

1 *Title page*

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

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14 **Running head:** Effects of different animal manures on soil ARGs

15

16 **Abstract**

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

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

42

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

45

46 **1 Introduction**

47 The increasing prevalence of antibiotic resistance genes (ARGs) in environmental
48 settings and their potential acquisition by human pathogens have become a global public
49 health concern in the 21st century (Rossolini et al., 2014; WHO, 2014). Antibiotic-resistant
50 infections are now responsible for more than half a million human deaths globally each year
51 (Dominey-Howes and Labbate, 2014), while over 10 million deaths in 2050 is predicted if the
52 antibiotic resistance issue is not tackled from now onwards. The over-prescription and abuse
53 of antibiotics in clinical environments, and especially in livestock production, is considered
54 the major cause leading to the global spread of antibiotic resistant bacteria (Sarmah et al.,
55 2006). Environmental ARGs are subjected to potential transmission to human pathogens and
56 commensals via the route of horizontal gene transfer (HGT; conjugation, transduction and
57 transformation) mediated by mobile genetic elements (e.g., plasmids, integrons, and
58 transposons) (Thomas and Nielsen, 2005; Heuer et al., 2011). Therefore, ARGs have been
59 recognized as a novel type of environmental contaminant (Pruden et al., 2006) attracting

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

62 The global consumption of antibiotics substantially increased from 50 billion to 70
63 billion standard units between 2000-2010, among which 70-80% are applied to livestock
64 industry, especially in poultry, swine, and cattle husbandry (Gelband et al., 2015).
65 Tetracyclines, macrolides, and sulfonamides are three most commonly used antibiotics in
66 livestock, and are also widely used in human medicines for treatment of infections (Jechalke
67 et al., 2014b; Gelband et al., 2015). Along with the increasing consumption of antibiotics, it
68 was found that veterinary antibiotics are poorly digested in the guts of animals, and a
69 significant amount (30-90%) of them are released into the environment through animal
70 urination and defecation (Zhang and Zhang, 2011). Use of veterinary antibiotics posed a
71 strong pressure on the gut microbiomes by selecting antibiotic resistant bacteria through
72 mutation or acquisition of ARGs (Tenover, 2006). An emerging body of studies have
73 reported that there is a positive correlation between dosage of veterinary antibiotics and the
74 occurrence of ARGs in manure and agricultural soils (Bibbal et al., 2007; Byrne-Bailey et al.,
75 2009; Heuer and Smalla, 2007; Hölzel et al., 2010). Therefore, it is imperative to understand
76 the impact of antibiotic usage on ARGs in veterinary settings and their fate after release into
77 the environment.

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

82 close to potential human pathogens (Heuer et al., 2011; Forsberg et al., 2012), manure can
83 also provide nutrients for favoring the occurrence of HGT (Smalla et al., 2000). These
84 manure-derived ARGs are in high risk of spreading into the food chain (Marti et al., 2013;
85 Zhu et al., 2017), and may pose a potential threat to public health when vegetables grown in
86 manured soils are consumed by humans. Recent studies have started to shed light on the
87 transmission of manure-derived ARGs in agricultural soils following land application of
88 animal manures (Jechalke et al., 2013, 2014a; Garder et al., 2014; Peng et al., 2015; Hu et al.,
89 2016a; Luby et al., 2016). Most of these studies, however, focused on only single type of
90 manure or a limited number of well-documented ARG types (Heuer and Smalla, 2007). To
91 our knowledge, no studies have attempted to systematically compare the impacts of different
92 sources of animal manures, in the presence or absence of antibiotics, on the temporal patterns
93 of a broad spectrum of ARGs. An improved understanding of this knowledge is critical to
94 prediction of ARG behaviours in soil environments, and development of appropriate manure
95 treatment approaches to minimise the spread of environmental ARGs.

96 Therefore, the objective of this study is to compare the effects of amendment of three
97 animal manure sources (swine, cattle, and poultry) on the temporal succession of a diverse
98 array of ARGs in soil microcosms incubated with agricultural soils. Tylosin, as a macrolide
99 antibiotic which is used in livestock industries for growth promotion and therapy (Sarmah et
100 al., 2006; Apley et al., 2012), was spiked into manure to test their synergistic selection effects
101 on antibiotic resistance. The high-throughput quantitative PCR array was performed to target
102 285 ARGs which confer resistance to all major classes of antibiotics; and 10 mobile genetic
103 elements (MGEs) as a proxy for HGT potential of ARGs. We hypothesized that: (i)

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

108 **2 Materials and Methods**

109 **2.1 Soil and manure sampling**

110 The soil used in this study was taken from a vegetable farm at Clyde (38°07'S,
111 145°19'E), Victoria, Australia. The soil in this site has a pH value of 7.2 and is classified as
112 loamy sand. Total carbon and total nitrogen are 3.75% and 0.43%, respectively. Cattle
113 manure and swine manure were collected from the Dookie Farm (36°25'S, 145°42'E),
114 University of Melbourne, and poultry manure was taken from a chicken farm in Mornington,
115 Melbourne. Manure samples were collected within three days after excretion without
116 composting, and had no known history of antibiotic treatment. Soil and manure samples were
117 kept on ice during transportation and stored at 4°C before analysis. The antibiotic tylosin
118 used in the experiment was purchased from Sigma-Aldrich company (St Louis, MO, USA).

