2007; Heuer et al., 2011). Once being transferred, the resistance traits are likely persistent in natural settings because native bacteria are generally better adapted to the soil environment (Chee-Sanford et al., 2009). Therefore, amendment of both manure and antibiotic is likely to interactively promote the HGT potential of ARGs in soils.

In this study, the abundances of both integrase and transposase genes were significantly and positively correlated with those of total ARGs and individual ARG types across all the treatments (Table 1), indicating that there is a risk for HGT of ARGs in the tested agricultural soils after manure/antibiotic application. Particularly, the relative abundances of MGEs were significantly higher in poultry manure treatment compared with untreated soil samples and other manure treatments, and this enrichment was even more remarkable in the presence of tylosin (Fig. 2). Therefore, ARGs in the poultry manure-treated soils may have greater HGT potential than those in other treatments. In addition, we found that the *intI1* gene (belonging to MGEs) had intensive connections with multiple ARGs conferring resistance to multiple classes of antibiotics in the co-occurrence network, suggesting that class 1 integron may play important roles in dissemination of these co-occurring ARGs, which is also reported in previous studies (Gillings et al., 2015). Altogether, the enormous diversity of ARGs and MGEs in manure-treated agricultural soils provides a high likelihood of dispersal and HGT of soil ARGs, the actual frequencies of which should be tested in future studies.

5 Conclusions

In conclusion, by combining HT-qPCR ARG arrays with soil microcosm incubations, we provide comprehensive evidence that poultry and swine manures have stronger impacts

on the diversity, abundance and HGT potential of a wide spectrum of soil ARGs than cattle manure. Such effects on soil resistome were enhanced by addition of the antibiotic tylosin, which selected for increased resistance to multiple categories of antibiotics and prolonged the persistence of ARGs during the incubation. Our findings have important implications for public health if these enriched ARGs following manure application can be transferred into the food chain through human consumption of the harvested vegetables, and necessitate the appropriate treatment of raw animal manures (especially poultry and swine manures) to minimise the dissemination of environmental ARGs

Acknowledgements

This work was financially supported by Australian Research Council (DP170103628, DE150100870).

References

Apley, M. D., Bush, E. J., Morrison, R. B., Singer, R. S., and Snelson, H. (2012). Use estimates of in-feed antimicrobials in swine production in the United States. *Foodborne pathogens and disease* **9**, 272-279.

Bastian, M., Heymann, S., and Jacomy, M. (2009). Gephi: an open source software for exploring and manipulating networks. *ICWSM* **8**, 361-362.

Bibbal, D., Dupouy, V., Ferré, J.-P., Toutain, P.-L., Fayet, O., Prere, M.-F., and Bousquet-Mélou, A. (2007). Impact of three ampicillin dosage regimens on selection of ampicillin

resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces. *Applied and Environmental Microbiology* **73**, 4785-4790.

Byrne-Bailey, K., Gaze, W. H., Kay, P., Boxall, A. B., Hawkey, P. M., and Wellington, E. M. (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrobial Agents and Chemotherapy* **53**, 696-702.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y.-F., Yannarell, A. C., Maxwell, S., and Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of environmental quality* **38**, 1086-1108.

Dominey-Howes, D., and Labbate, M. (2014). The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in public health* **2**, 145.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., and Dantas, G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**, 1107-1111.

Garder, J. L., Moorman, T. B., and Soupir, M. L. (2014). Transport and Persistence of Tylosin-Resistant Enterococci, Genes, and Tylosin in Soil and Drainage Water from Fields Receiving Swine Manure. *Journal of environmental quality* **43**, 1484-1493.

Gelband, H., Molly Miller, P., Pant, S., Gandra, S., Levinson, J., Barter, D., White, A., and Laxminarayan, R. (2015). The state of the world's antibiotics 2015. *Wound Healing Southern Africa* **8**, 30-34.

469 Gillings, M.R., Gze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., and Zhu, Y.G. (2015) Using
470 the class 1 integron-integrase gene as a proxy for anthropogenic pollution. The ISME
471 Journal 9, 1269-1279.
472 Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure
473 application on agricultural fields. *Current opinion in microbiology* **14**, 236-243.
474 Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial
475 antibiotic resistance in soil over at least two months. *Environmental Microbiology* **9**,
476 657-666.
477 Hölzel, C. S., Harms, K. S., Küchenhoff, H., Kunz, A., Müller, C., Meyer, K., Schwaiger, K.,
478 and Bauer, J. (2010). Phenotypic and genotypic bacterial antimicrobial resistance in
479 liquid pig manure is variously associated with contents of tetracyclines and
480 sulfonamides. *Journal of applied microbiology* **108**, 1642-1656.
481 Hu, H.W., Han, X.M., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., and He, J.Z. (2016a)
482 Temporal changes of antibiotic resistance genes and bacterial communities in two
483 contrasting soils treated with cattle manure. FEMS Microbiology Ecology 92, fiv196.
484 Hu, H. W., Wang, J. T., Li, J., Li, J. J., Ma, Y. B., Chen, D., and He, J. Z. (2016b). Field-based
485 evidence for copper contamination induced changes of antibiotic resistance in
486 agricultural soils. *Environmental microbiology* **18**, 3896-3909.
487 Jackson, C. R., Fedorka-Cray, P. J., Barrett, J. B., and Ladely, S. R. (2004). Effects of tylosin
488 use on erythromycin resistance in enterococci isolated from swine. *Applied and*
489 *environmental microbiology* **70**, 4205-4210.

