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Phyllosphere of staple crops under pig manure fertilization, a reservoir of antibiotic resistance genes

Shuyidan Zhou,†,‡, # Dong Zhu,†,‡, # Madeline Giles§, Xiao-Ru Yang,† Tim Daniell||,§, Roy Neilson§, Yong-Guan Zhu,*, †,‡,⊥

† Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China.

‡ University of the Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China.

§ Ecological Sciences, The James Hutton Institute, Dundee, DD2 5DA, Scotland, UK

|| Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, S10 2TN, UK

⊥ State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

# Shuyidan Zhou and Dong Zhu contributed equally to this paper

* Corresponding author: Yong-Guan Zhu

Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China

E-mail address: ygzhu@iue.ac.cn (Y.G. Zhu)

Abstract

In China, the common use of antibiotics in agriculture is recognized as a potential public health risk through the increasing use of livestock derived manure as a means of fertilization. By doing so this may increase the transfer of antibiotic resistance genes (ARGs) from animals, to soils and plants. In this study two staple crops (rice and wheat) were investigated for ARG enrichment under differing fertilization regimes. Here, we applied 4 treatments, no fertilizer, mineral fertilizer, clean (reduced antibiotic practice) and dirty (current antibiotic practice) pig manure, to soil microcosms planted with either rice or wheat, to investigate fertilization effects on the abundance of ARGs in the respective phyllospheres. For both rice and wheat, samples were collected after two separate fertilization periods. In total 162 unique ARGs and 5 mobile genetic elements (MGEs) were detected from all rice and wheat samples. The addition of both clean and dirty manure, enhanced ARGs abundance significantly when compared to no fertilizer application ($P<0.001$), though clean manure enriched ARGs to a lesser extent than dirty manure, in all rice and wheat samples ($P<0.001$). The classes of ARGs recorded were different between crops and wheat samples had a higher ARG diversity than rice. These results revealed that staple crops in China such as rice and wheat may be a reservoir for ARGs when clean and dirty pig manure is used for fertilization.

Capsule: Pig manure can enrich the antibiotic resistance genes in the phyllsophere of rice and wheat.

Key words: Phyllosphere, Pig manure, Antibiotic resistance genes, Staple crops,

43 Leaf-microbiome

44

45 **Introduction**

46 Organic fertilizers are increasingly being used as a cost-effective alternative to mineral
47 fertilizers, as their high organic matter and mineral nutrient content generates similar
48 improvement in crop productivity to that of mineral fertilizer (Hao et al., 2008; Cheng
49 et al., 2013). However, organic fertilizers frequently include animal manure or activated
50 sludge, the use of which is an efficient way to reduce agricultural waste, but manure
51 has been shown to increase the abundance of ARGs in soils (Joy et al., 2013; Xie et al.,
52 2016). Both manure and activated sludge can contain incompletely metabolized
53 antibiotics, antibiotic resistant bacteria and antibiotic resistant genes (ARGs).
54 The continued increase in the abundance of antibiotic resistance genes in the
55 environment is a recognized global public health issue (Martinez, 2008). Although
56 ARGs generally occur within the genetic material of the carrier organisms (D'Costa et
57 al., 2011), the anthropogenic spread of antibiotics has led to an enrichment of ARGs in
58 the environment (Pruden et al., 2006) with a concomitant concern that such ARGs can
59 have an impact on human and animal health.
60 ARGs enter the food chain through ARG contaminated crops leading to a risk of
61 antibiotic resistance related problems in humans (Gillings, 2017). For example, studies
62 have shown that ARGs can enter the human food chain through chicken and pork
63 products sold at local markets (Leverstein-van Hall et al., 2011; Zhang et al., 2018),

64 fish products (Antunes et al., 2018) and vegetables (Marti et al., 2013; Rahube et al.,
65 2014; Wu et al., 2018). Antibiotics are used widely for medical purposes, and regularly
66 in agricultural production systems, consequently large amounts of antibiotics pass
67 through waste streams into the environment (Allen et al., 2010; Marshall and Levy,
68 2011; Zhou et al., 2013). Antibiotics released into the environment have been shown to
69 impact the regulation of antibiotic resistance genes (ARGs) and trigger a microbial
70 response that increases the mutation rate in bacteria and the sharing, through horizontal
71 gene transfer, of ARGs from antibiotic resistant bacteria (ARB) to those without
72 antibiotic resistance (Zhu et al., 2013). Consequently, individual bacteria and the wider
73 microbial community adapt to the changing presence of antibiotics by increasing the
74 number of ARGs or selecting antibiotic resistant bacteria that match the soil antibiotic
75 profile in the wider environment (Tello et al., 2012; Gillings, 2017; Zhu et al., 2017).
76 The plant phyllosphere provides a large surface area for microorganisms to inhabit
77 (Woodward and Lomas, 2004). It has been estimated that the total surface area of leaves
78 on the planet approaches approximately twice that of the land surface and can contain
79 bacterial populations of up to 10^{26} cells (Lindow and Brandl, 2003; Vorholt, 2012). Thus,
80 the phyllosphere provides a potential reservoir for micro-organisms and ARGs and
81 allows them to come in direct contact as a result of aerial spreading of animal-derived
82 manures on crops. Senescence of deciduous leaves facilitates the transfer of
83 phyllosphere adhering microorganisms to soil through leaf drop, while the exposure of
84 the phyllosphere to the atmosphere allows it to be a receiver of wind-borne
85 microorganisms (Berlec, 2012; Mhuireach et al., 2016), highlighting the transfer of