119 **2.2 Soil microcosm incubations**

120 Three sets of soil microcosm incubation experiments were established depending on the
121 amendment of different animal manure sources, and each set included two treatments (Fig.
122 S1). In the first treatment, manure was mixed thoroughly with sieved soils (<2 mm) to reach
123 a final concentration of 80 mg g⁻¹ soil, which is corresponded to a typical agricultural amount
124 of 60 m³ manure per hectare. In the second treatment, tylosin in aqueous solutions was spiked
125 to the manure and the tylosin-spiked manure was mixed with soil to reach a concentration of

126 0.1 mg tylosin g⁻¹ soil and 60 m³ manure per hectare. The untreated soil sample with same
127 water content was used as the control treatment. Soil microcosms were established in 250 ml
128 vials with 20 g of soil or manured soil, loosely covered, and incubated in the dark at 25°C.
129 The aerobic condition in the microcosms was maintained by opening the vials for air
130 refreshing every three days. Soil moisture contents were maintained at 60% of the water
131 filled pore space by adding sterilized water regularly. Soil microcosms were destructively
132 sampled at eight time points on days 1, 7, 20, 35, 50, 70, 100, and 130 after manure
133 application.

134 **2.3 Soil physicochemical analysis and genomic DNA extraction**

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

144 **2.4 High-throughput quantitative PCR**

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

148 5184 reactions with each volume of 100 nl using the SensiMix SYBR No-ROX reagent
149 (Bioline, London, UK). Primers, DNA and reagents were dispensed into the 5184-nanowell
150 SmartChip using a Multisample NanoDispenser (Fremont). The HT-qPCR array contained a
151 total of 296 primer sets (Su *et al.*, 2015; Hu *et al.*, 2016b), including 285 primer sets targeting
152 eight major classes of ARGs, 10 primer sets targeting MGEs, and one 16S rRNA gene as the
153 internal control (Table S1). The specificity of all primers was verified through amplicon
154 sequencing, BLAST search and amplification efficiency check (Looft *et al.*, 2012; Johnson *et*
155 *al.*, 2016). The SmartChip was loaded into the Wafergen SmartChip Real-Time PCR Cycler
156 using the thermal-cycling conditions as follows: 95°C for 10 min, followed by 40 cycles of
157 95°C for 30s and 60°C for 30s. Only well data with the amplification efficiencies within 1.7-
158 2.3 were remained. A threshold cycle (C_T) value of 31 was used as the detection limit (Su *et*
159 *al.*, 2015). A comparative C_T method, also referred to as the $\Delta\Delta C_T$ method of relative
160 profiling, was performed to assess the temporal changes of ARGs across all the samples
161 (Schmittgen and Livak, 2008).

162 **2.5 Quantitative PCR analysis of the bacterial 16S rRNA gene**

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

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

172 **2.6 Co-occurrence analysis and network generation**

173 The CoNet Cytoscape plug-in method was used to visualize the co-occurrence patterns
174 of ARGs and MGEs in the network interface (Soffer et al., 2015). ARGs with a minimum
175 occurrence of 3 across all soil samples were regarded as poorly represented ARG subtypes
176 and discarded to reduce artificial correlation bias. The *P*-values were calculated based on two
177 dissimilarity methods (Bray-Curtis and Kullback-Leibler) and two correlation methods
178 (Spearman and Pearson) to control the potential of obtaining false-positive results. The
179 significant pairwise correlations (*P*-value < 0.05) between the ARG and MGE subtypes were
180 utilized to form their co-occurrence networks. The resultant co-occurrence network was
181 visualized using the Frucherman Reingold algorithm on the interactive platform of Gephi
182 (Bastian et al., 2009). A correlation with a correlation coefficient (ρ -value) > 0.8 and a
183 significant level (*P*-value) < 0.05 were considered statistically robust (Junker and Schreiber,
184 2008).