Jechalke, S., Focks, A., Rosendahl, I., Groeneweg, J., Siemens, J., Heuer, H., and Smalla, K. (2014a). Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. *FEMS microbiology ecology* **87**, 78-88.

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., and Smalla, K. (2014b). Fate and effects of veterinary antibiotics in soil. *Trends in microbiology* **22**, 536-545.

Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krögerrecklenfort, E., Brandes, N., Nordwig, M., Ding, G.-C., and Siemens, J. (2013). Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. *Applied and environmental microbiology* **79**, 1704-1711.

Johnsen, P. J., Townsend, J. P., Bøhn, T., Simonsen, G. S., Sundsfjord, A., and Nielsen, K. M. (2009). Factors affecting the reversal of antimicrobial-drug resistance. *The Lancet infectious diseases* **9**, 357-364.

Johnson TA, Stedtfeld RD, Wang Q, Cole JR, Hashsham SA, Looft T, *et al.* (2016) Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. *mBio* **7**, e02214-15.

Junker, B., and Schreiber, F. (2008). Correlation networks. *Analysis of Biological Networks*.

Kolz, A., Moorman, T., Ong, S. K., Scoggin, K., and Douglass, E. (2005). Degradation and metabolite production of tylosin in anaerobic and aerobic swine-manure lagoons. *Water Environment Research* **77**, 49-56.

Knapp, C.W., Dolfing, J., Ehlert, P.A., and Graham, D.W. (2010) Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environmental Science and Technology 44, 580-587.

513 Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J.,
514 Stedtfeld, T. M., Chai, B., and Cole, J. R. (2012). In-feed antibiotic effects on the swine
515 intestinal microbiome. *Proceedings of the National Academy of Sciences* **109**, 1691-
516 1696.
517 Luby, E. M., Moorman, T. B., and Soupir, M. L. (2016). Fate and transport of tylosin-resistant
518 bacteria and macrolide resistance genes in artificially drained agricultural fields
519 receiving swine manure. *Science of the Total Environment* **550**, 1126-1133.
520 Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., and Topp, E. (2013)
521 Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and
522 frequency of detection of antibiotic resistance genes in soil and on vegetable at harvest.
523 Applied and Environmental Microbiology 79, 5701-5709.
524 Peng, S., Wang, Y., Zhou, B., and Lin, X. (2015). Long-term application of fresh and
525 composted manure increase tetracycline resistance in the arable soil of eastern China.
526 *Science of the Total Environment* **506**, 279-286.
527 Pruden, A., Pei, R., Storteboom, H., and Carlson, K.H. (2006) Antibiotic resistance genes as
528 emerging contaminants: studies in Northern Colorado. Environmental Science and
529 Technology 40, 7445-7450.
530 Rollins, L., Lee, L., and LeBlanc, D. (1985). Evidence for a disseminated erythromycin
531 resistance determinant mediated by Tn917-like sequences among group D streptococci
532 isolated from pigs, chickens, and humans. *Antimicrobial agents and chemotherapy* **27**,
533 439-444.

534 Rossolini, G. M., Arena, F., Pecile, P., and Pollini, S. (2014). Update on the antibiotic
535 resistance crisis. *Current opinion in pharmacology* **18**, 56-60.
536 Saribas, Z., Tunckanat, F., and Pinar, A. (2006). Prevalence of erm genes encoding macrolide-
537 lincosamide-streptogramin (MLS) resistance among clinical isolates of Staphylococcus
538 aureus in a Turkish university hospital. *Clinical microbiology and infection* **12**, 797-
539 799.
540 Sarmah, A. K., Meyer, M. T., and Boxall, A. B. (2006). A global perspective on the use, sales,
541 exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the
542 environment. *Chemosphere* **65**, 725-759.
543 Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative
544 CT method. *Nature protocols* **3**, 1101-1108.
545 Smalla, K., Heuer, H., Götz, A., Niemeyer, D., Krögerrecklenfort, E., and Tietze, E. (2000).
546 Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries
547 reveals a high prevalence and diversity of IncQ-like plasmids. *Applied and*
548 *Environmental Microbiology* **66**, 4854-4862.
549 Soffer, N., Zaneveld, J., and Vega Thurber, R. (2015). Phage–bacteria network analysis and its
550 implication for the understanding of coral disease. *Environmental microbiology* **17**,
551 1203-1218.
552 Su, J. Q., Wei, B., Xu, C. Y., Qiao, M., and Zhu, Y. G. (2014). Functional metagenomic
553 characterization of antibiotic resistance genes in agricultural soils from China.
554 *Environment international* **65**, 9-15.