86 microorganisms across the critical zone i.e. the atmosphere, phyllosphere and soil. Thus,
87 ARGs can be potentially ubiquitous on the plant phyllosphere, for example, on the
88 phyllosphere of field grown vegetables (Zhu et al., 2016; Chen et al., 2017b). However,
89 it is noted that to date the phyllosphere has not been recognized as an explicit source of
90 ARGs in agricultural systems (Chen et al., 2018). Therefore, it is critical to establish a
91 knowledge base about ARGs in the plant phyllosphere especially in an agricultural
92 context.
93 Questions remain as to whether staple crops such as rice and wheat which are grown in
94 a continuous rotation in China can transfer ARGs to the environment. Rice and wheat
95 have contrasting agronomic management (flooded or not-flooded) which may drive
96 differences in the structure of phyllosphere microbial communities and abundance of
97 ARGs. As these crops contribute a high proportion of the global human diet (Tilman et
98 al., 2002) there is an imperative to identify their interactions, if any, with antibiotic
99 resistant bacteria (ARB) /ARGs. More than 25% of farmland in China is under rice
100 cultivation (Rahman et al., 2018). Current studies on rice focus on soil and hydrological
101 systems and typically overlook the risk of phyllosphere ARGs.
102 The aims of this study were therefore to 1) characterize the abundance and diversity of
103 ARGs in the phyllosphere of rice and wheat; 2) investigate the effects of manure
104 application on phyllosphere ARG communities; 3) determine if there is a difference in
105 ARG abundance between manure from farms with standard and reduced antibiotic use;
106 4) identify differences, if any, in ARG abundances between rice and wheat; 5) explore
107 the contribution of the bacterial community to shifts, if any, in phyllosphere ARGs. We

hypothesize that 1) rice and wheat phyllospheres would exhibit different ARG profiles; 2) the addition of manure from farms with reduced antibiotic use results in lower phyllosphere ARG enrichment than the addition of manure from farms using standard antibiotic use; and 3) Shifts in the community composition of the bacterial community can be accounted for by changes in ARG composition.

2. Materials and methods

2.1 Soil and manure properties

Soil, a sandy loam, was collected to a depth of 20 cm from a farm under a rice and wheat rotation in Ningbo, Zhejiang, China (29º 47′N, 121º 21′E). Soil characteristics are listed in Table S2. Soil was sieved through a 5 mm sieve to remove stubble, roots and stones. Mineral fertilizer treatments comprised of pure nitrogen fertilizer (Urea) (21 g m^{-2}), P_2O_5 (7.5 g m^{-2}) and K_2O (12 g m^{-2}) for rice, while urea (12.6 g m^{-2}), P_2O_5 (2.4g m^{-2}) and K_2O (10.0 g m^{-2}) were applied before wheat planting, and then urea N (5.4 g m^{-2}) was applied during shoot elongation stage.

Two organic fertilizer treatments were applied, a “dirty” slurry comprising pig manure from a farm using standard antibiotic practice and a “clean” slurry comprising pig manure from a farm using reduced antibiotic practice. The properties of each slurry are listed in Table S3. The clean slurry was added 9.47 g/pot (N%: 3.36) and the dirty slurry was added 11.007 g/pot (N%: 2.89).

2.2 Experimental design and crop cultivation

Sixteen treatments were established: no fertilizer, mineral fertilizer, clean manure and dirty manure for each of two crops (rice and wheat) grown in rotation and two fertilization periods per crop rotation. For each fertilization period there were 5 replicates per treatment. Phyllosphere samples were collected twice for each crop rotation: at grain filling stage and before harvest. Eighty microcosms were established each containing 3.5 kg wet soil (water holding capacity for rice 100%, for wheat 65%), Microcosms were made of polyvinyl chloride with a diameter of 15 cm and height of 23 cm, with water drainage effected through a small hole in the bottom of pot.

Rice (cv. Yonguou 12) and wheat (cv. Yangmai 20) were germinated before planting in microcosms using the following methods: rice seeds were field grown and transplanted to the microcosms 28 days after sowing. Microcosms were flooded, seedlings planted, and the soil held at 100% water holding capacity (WHC). At rice tilling, approximately 2 weeks before harvest, soil in the microcosms was dried through to harvest. Wheat seeds were sterilized in a 10 % hydrogen peroxide solution for 15 min and stored at 4°C for 1 week prior to planting in the microcosms. Wheat was grown at 65 % WHC. Yongyou 12 is a three-line indica–japonica hybrid super rice and Yangmai 20, a main middle-early mature wheat variety.

2.3 DNA extraction from the phyllosphere of rice and wheat

DNA was extracted according to the method described in (Zhu et al., 2016). Prior to DNA extraction, 5 g of either rice or wheat leaf was weighed into a 250 ml conical flask

containing 100 ml of 0.01 M, phosphate-buffered saline (pH=7.4), flasks were sonicated for 7 minutes before being shaken for 1 hour at 180 rpm, with samples held at 30 °C during this process. Phosphate buffer was initially filtered through a nylon gauze followed by filtration through a 0.22 μM cellulose membrane. Target DNA was extracted from the filters using a FastDNA Spin Kit for Soil (MP Biomedicals, CA) and quality checked using spectrophotometer analysis (NanoDrop ND-1000, Thermo Scientific, Waltham, MA). DNA was stored at -20 °C prior to analysis.