185 **2.7 Statistical analysis**

186 One-way analysis of variance (ANOVA) was performed to analyse the effects of
187 manure application and tylosin addition on the diversity, relative and absolute abundances of
188 ARGs across different treatments. Pearson's correlation test was performed to test the
189 correlations between the relative abundance of ARGs and MGEs. Non-metric
190 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

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

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

223 **3.2 Abundance of ARGs and MGEs in different treatments**

224 The comparative C_T method was used to calculate the relative abundances of ARGs and
225 MGEs by normalizing to 16S rRNA gene in the same HT-qPCR array (Schmittgen and Livak,
226 2008). The relative and absolute abundances of ARGs in cattle manure samples were
227 significantly lower compared with those in swine and poultry manure samples, with swine
228 manure harbouring the highest abundance of ARGs (Figs. 2a and 2c). When animal manure
229 was applied to the soil, the relative and absolute abundances of ARGs in soil samples treated
230 with cattle and swine manure were significantly lower than those in poultry manure-treated
231 samples (Figs. 2b and 2d). In manure-treated samples spiked with tylosin, the ARG
232 abundances were consistently higher than those in samples without tylosin addition (Figs. 2b

233 and 2d). The pattern of MGE abundances was quite similar to that of ARG abundances, with
234 the lowest abundances found in cattle manure and cattle manure-treated soil samples (Fig.
235 2).

236 **3.3 Temporal changes of ARGs and MGEs in manure-treated soils**

237 Soil microcosms were destructively sampled at eight time points (days 1, 7, 20, 35, 50,
238 70, 100, and 130) during the incubation to explore the temporal changes of ARGs and MGEs
239 in soils treated with three sources of animal manures with or without tylosin (Fig. 3). The
240 numbers of detected ARGs in manure-treated soils gradually decreased over time during the
241 incubation, regardless of whether or not tylosin was added (Fig. 3a). Interestingly, the ARG
242 numbers in soils treated with cattle manure approached the background levels of ARGs in
243 untreated soils at the end of incubation (day 130), but swine and poultry manure-amended
244 soils had a significantly higher level of ARGs than untreated soils at day 130 (Fig. 3a). The
245 relative abundances of ARGs in soils treated with poultry and cattle manures declined over
246 time but were still higher than that in untreated soils, with a more pronounced selection effect
247 observed in tylosin-amended samples (Fig. 3b). The tylosin-amended manure application
248 showed consistently higher relative abundances of ARGs compared with their corresponding
249 no-tylosin treatments (Fig. 3b). The ARG abundances in swine manure-treated samples
250 declined sharply to background levels in untreated soils within 20 days of incubation, and
251 then remained largely unchanged till day 130 (Fig. 3b).

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

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

259 **3.4 Temporal changes of individual subtypes of ARGs**

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

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

275 in soils treated with manure and tylosin, especially in the poultry and swine manure
276 treatments; (ii) Some genes conferring resistance to multidrug (*yceL/mdtH-03*, *yidY/mdtL-01*,
277 *yidY/mdtL-02*) and tetracycline (*tetA-02*, *tetB-01*, *tetB-02*, *tetC-01*), which were absent in
278 untreated soil samples, appeared in manure and tylosin treated samples, and persisted until
279 the end of the incubation; (iii) Several multidrug resistance genes (*qacH-01*, *qacH-02*, *rarD-*
280 *02*, *tolC-01*, *tolC-02* and *tolC-03*) and vancomycin resistance genes (*vanRA-02*, *vanRB*) were
281 abundant in three types of manures, however, decreased over time in all the manured soils
282 and became undetected at the end of the incubation; (iv) Most β -lactam resistant genes kept
283 relatively unchanged in soils amended with manure in the presence or absence of tylosin.

284 **3.5 Co-occurrence patterns among ARGs and MGEs**

285 The network analysis was performed to explore the co-occurrence patterns of ARGs
286 and MGEs based on strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations. The resultant
287 network was composed of 73 nodes (ARG subtypes) and 209 edges (pairwise correlations)
288 (Fig. 7a), and could be clearly separated into six modules (Fig. 7b). Each module consisted of
289 different types of ARGs, except module IV which included exclusively five tetracycline
290 resistance genes. The most densely connected node in each module was defined as the ‘hub’,
291 for example, the ‘*tolC-03*’ (multidrug resistance) and ‘*aadA2-01*’ (aminoglycoside resistance)
292 were the hubs for modules I and II, respectively (Fig. 7b). The module III had three
293 equivalent hub genes *vgb-01*, *acrA-05* and *yidY/mdtL-01*. It has been suggested that hubs
294 could be regarded as indicator ARGs to represent the quantity of other co-occurring ARGs in
295 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

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

370 Erythromycin ribosome methylation (*erm*) genes encoding tylosin resistance by
371 reducing the ability of tylosin from binding to the 50S ribosomal subunit (Leclercq and
372 Courvalin, 1991; Weisblum, 1998), and they are detected in bacteria isolates of human origin
373 which may constitute a health risk for human (Rollins et al., 1985). Three main *erm* genes
374 have been described in *staphylococci*: *erm(A)* gene is located on transposon Tn554, *erm(B)*
375 gene on transposon Tn551 and *erm(C)* gene on a plasmid (Saribas et al., 2006), suggesting
376 the HGT potential of the *erm* genes. We detected 11 *erm* genes in this study, and these genes
377 were obviously more abundant in the tylosin-amended treatments, especially in the poultry
378 manure and swine manure treatments (Fig. 5). In the swine manure treatments, the relative
379 abundances of *erm* genes were significantly higher in the tylosin-amended treatments
380 compared with the tylosin-absent treatments (Fig. S3). Therefore, the presence of tylosin in
381 animal manures can impose strong selection pressure on soil microbiome, and increase the
382 propagation of tylosin-resistant bacteria.