555 Suzuki, M.T., Taylor, L.T., and Delong, E.F. (2000) Quantitative analysis of small-subunit
556 rRNA genes in mixed microbial populations via 5'-nuclease assays. Applied and
557 Environmental Microbiology 66, 4605-4614.

558 Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *The American*
559 *journal of medicine* **119**, S3-S10.

560 Thomas, C. M., and Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene
561 transfer between bacteria. *Nature reviews microbiology* **3**, 711-721.

562 Udikoviv-Kolic, N., Wichmann, F., Broderick, N.A., and Handelsman, J. (2014) Bloom of
563 resident antibiotic-resistant bacteria in soil following manure fertilization. Proc Natl
564 Acad Sci USA 111, 15202-15207.

565 Wang, F.-H., Qiao, M., Chen, Z., Su, J.-Q., and Zhu, Y.-G. (2015). Antibiotic resistance genes
566 in manure-amended soil and vegetables at harvest. *Journal of Hazardous Materials* **299**,
567 215-221.

568 Weisblum, B. (1998). Macrolide resistance. *Drug Resistance Updates* **1**, 29-41.

569 World Health Organization. (2014). *Antibiotic Resistance: Global Report on Surveillance*;
570 World Health Organization: Geneva, Switzerland.

571 Zhang, X.X., and Zhang, T. (2011) Occurrence, abundance, and diversity of tetracycline
572 resistance genes in 15 sewage treatent plants across China and other global locations.
573 Environmental Science and Technology 45, 2598-2604.

574 Zhu, B., Chen, Q., Chen, S., and Zhu, Y. (2017) Does organically produced lettuce harbor
575 higher abundance of antibiotic resistance genes than conventionally produced?
576 Environment International 98, 152-159.

577 Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., et al. (2013)
578 Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl
579 Acad Sci USA 110: 3435-3440.

580

Table 1 Pearson correlations between the relative abundance of ARGs and MGEs in all the samples.

	Relative abundance of MGEs	
	Transposase	Integrase
Total ARGs	**0.617 (< 0.001)**	**0.733 (< 0.001)**
Aminoglycoside	**0.926 (< 0.001)**	**0.826 (< 0.001)**
β-lactamase	**0.23 (< 0.001)**	**0.477 (< 0.001)**
FCA	**0.482 (< 0.001)**	**0.473 (< 0.001)**
MLSB	0.242 (0.088)	**0.239 (< 0.001)**
Multidrug	**0.885 (< 0.001)**	**0.943 (< 0.001)**
Sulfonamide	**0.840 (0.015)**	**0.194 (0.001)**
Tetracycline	**0.305 (< 0.001)**	**0.486 (< 0.001)**
Vancomycin	**0.269 (< 0.001)**	0.338 (0.072)
Other	**0.514 (< 0.001)**	**0.194 (0.001)**

Values showed in the table are the r-values derived from the Pearson analysis, and the *P*-values are showed in the brackets. The bold numbers represent significant correlations ($P < 0.05$).

Figure captions

Figure 1. (a) Classification of the 185 ARGs detected in all the samples based on the classes of antibiotic to which they confer resistance. (b) The average number of ARGs and MGEs detected in each treatment. Different letters above the bars indicate a significant difference ($P < 0.05$). Venn diagram shows the number of unique and shared ARGs among the three types of manures (c) and among the untreated soils and manured soils (d). (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance. CK, untreated soil samples; PM, poultry manure treatment; PMA, tylosin-amended poultry manure treatment; CM, cattle manure treatment; CMA, tylosin-amended cattle manure treatment; SM, swine manure treatment; SMA: tylosin-amended swine manure treatment; UPM, untreated poultry manure; UCM, untreated cattle manure; USM, untreated swine manure)

Figure 2. (a) Relative abundance of ARGs and MGEs detected across different treatments. (b) Absolute abundance of ARGs and MGEs detected across different treatments.

Figure 3. (a) The temporal changes of the average number of detected ARGs across the different treatments. (b) The temporal changes of the relative abundance of ARGs across the different treatments.