2.4 Illumina Sequencing and bioinformatics analysis

The V4-V5 hypervariable region of the 16S rRNA gene was used to analyse the structure of the phyllosphere bacterial community, using primers 515F: GTGCCAGCMGCCGCGG and 907R: CCGTCAATCMTTTRAGTTT (Turner et al., 1999). To each PCR tube 1μL 10 μM 515F primer, 1μL 10 μM 907R primer, 0.81μL bovine serum albumin (BSA, 20 mg mL^{-1}), 21.2 μL sterile water，25μL TAKARA Premix Taq™ (Ex Taq™ Version 2.0 plus dye, No. RR902A) and 1μL 20ng μL^{-1} DNA template was added. PCR conditions were 95°C for 5 minutes, followed by amplification for: 25 cycles of 30s at 94°C, 35s at 58°C and 30s at 72°C. TIANGEN universal DNA purification kits (TIANGEN biotech, Beijing, China) were used to clean PCR products. Purified products were then normalized to 200 ng DNA before being sequenced by Novogene (Beijing, China) using a Illumina Hiseq 2500 platform. The Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010b; Chen et al., 2017c) was used to produce OTUs, with 97 % OTU using UCLUST (Edgar,

2010). OTUs were aligned using one representative sequence per OTU, using PyNAST aligner (Caporaso et al., 2010a). RDP classifier which uses the Greengenes data base (version 13.8, 16S rRNA gene database) (McDonald et al., 2012; Langille et al., 2013), was used to assigned taxonomic identity and relative abundance of OTUs. Alpha diversity of samples was calculated using QIIME.

2.5 HT-qPCR quantification of Antibiotic resistant genes.

We investigated the diversity and abundance of ARGs using High-throughput qPCR with a Wafergen SmartChip Real-time PCR system (Wafergen, Fremont, CA). All samples were diluted to 20 ng μL^{-1} and run in triplicate. 285 ARG genes were targeted using 296 primer sets (Su et al., 2015) (Table S1). Additionally these primer sets can also detect 16S rRNA gene, 8 transposases, class 1 integron-integrase gene (*intl1*) (Stokes et al., 2006) and the clinical related class 1 integron-integrase gene (*cintl1*) (Gillings et al., 2014). A total of 5184 Nano-wells on the Smart Chip platform provided reaction sites for PCR. A 100nl PCR reaction mix was used per well, this mix contained 1 × LightCycler 480 SYBR Green I Master, 1mg mL^{-1} bovine serum albumin, nuclease-free PCR-grade water, 500 nM of each 296 primers and 20 ng μL^{-1} of each DNA sample. PCR conditions were: enzyme activation at 95°C for 10 minutes followed by 40 cycles of amplification: denaturation at 95°C for 30s, annealing at 60°C for 30s (Chen et al., 2017). An amplicon range of 1.8 to 2.2 was set as the efficiency range, with anything outside this range discarded, including those with multiple melting peaks. qPCR results were analyzed using Smartchip qPCR software (Wafergen, Fremont, CA). The

194 detection limit was set at a threshold cycle of 31 (C_T) and amplification only considered
195 as positive if all three technical replicates showed a positive result. Relative gene copy
196 number and a normalized gene copy per bacteria were calculated by the following
197 equations (Chen et al., 2016):
198
199 Relative gene copy number = $10^{(31\text{-}CT)/\,(10/3)}$
200 Normalized ARG copy number
201 = (Relative ARG copy number / Relative 16S rRNA gene copy number) × 4.1
202
203 4.1 was considered to be the average number of 16S rRNA gene copies per bacterium
204 based on the Ribosomal RNA Operon Copy Number Database (Klappenbach et al.,
205 2001).
206

207 2.6 Statistical analysis

208 Statistical analysis was performed in the R environment. The R package, Vegan
209 (Oksanen et al., 2018) was used for PERMANOVA (Adonis), Principal Coordinate
210 Analysis (PCoA), Canonical Correlation Analysis (CCA), Redundancy Analysis
211 (RDA), Variation partitioning analysis (VPA), Inverse Simpson index and Shannon
212 evenness score. The protocol for choosing either CCA or RDA was based on the axis
213 lengths of 4 iterations. If the first four axis lengths were shorter than 3, RDA was
214 selected. However, if the first four axis lengths were longer than 4, CCA was selected.
215 Both analyses can be chosen when the first four axis lengths were between 3 and 4.

Graphs were constructed using ggplot 2 3.1 (Wickham et al., 2018) and heatmaps were built by using the R heatmap package (Galili et al., 2018) Pearson correlation coefficient analysis and analysis of variance (ANOVA) was conducted using SPSS 21. Pie charts and bar charts were produced in Excel 2016 and originlab 2018, respectively. Bar charts show the mean value of 5 replicates with standard errors (SE) calculated in Excel 2016. PD whole tree, observed species, chao1 and Shannon analysis of the bacterial diversity among rice and wheat samples was calculated using QIIME and visualized with orginlab 2018.

225 **3. Results**

226 3.1 Diversity and abundance of ARGs in the phyllosphere of rice and wheat.

227 Across all rice and wheat leaf samples a total of 162 unique ARGs and 5 mobile genetic
228 elements MGEs were detected. In each sample the total number of ARGs detected
229 ranged from 32 to 105 (Figure 1A), while on average there were 3 MGEs per sample,
230 these included major antibiotic resistance classes. Four types of antibiotic resistant
231 mechanisms were detected: antibiotic deactivate (42%), cellular protection (17%),
232 efflux pump (34%) and other unknown mechanisms (7%) (Figure 1B).
233 The normalized abundance of ARGs in rice and wheat samples ranged from 0.0044 to
234 0.047 and 0.03 to 0.29 copies per cell, respectively (Figures 2A, B). ARG composition
235 for both rice and wheat varied between, organic and mineral fertilizer treatments along
236 the x axis which accounted for 37.76% and 29.26% of the variation for rice and wheat
237 samples, respectively. (Figures 2C, D).
238 The composition of ARGs varied significantly between the phyllospheres of rice and
239 wheat ($P < 0.001$, PERMANOVA). Aminoglycoside, MGEs, multidrug and others
240 resistance genes were increased in samples ($P < 0.05$, ANOVA) from the first rice
241 fertilization, compared to the second rice fertilization. While the abundance of Beta-
242 Lactamase and multidrug resistant dominated in the second fertilization of rice (Figure
243 S1A). In wheat, MGEs ($P < 0.05$, ANOVA) and Beta Lactamase ($P < 0.001$, ANOVA)
244 were significantly raised in wheat samples from the first fertilization. Aminoglycoside
245 abundance also increased between first and second fertilizations of wheat (Figure S1B)
246 ($P < 0.001$, ANOVA).