383 Beyond the selection effect of tylosin on MLSB resistance genes, it is interesting to find
384 that ARGs encoding resistance to almost all the major classes of ARGs were clearly enriched
385 in tylosin-amended treatments compared with manured soils without tylosin (Fig. 2). This no-
386 targeted selection phenomenon might be explained by the co-selection mechanisms (Hu et al.,
387 2016b) in which genes encoding resistance to different antibiotics may reside in the same
388 MGEs (plasmid, integrin, or transposon) or single genes can encode resistance to various
389 classes of ARGs. As shown in the co-occurrence network (Fig. 7), genes conferring
390 resistance to different categories of antibiotics were shared in the same module, suggesting
391 that these ARGs might be carried by the same bacterial cells or MGEs, and they can change
392 and transfer together under the selection pressure imposed by tylosin. Therefore, our findings
393 caution that the addition of tylosin can select for a broad range of ARGs apart from MLSB
394 resistance, and elongate the lifespan of ARGs throughout the whole incubation period (in
395 poultry and swine manured soils).

396 **4.3 The HGT potential of ARGs in soils treated with manure and tylosin**

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

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

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

424 **5 Conclusions**

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

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

435

436 **Acknowledgements**

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

439

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580

581 **Table 1** Pearson correlations between the relative abundance of ARGs and MGEs in all the
 582 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)

583 Values showed in the table are the r-values derived from the Pearson analysis, and the *P*-
 584 values are showed in the brackets. The bold numbers represent significant correlations (*P* <
 585 0.05).

586

587

588 **Figure captions**

589 **Figure 1.** (a) Classification of the 185 ARGs detected in all the samples based on the classes
590 of antibiotic to which they confer resistance. (b) The average number of ARGs and MGEs
591 detected in each treatment. Different letters above the bars indicate a significant difference (P
592 < 0.05). Venn diagram shows the number of unique and shared ARGs among the three types
593 of manures (c) and among the untreated soils and manured soils (d). (Abbreviations: FCA,
594 fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes;
595 MLSB, Macrolide-Lincosamide-Streptogramin B resistance. CK, untreated soil samples; PM,
596 poultry manure treatment; PMA, tylosin-amended poultry manure treatment; CM, cattle
597 manure treatment; CMA, tylosin-amended cattle manure treatment; SM, swine manure
598 treatment; SMA: tylosin-amended swine manure treatment; UPM, untreated poultry manure;
599 UCM, untreated cattle manure; USM, untreated swine manure)

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

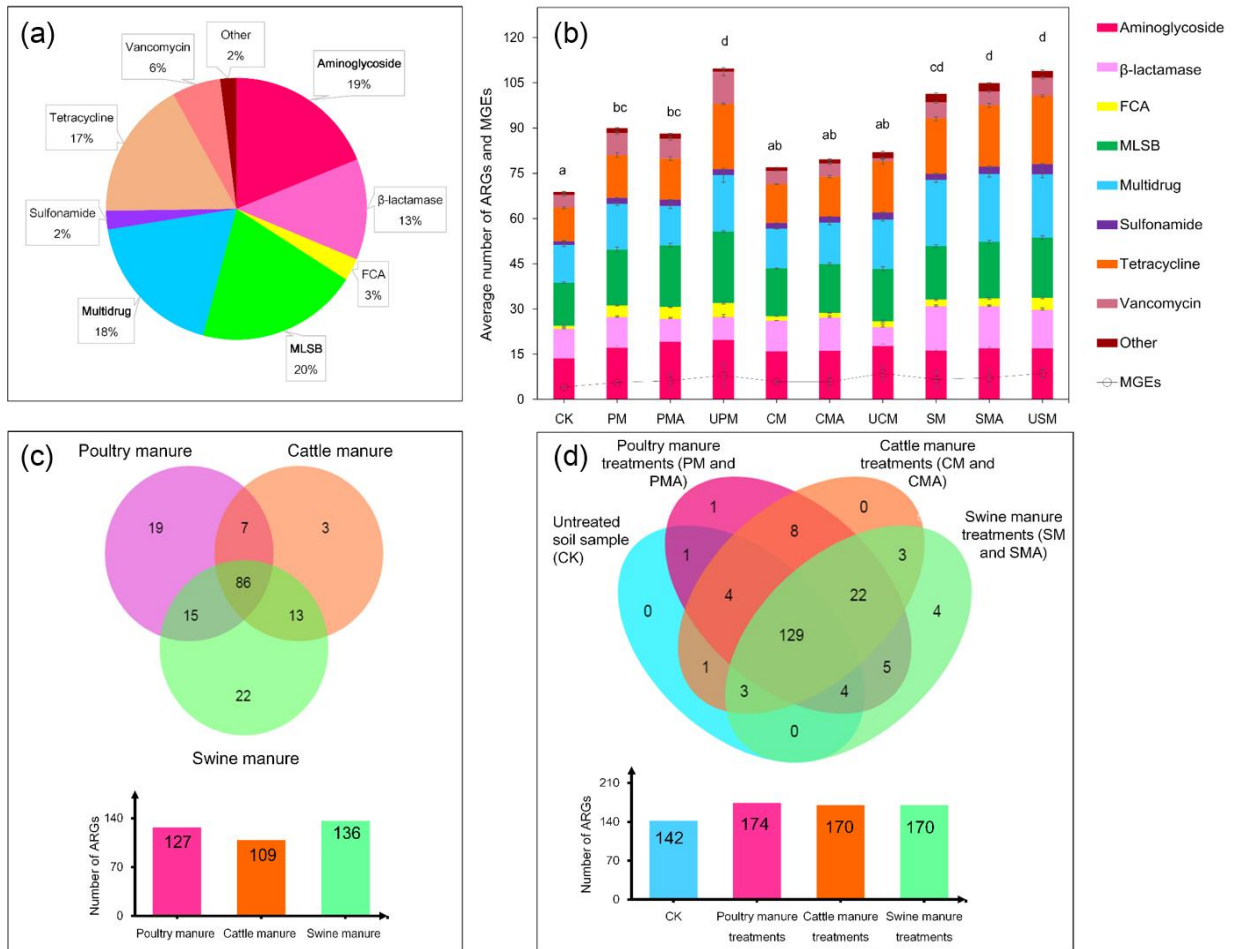
602 **Figure 3.** (a) The temporal changes of the average number of detected ARGs across the
603 different treatments. (b) The temporal changes of the relative abundance of ARGs across the
604 different treatments.