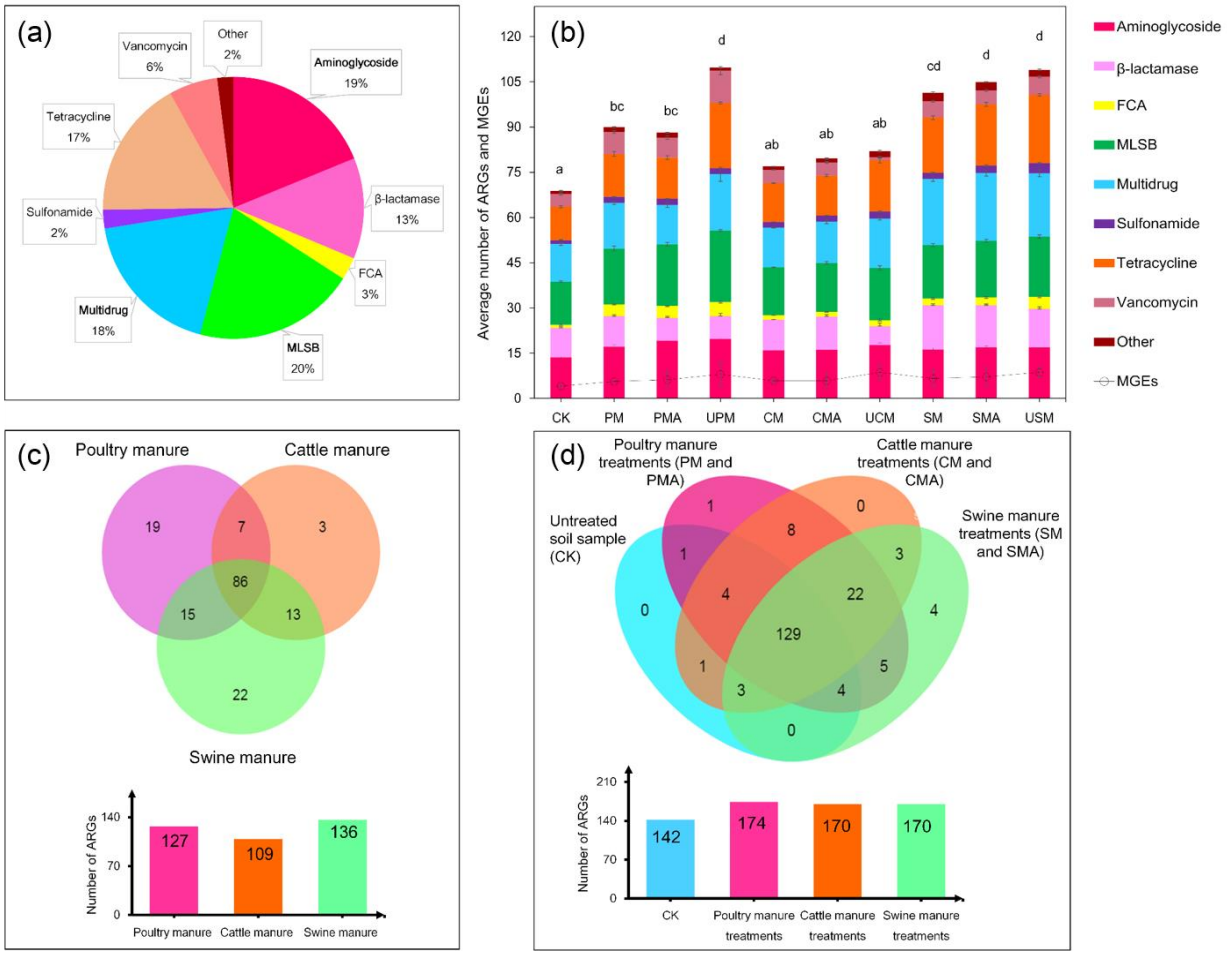
Figure 4. Correlation between the relative abundance of total ARGs and the relative abundances of transposase genes or integrase genes.

Figure 5. The heat map showing the temporal changes of the log-transformed relative abundance of ARGs across different treatments. Three columns at each sampling time point represent three independent replicates, and each row reprents a specfic ARG subtype.

Figure 6. Non-metric multidimensional scaling ordination plot depicts the Bray-Curtis dissimilarity matrices between soils based on the relative abundance of ARGs. The 2D stress value is 0.14, which indicated that the two-dimensional ordinations could well represent the data.

Figure 7. The networks depicting the co-occurrence patterns among the detected ARGs and MGEs. The nodes coded with different colors represent different classes of ARGs (a) and different modules (b). The edges connecting nodes correspond to statistically significant correlations between nodes. Node size is proportional to the number of connections between nodes (degree).