247 Inverse Simpson and Shannon indices for ARGs of first fertilization samples of rice
248 were higher than those from the second fertilization (Figure 2, P <0.001), while the
249 diversity from the wheat phyllosphere was higher after the second fertilization than the
250 first (Figure S1, P <0.001). Overall, wheat phyllosphere samples had a greater diversity
251 of ARGs than the rice phyllosphere (Figure S2).
252 According to heatmap analysis (Figure 3), the total abundance of ARGs in rice samples
253 were lower than in wheat. However, the abundance of ARGs in rice after the first
254 fertilization (Rice no fertilization 1 - RCF, Rice mineral fertilization 1 - RMF, Rice
255 clean manure fertilization 1 - RCM and Rice dirty manure fertilization 1 -RDM) was
256 higher than those after the second fertilization (Rice no fertilization 2 - RCF2, Rice
257 mineral fertilization 2 - RMF2, Rice clean manure fertilization 2 - RCM2 and Rice dirty
258 manure fertilization 2 - RDM2) (P < 0.001, PERMANOVA).

259 3.2 The effect of antibiotic content of manures on phyllosphere ARGs abundance

260 Both clean and dirty manure significantly increased the abundance of ARGs and MGEs
261 in the phylloshere of both wheat and rice (Figure 2A, B; P < 0.01; RCM, P < 0.05)
262 when compared to mineral fertilizer and control treatments.. The abundance of ARGs
263 under clean manure application was lower than the abundance under dirty manure
264 application. In rice samples, Tetracycline (P <0.001), Aminoglycoside (P <0.001), Beta
265 Lactamase (P =0.001), MGEs (P <0.001), MLSB (P <0.05), Multidrug (P <0.05) and
266 Vancomycin (P <0.05) were enriched in the dirty manure compared to the clean manure
267 treatment (Figure S1). Whereas, Tetracycline (P <0.001), Beta Lactamase (P <0.001),
268 Aminoglycoside (P <0.05) and MLSB (P <0.05) were more abundant in wheat samples

269 treated with dirty than clean manure (Figure S1). Specifically, the abundance of ARGs
270 such as, *aadA, aadA1, ampC, ttgA, tnpA, blaSHV, fosX* and *qacEdelta1* were increased
271 in rice phyllosphere samples when dirty manure was applied ($P < 0.001$, ANOVA). In
272 the wheat phyllosphere *tnpA, acrR, aadA2, aadA5, ampC, cmlA1, vanSB, blaOXY and*
273 *acrA* were enriched in both clean and dirty manure treatments ($P < 0.05$, ANOVA)
274 (Figure 3).
275

276 3.3 Correlation between phyllosphere ARGs and bacterial communities.

277 2,891,103 high-quality sequences were detected from 80 samples and a total of 19,013
278 operational taxonomic units (OTUs) obtained. Proteobacteria (70.6%), Firmicutes
279 (13.5%), Actinobacteria (0.8%) and unassigned OTUs (11.6%) were the major phyla in
280 both rice and wheat phyllosphere samples. PD whole tree, observed species, chao1 and
281 Shannon analysis of the bacterial communities demonstrated that the diversity of the
282 second wheat fertilization samples was the highest amongst all four groups with the
283 lowest associated with the second rice fertilization (Figure S3). Among the
284 Proteobacteria, alpha and gamma Proteobacteria were the most abundant classes,
285 ranging in relative abundance from 0% to 38.9% and 0.4% to 99.9%, respectively.
286 A correlation existed between ARGs and the composition of bacterial communities
287 based on the Bray-Curtis dissimilarity metrics by Procrustes analysis for rice
288 (Procrustes sum of squares M^2=0.78, r=0.17, P <0.001) and wheat (Procrustes sum of
289 squares M^2=0.87, r=0.34, P <0.001) samples (Figure S4).
290 PCoA analysis showed differences in the composition of bacterial communities

291 between rice and wheat phyllospheres (Figure 4). Samples from the first and second
292 fertilization of rice clustered together while wheat samples from the two fertilization
293 periods separated along the secondary coordinate, which accounted for 19.7% of
294 variation (Figure 4).
295
296 Canonical correlation analysis (CCA) (Figure 5A) (First axis length 4.3481) was
297 conducted for rice samples, and highlighted that the dominant families (>1%)
298 (Enterobacteriaceae, Bacillaceae, Pseudomonadaceae, Rhizobiaceae, Moraxellaceae)
299 and MGEs were the main drivers. ARGs in the dirty manure treatments from rice for
300 the second fertilization was positively correlated with the abundance of MGEs (*P*
301 =0.001, R^2 0.68) and Pseudomonadaceae respectively (*P* =0.016, R^2 0.26).
302 Redundancy analysis (RDA) (First four axis length shorter than 3) was carried out for
303 wheat samples (Figure 5B), where ARGs in both manure (clean and dirty) treatments
304 at both first and second fertilizations were positively correlated with MGEs (*P* =0.001,
305 R^2 0.70) and Pseudomonadaceae (*P* =0.001, R^2 0.72), but negatively correlated with
306 Moraxellaceae (*P* =0.0047, R^2 0.22). In particular, ARGs in the clean and dirty manure
307 treatments at the first fertilization of wheat were influenced by Moraxellaceae. In
308 contrast, ARGs in the clean manure treatment at the second fertilization of wheat were
309 influenced by Pseudomonadaceae. Both MGEs and Pseudomonadaceae affected the
310 ARGs in the dirty manure treatment at the second fertilization of wheat.
311 Variation partitioning analysis (VPA) (Figure 6), showed that the total variation of
312 ARGs in rice from bacterial communities and MGEs was 47.7%, with a greater