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

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

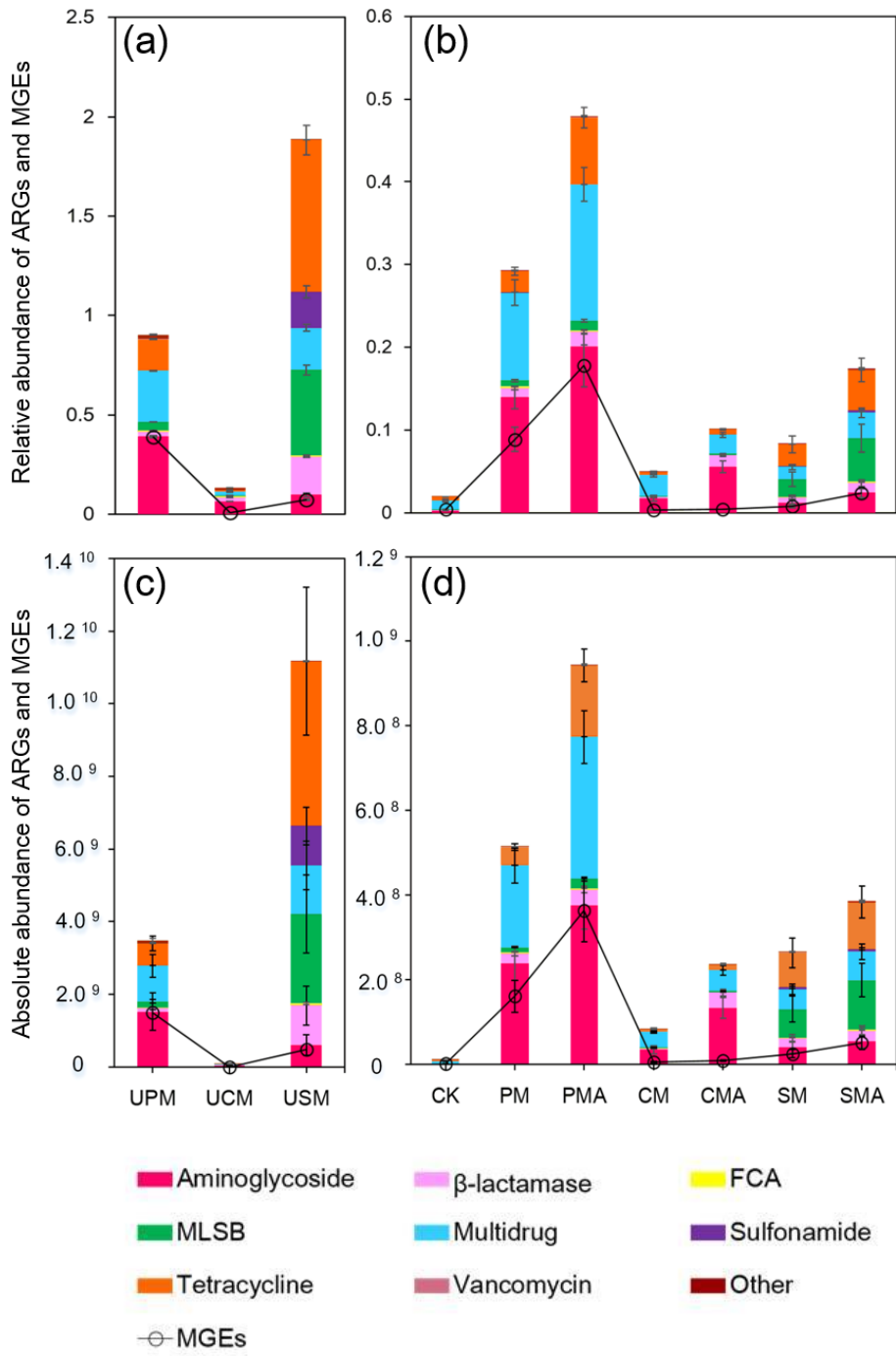
610 **Figure 6.** Non-metric multidimensional scaling ordination plot depicts the Bray-Curtis
611 dissimilarity matrices between soils based on the relative abundance of ARGs. The 2D stress
612 value is 0.14, which indicated that the two-dimensional ordinations could well represent the
613 data.