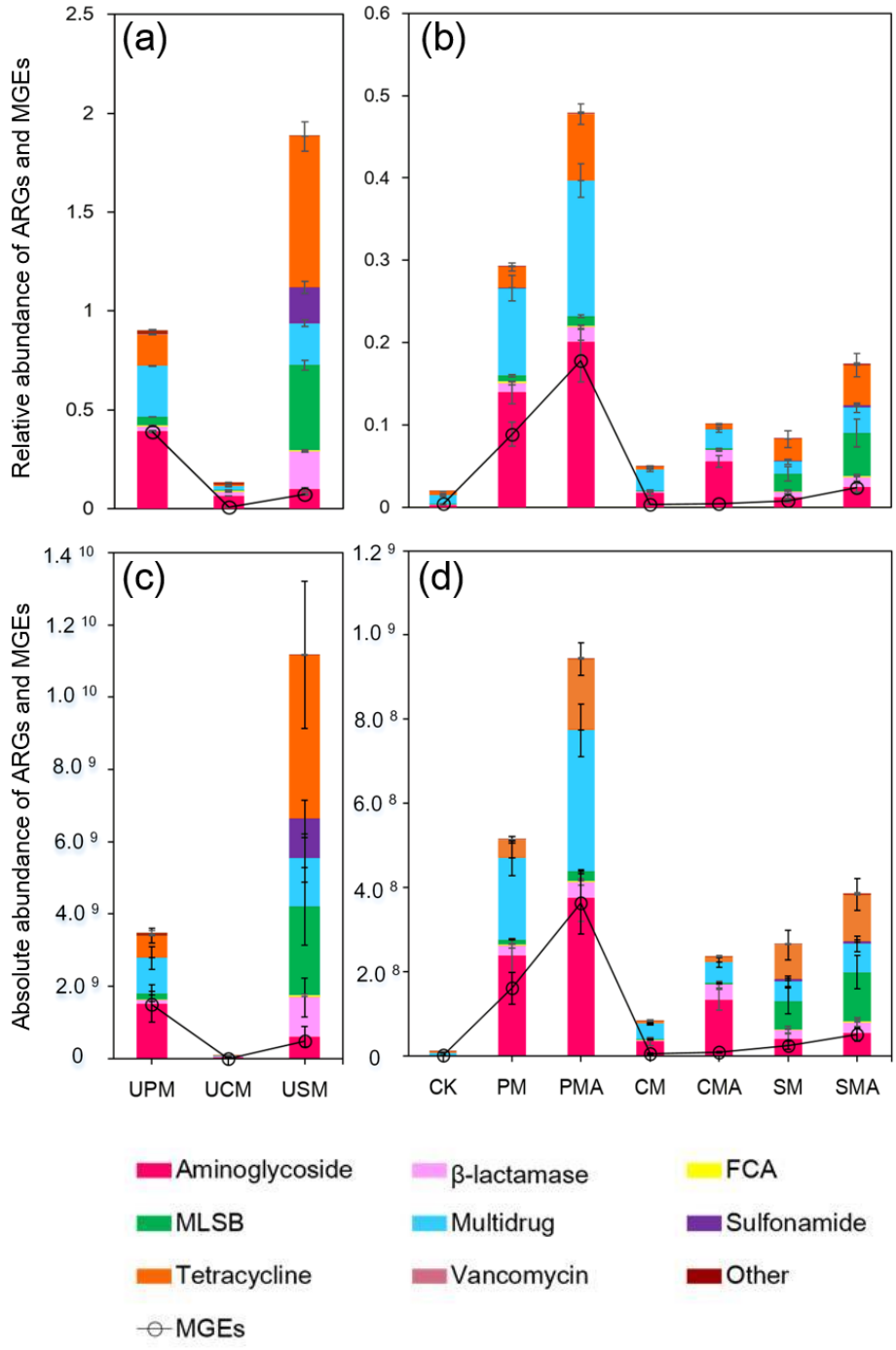
620 **Fig. 1**



621

622

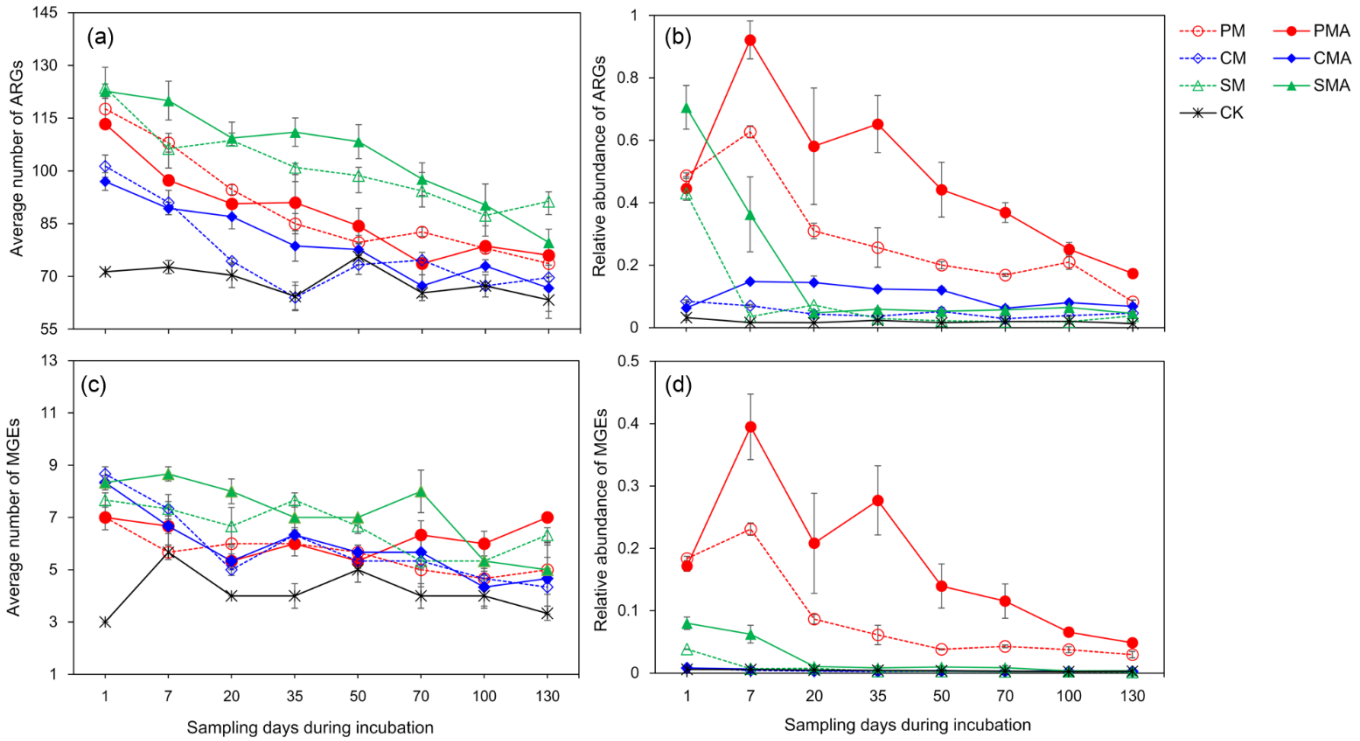
623 **Fig. 2**



624

625

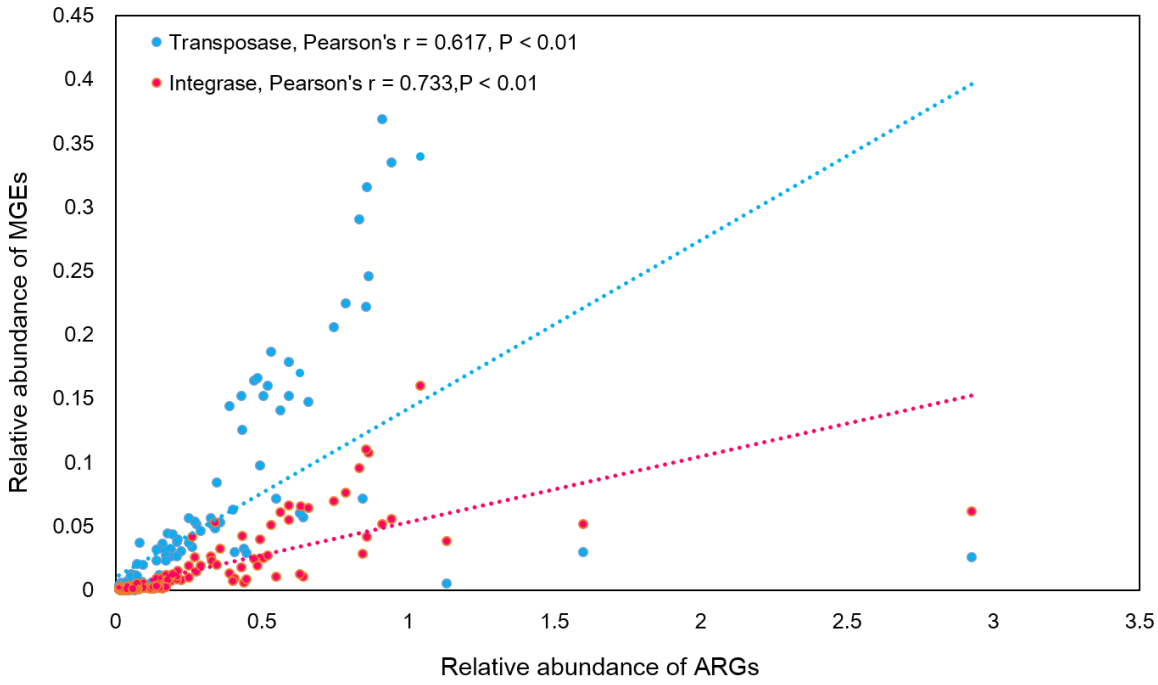
626 **Fig. 3**



627

628

629 **Fig. 4**



630

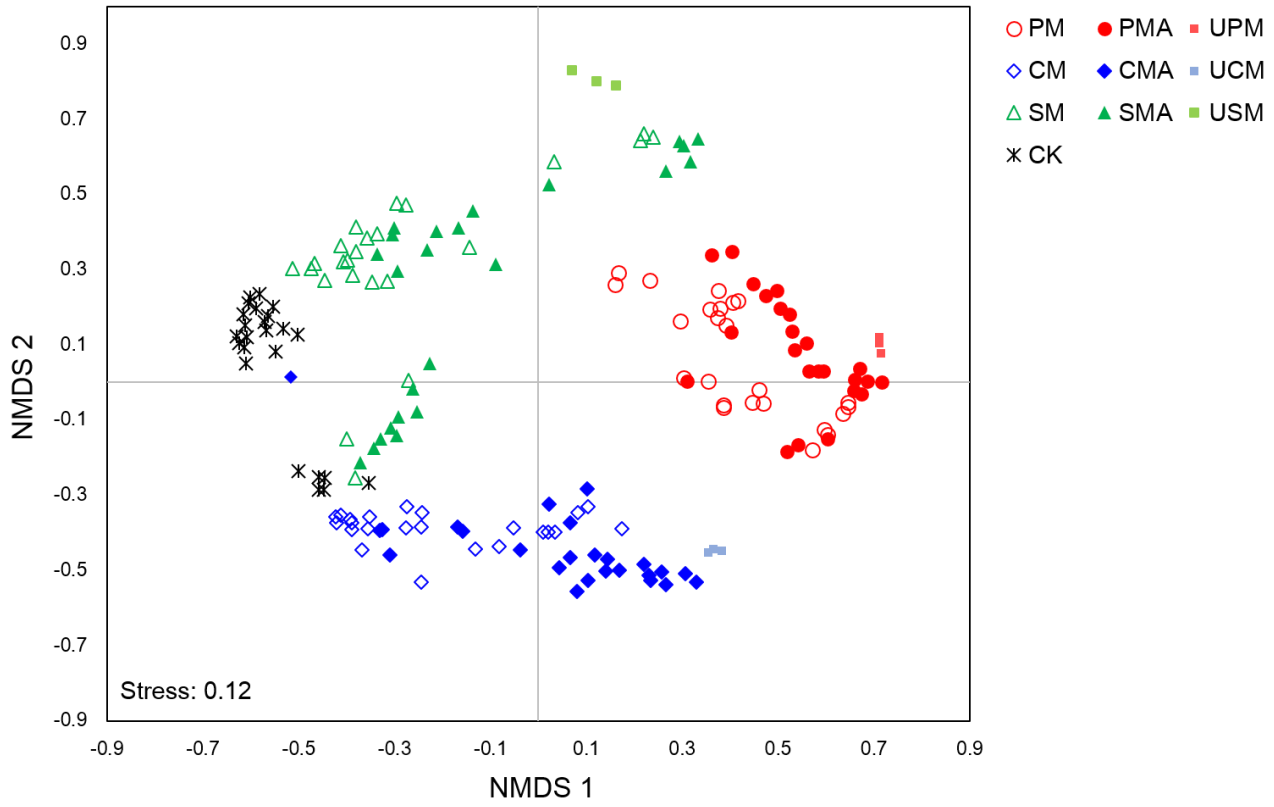
631

632 **Fig. 5**

633

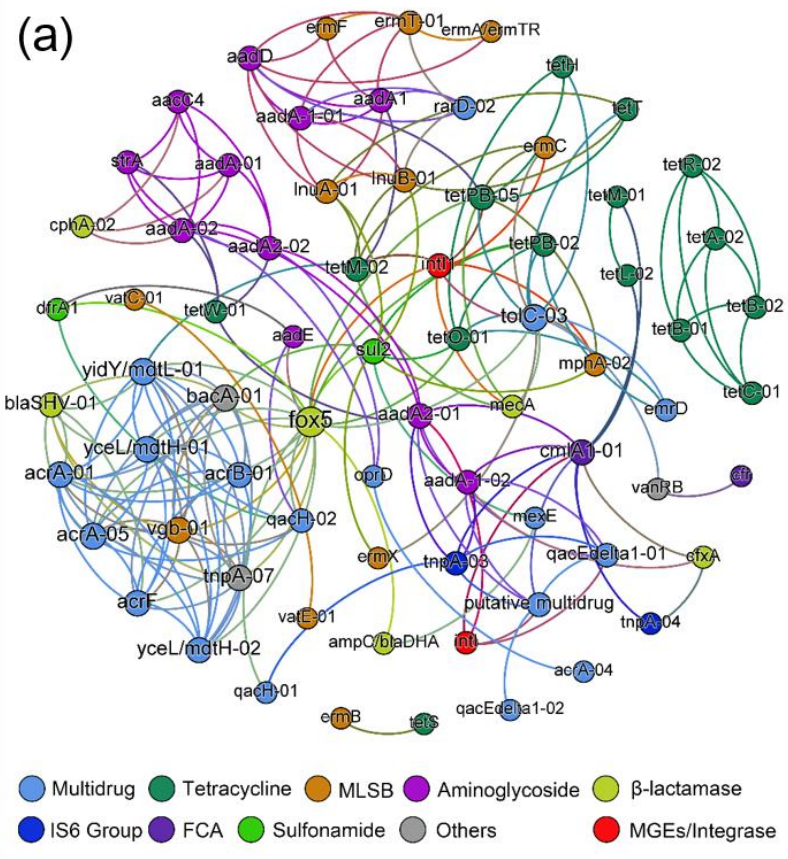
634

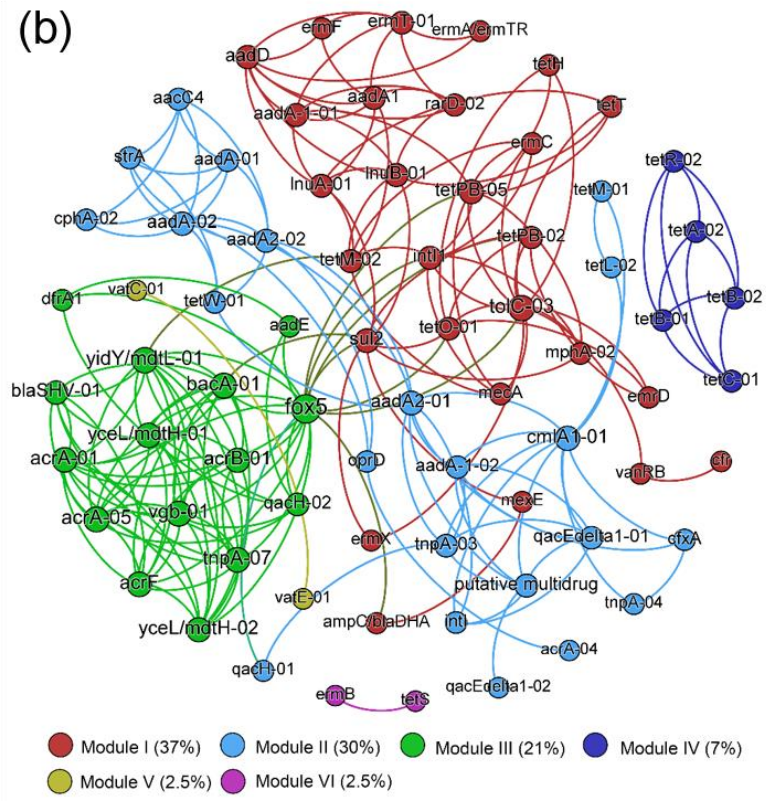
635 **Fig. 6**


PM
PMA
UPM
CM
CMA
UCM
SM
SMA
USM
CK
NMDS 2
NMDS 1
Stress: 0.12