313 contribution from bacterial (43.6%) communities than by MGEs (4.1%) The coefficient
314 between bacterial and MGEs accounted for 2.6% and 8.6% in rice and wheat samples,
315 respectively. For the wheat phyllosphere, shifts in ARG composition could similarly be
316 explained by interactions between bacterial communities and MGEs, with relative
317 contributions of each group being 30.3% and 2.7%, respectively.
318

4. Discussion

4.1 Rice and wheat phyllospheres have significantly different ARG patterns.

As we hypothesized, the composition of ARGs associated with rice and wheat phyllospheres were different. Although we applied the same treatments to both rice and wheat (no fertilizer, mineral fertilization, clean manure and dirty manure), the pattern of ARGs between these two crops were distinct. Moreover, differential selection between rice and wheat formed distinct bacterial communities which further affected the structure of ARGs in the phyllosphere. The diversity of the bacterial community in the rice phyllosphere was lower than that of the wheat phyllosphere. The high soil water content during rice cultivation may be a factor in this reduced diversity. Paddy fields are rain fed and the high water content can lead to both aerobic and anerobic soil conditions, which may have impacted the diversity of ARGs (Wang et al., 2018). Furthermore, flooded water may block the contact pathway from manure amended soil to the phyllosphere, and thus may affect the spread of ARGs. In addition to soil water content, supply of nutrients such as nitrogen (Ikeda et al., 2011), carbon (Wilson and Lindow, 1994), phosphate and sulphate (Delmotte et al., 2009), changes in growth stage and leaf age (Kadivar and Stapleton, 2003; Yutthammo et al., 2010), may all cause shifts in ARGs. Therefore, ARG composition is likely to be driven by multiple factors.

4.2 Manure enhances ARGs in the phyllosphere of rice and wheat.

In our study, organic fertilization enriched ARGs in the phyllosphere of rice and wheat, which is supported by previous studies (Chen et al., 2018; Marti et al., 2013).

341 Additionally it has been reported that some ARGs originate from manure which may
342 enhance ARGs in the wider environment (Wang et al., 2018). The application of organic
343 fertilizers such as sewage sludge and pig manure can enrich the abundance of ARGs
344 which may reach the phyllosphere (Jadhav et al., 2014; Rahube et al., 2014). The
345 phyllosphere provides a habitat for microbial communities that originate from soil,
346 water and air (Bulgarelli et al., 2013). As there is a clear correlation between the soil
347 and phyllosphere resistome, the use of manures on soil is likely to affect the microbial
348 structure on the leaf surface (Chen et al., 2017a; Chen et al., 2017b).
349
350 This study further found that the enrichment of ARGs in the phyllosphere was lower in
351 clean (reduced antibiotic burden) than dirty (current antibiotic practice) manure,
352 supporting our hypothesis that dirty manure can be a source of ARGs. While mutation
353 of animal gut microbiota, creating antibiotic resistance will exist even in clean manure,
354 production of ARGs will be lower than that of dirty manure (Zhao et al., 2018). It has
355 been reported that wild mammals harbour ARGs, which also indicates that ARGs occur
356 in wild populations where antibiotics in comparison to managed livestock are rare
357 (Mallon et al., 2002; Poeta et al., 2007; Tsukayama et al., 2018). This suggests that even
358 the use of clean manure can also increase the risk of ARGs spreading. As a result,
359 organic manure should be pretreated before application in order to mitigate the risk of
360 ARG transfer (Burch et al., 2017). Alternatively, composting and biochar have been
361 reported to effectively mitigate the risk of the antibiotic resistome (An et al., 2018; Gao
362 et al., 2019).

363 Pathways such as the food chain and air circulation may account for the spread of ARGs
364 into the environment. For example, after harvest, residual wheat leaves are used in
365 animal feeds (Khush, 1997), which provides possible pathways for ARGs to enter the
366 food chain and interact with the gut microbiome of livestock. The possible exchange of
367 ARGs between the phyllosphere and atmosphere may also exist due to air movement
368 (Bringel and Couee, 2015) and thus the phyllosphere may provide a new pathway of
369 spreading ARGs to the wider environment.
370

371 4.3 Contribution of bacterial communities to ARGs composition in the phyllosphere

372 This study showed that changes in the composition of bacterial communities may be
373 responsible for shifts in ARGs in both rice and wheat samples, a finding supported by
374 Chen et al., (2018) and Zhao et al., (2018). These studies found that the application of
375 manure could significantly alter the composition of bacterial communities in both the
376 soil and phyllosphere as well as increase the diversity of the resistome. While part of
377 the change in ARGs remains unexplained, it is probable that environmental factors that
378 change during cultivation and the aerial deposition of bacteria induced compositional
379 changes in the bacterial communities of the phyllosphere. There are various channels
380 for bacteria to reach the phyllosphere, including soil, rain and air (Delmotte et al., 2009;
381 Vorholt, 2012). The composition of bacterial communities are also associated with
382 geographic and climatic factors (Ren et al., 2014) as well as differences in leaf
383 construction between species. Machine learning may be used in the future for fast
384 recognition of potential microbial communities which could affect the environmental

385 resistome (Camacho et al., 2018).