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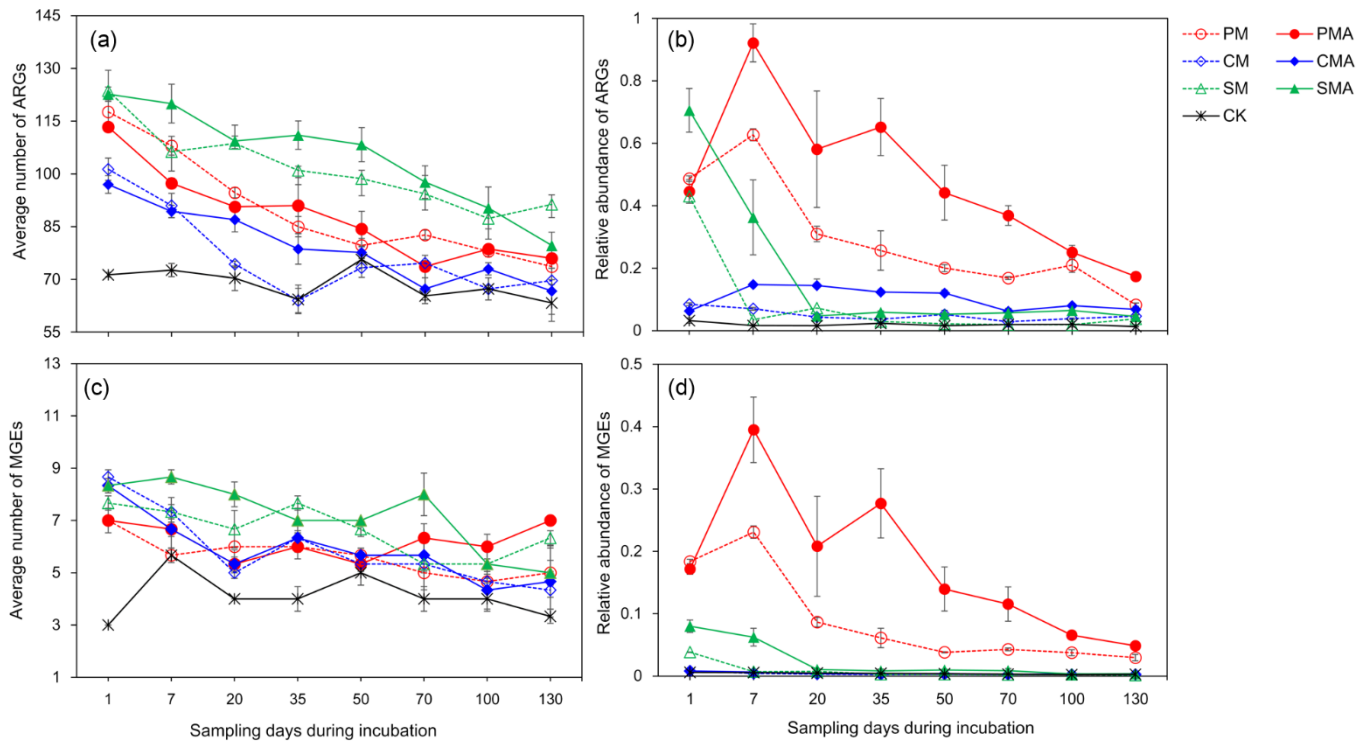