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
-0.9

636

637 **Fig. 7**


(a)
ermF
ermT-01
ermA/ermTR
aadD
tetH
aacC4
aadA1
rarD-02
tetT
aadA-1-01
ermC
strA
aadA-01
lnuA-01
lnuB-01
tetPB-05
tetM-01
tetR-02
cphA-02
aadA-02
aadA2-02
tetPB-02
tetA-02
tetM-02
intI
tetL-02
drrA1
vatC-01
tetW-01
tolC-03
tetB-02
aadE
tetO-01
tetB-01
sul2
mphA-02
yidY/mdtL-01
tetC-01
blaSHV-01
bacA-01
fox5
aadA2-01
mecA
emrD
yceL/mdtH-01
cmlA1-01
acrA-01
acrB-01
oprD
aadA-1-02
vanRB
qacH-02
mexE
acrA-05
vgb-01
ermX
tnpA-03
qacEdelta1-01
cfxA
tnpA-07
putative multidrug
acrF
vatE-01
tnpA-04
yceL/mdtH-02
ampC/blaDHA
intI
acrA-04
qacH-01
ermB
tetS
qacEdelta1-02
Multidrug
Tetracycline
MLSB
Aminoglycoside
β-lactamase
IS6 Group
FCA
Sulfonamide
Others
MGEs/Integrase


(b)
ermF
ermT-01
ermA/ermTR
aadD
tetH
aacC4
aadA1
rarD-02
tetT
aadA-1-01
ermC
strA
aadA-01
lnuA-01
lnuB-01
tetPB-05
tetM-01
tetR-02
cphA-02
aadA-02
aadA2-02
tetPB-02
tetA-02
tetM-02
intI
tetL-02
drrA1
vatC-01
tetW-01
tolC-03
tetB-02
aadE
tetO-01
tetB-01
sul2
mphA-02
yidY/mdtL-01
tetC-01
blaSHV-01
bacA-01
fox5
aadA2-01
mecA
emrD
yceL/mdtH-01
cmlA1-01
acrA-01
acrB-01
oprD
aadA-1-02
vanRB
qacH-02
mexE
acrA-05
vgb-01
ermX
tnpA-03
qacEdelta1-01
cfxA
tnpA-07
putative multidrug
acrF
vatE-01
tnpA-04
yceL/mdtH-02
ampC/blaDHA
intI
acrA-04
qacH-01
ermB
tetS
qacEdelta1-02
Module I (37%)
Module II (30%)
Module III (21%)
Module IV (7%)
Module V (2.5%)
Module VI (2.5%)