386

387 **Conclusion**

388 In this study, a total of 162 unique ARGs and 5 MGEs were detected through HT-qPCR.
389 Rice and wheat phyllospheres had differing patterns of ARGs and bacterial
390 communities, indicating that multiple factors, such as plant species, diverse growth
391 conditions, nutrient supply and atmospheric movement, may affect diversity in the
392 phyllosphere. Both clean and dirty manures enhanced ARGs in the phyllosphere, with
393 dirty manure in particular causing the greatest enrichment of ARGs. As rice and wheat
394 are staple crops globally, the application of both clean and dirty manure that deliver
395 ARGs and MGEs to the food chain may pose a significant risk to human health and act
396 as a conduit for ARGs to reach the environment.

397

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Figure Legend

Figure 1. a: Number of ARGs and MGEs detected from different samples. b: The percentage of antibiotic resistant mechanisms within all samples. ARGs were separated into 10 classes based on the following reference antibiotic resistant genes: Aminoglycosides, beta-lactams, chloramphenicol, MGEs, MLSB, Multidrug, sulfonamides, tetracycline, vancomycin and other unknown. For rice samples, RCF, RMF, RCM and RDM represent no fertilizer, mineral fertilization, clean and dirty manure fertilization. RCF2, RMF2, RCM2 and RDM2 represent second fertilization samples. Similar nomenclature applies to wheat samples.

Figure 2. Characteristics of ARGs in rice and wheat samples. a and b represent patterns of normalized abundance (copy/cell) of rice and wheat, respectively. c and d depict the PCoA analyses of ARGs. “R” represents Rice samples, “W” represents wheat samples. (1), (2), (3), (4) represent ARGs in the manure treatments at the first fertilization of rice (RCM, RDM), second fertilization of rice (RCM2, RDM2), first control (no fertilizer) samples and mineral ferilitzation treatments (RCF, RMF) and second control (no fertilizer) samples and mineral fertilization (RCF2, RMF2). (5), (6), (7), (8) represented ARGs in manure treatments from the first fertilization of wheat (WCM, WDM), second fertilization of wheat sample (WCM2, WDM2), first control (no fertilizer) and mineral fertilization treatments (WCF, WMF) and second control (no fertilizer) and mineral fertilization (WCF2, WMF2).

Figure 3. Heatmap analysis of ARGs in rice and wheat samples. The vertical axis lists the detected ARGs found in this study. For rice, RCF, RMF, RCM and RDM represent no fertilizer, mineral fertilization, clean and dirty manure fertilization respectively. RCF2, RMF2, RCM2 and RDM2 represent the second rice fertilization. Similar nomenclature applies to wheat samples. The order of the genes was based on their similarity abundance.

Figure 4. PCoA analysis of bacterial communities based on Bray-Curtis distance. a) represents the first and second fertilization of phyllosphere samples from rice; b) and c) represent the first and second wheat fertilization, respectively.

Figure 5. CCA (a) and RDA (b) analysis of the correlation among ARGs in rice and wheat samples, major microbial families (>1%) (Enterobacteriaceae, Bacillaceae, Pseudomonadaceae, Rhizobiaceae, Moraxellaceae) and MGEs in rice and wheat respectively. Label (1), (2), (3), (4) represent the ARGs in the first and second fertilization of rice and wheat samples respectively. Pseudomonadaceae and MGEs present a positive correlation in both rice and wheat samples, whereas Moraxellaceae in wheat samples shows a negative correlation.

Figure 6. Variation partitioning analysis highlighting the influence of the bacterial community and mobile genetic elements to the change in ARGs. For rice samples, 50.3% of changes in ARGs were explained through the bacterial community, MGEs and their coefficient, whereas in wheat samples this was 41.6%.