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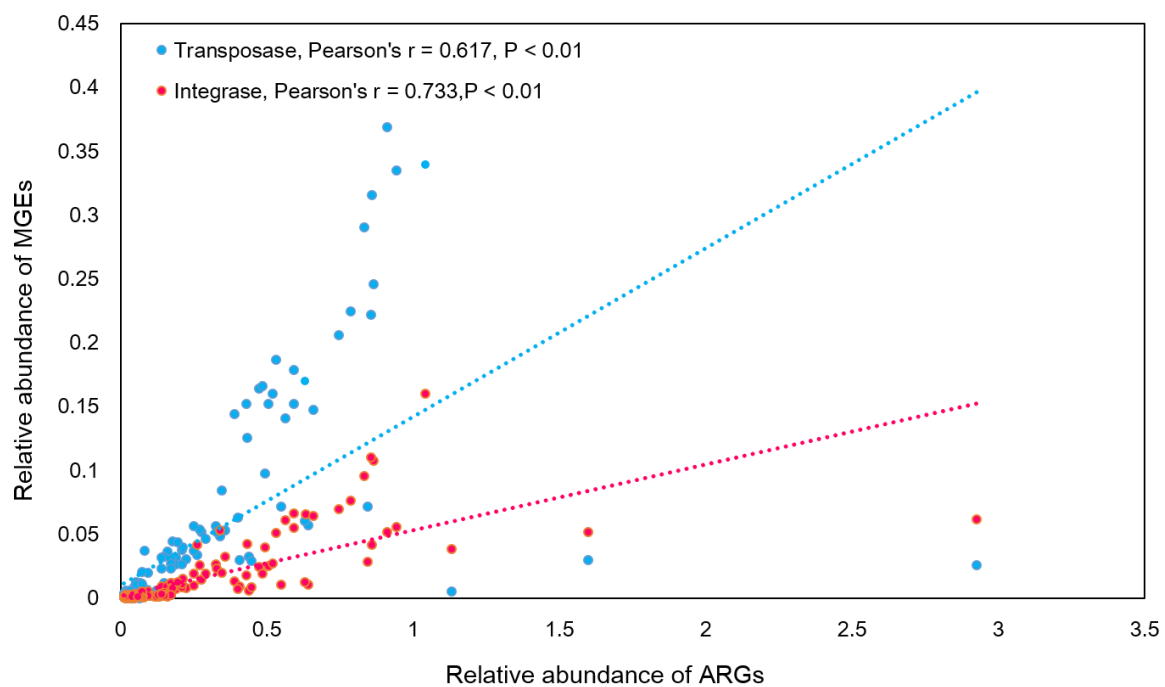
626 **Fig. 3**



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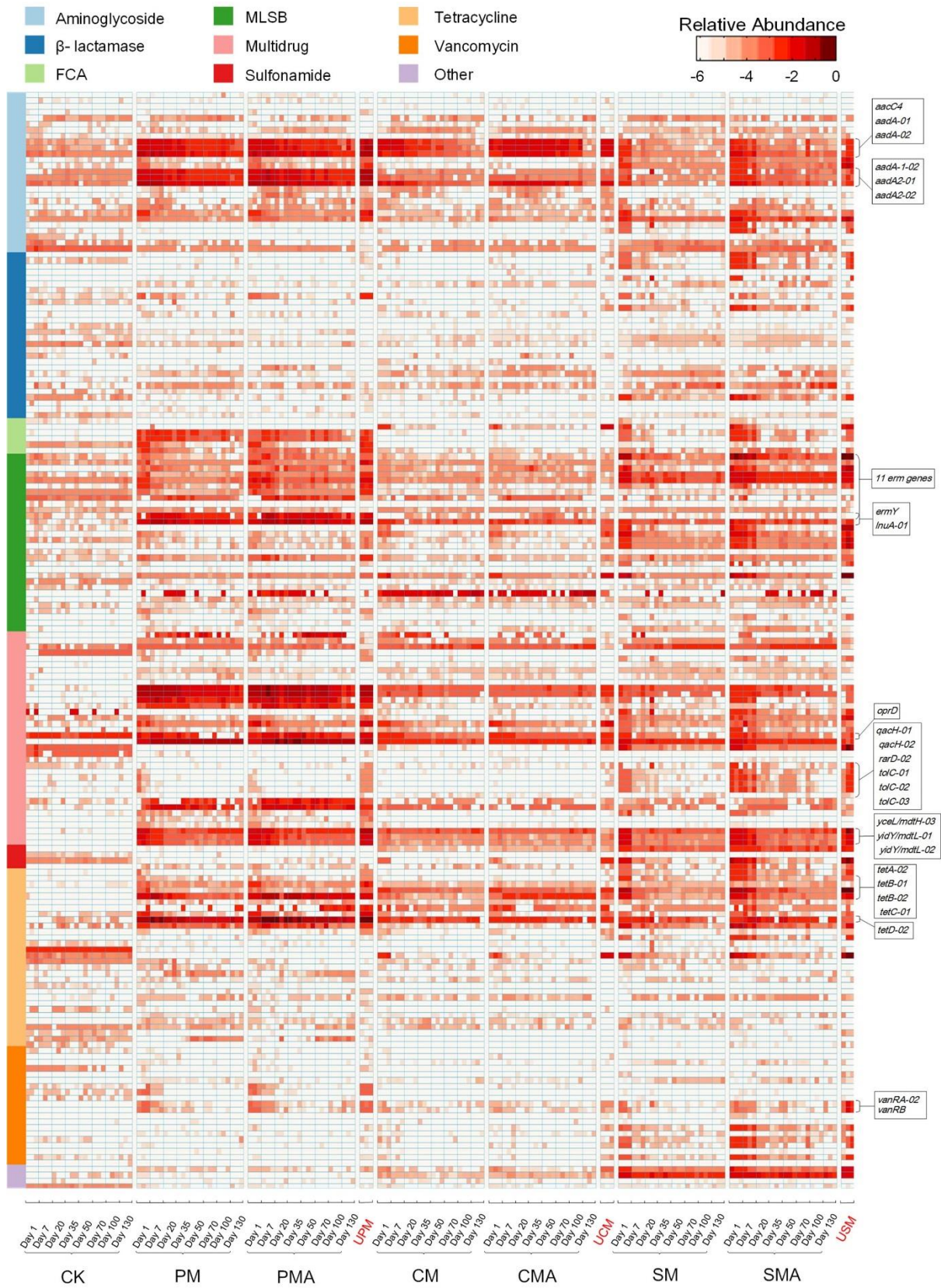
629 **Fig. 4**