Figure 1

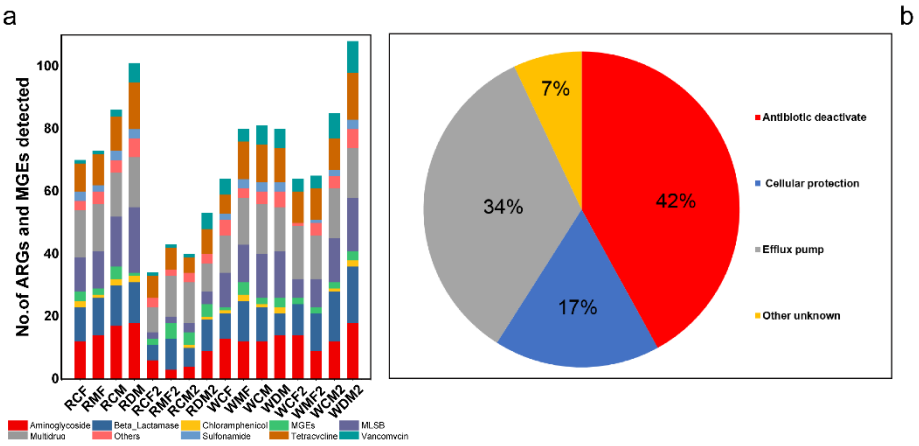

Figure 2

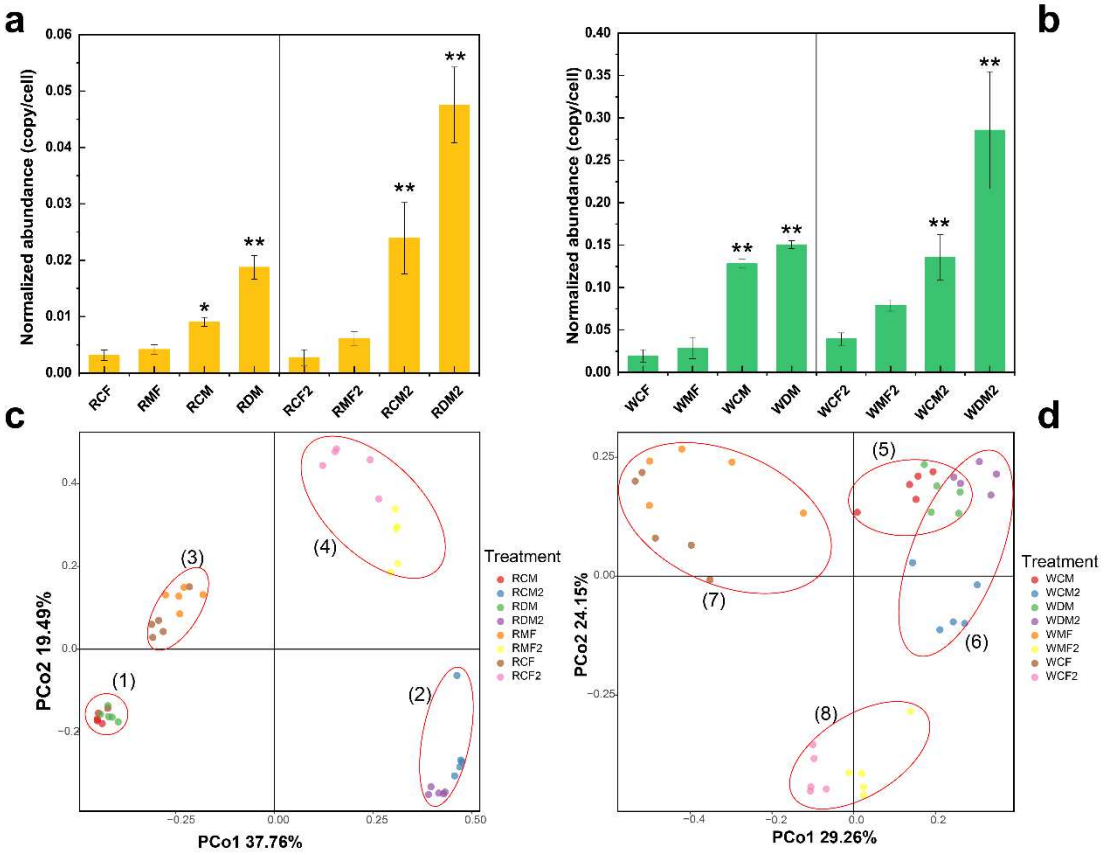

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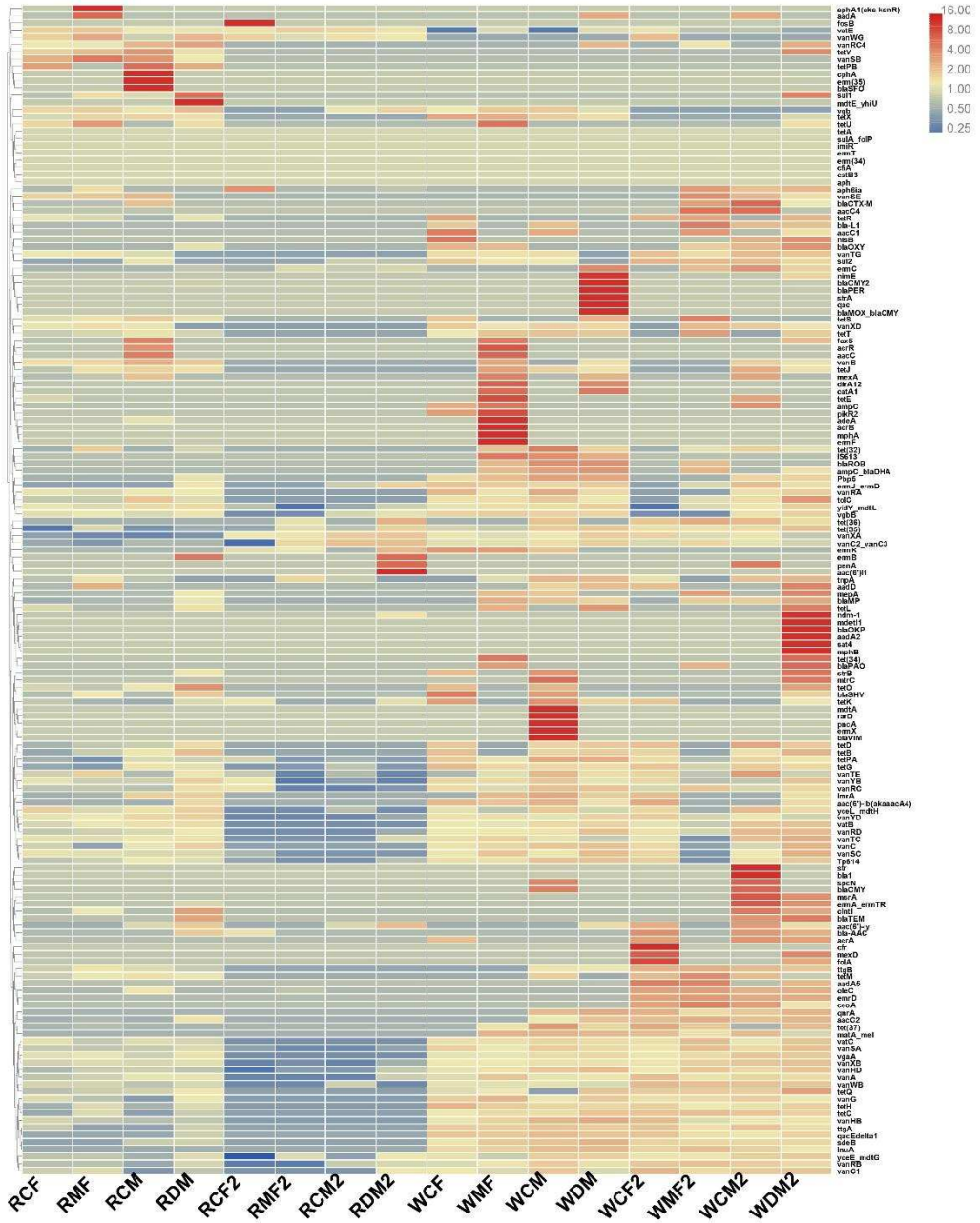
Figure 3