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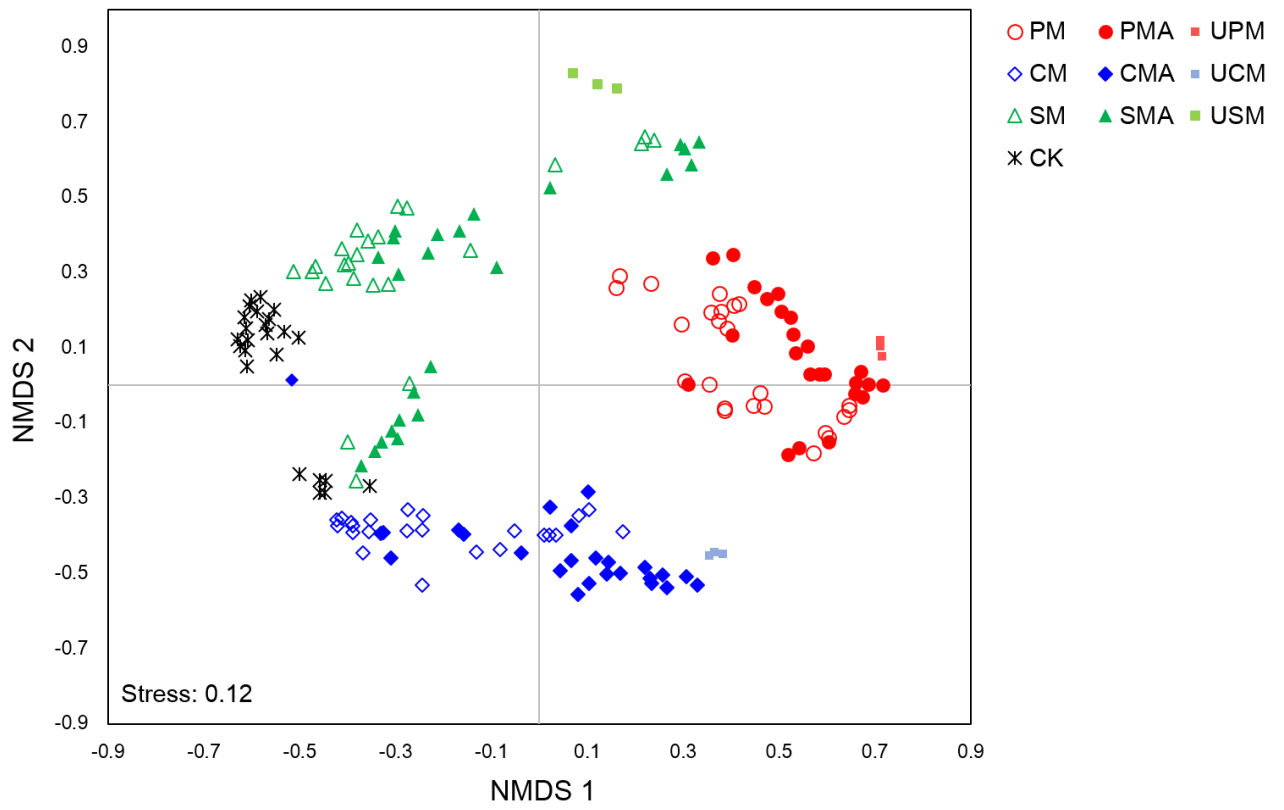
632 **Fig. 5**



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634

635 **Fig. 6**



636