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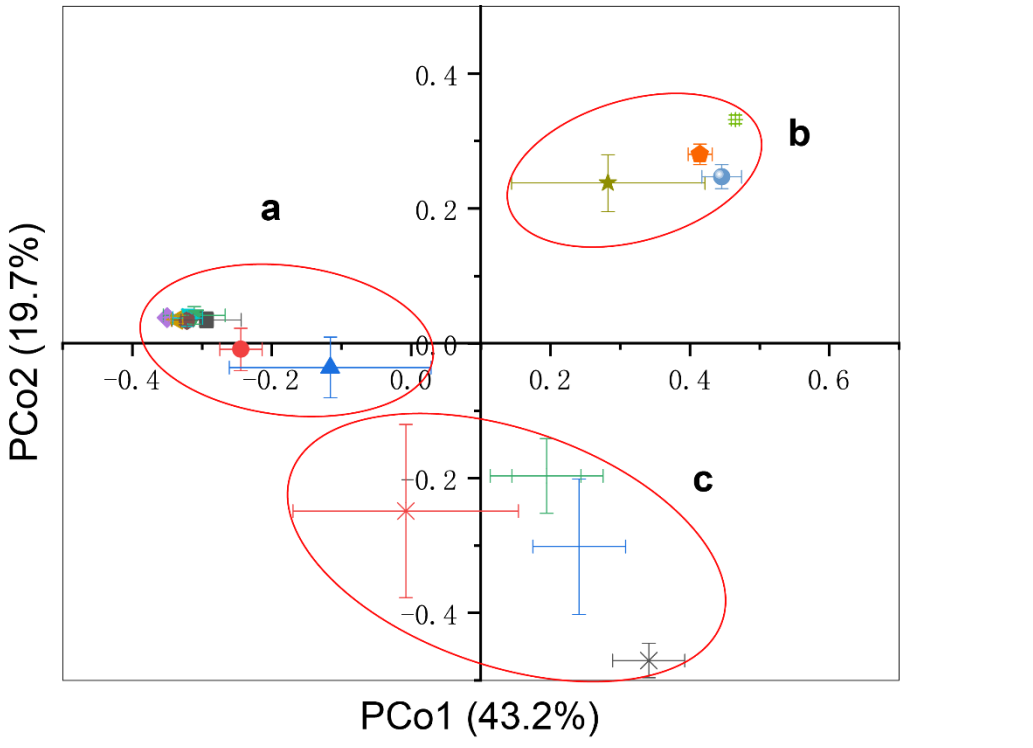
646 **Figure 4**



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649 **Figure 5**

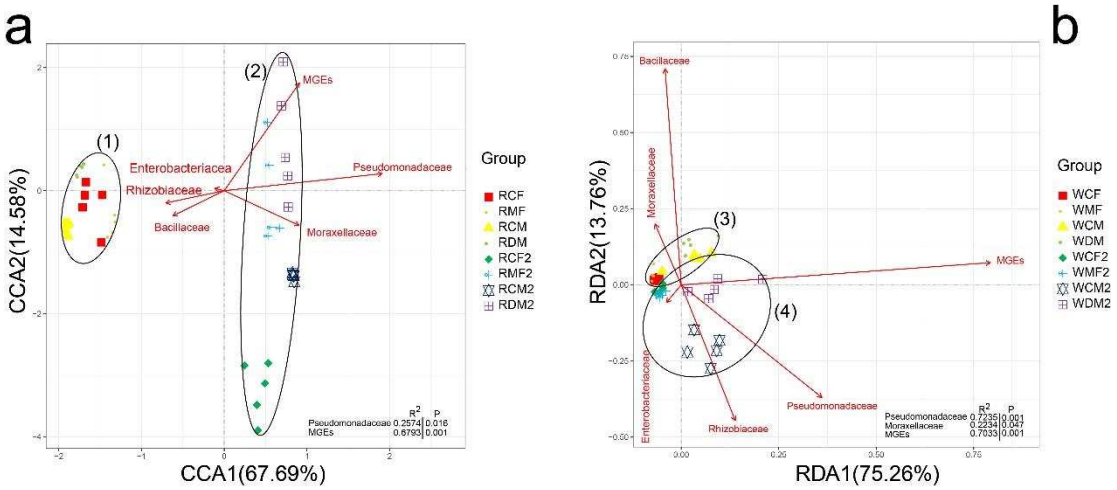

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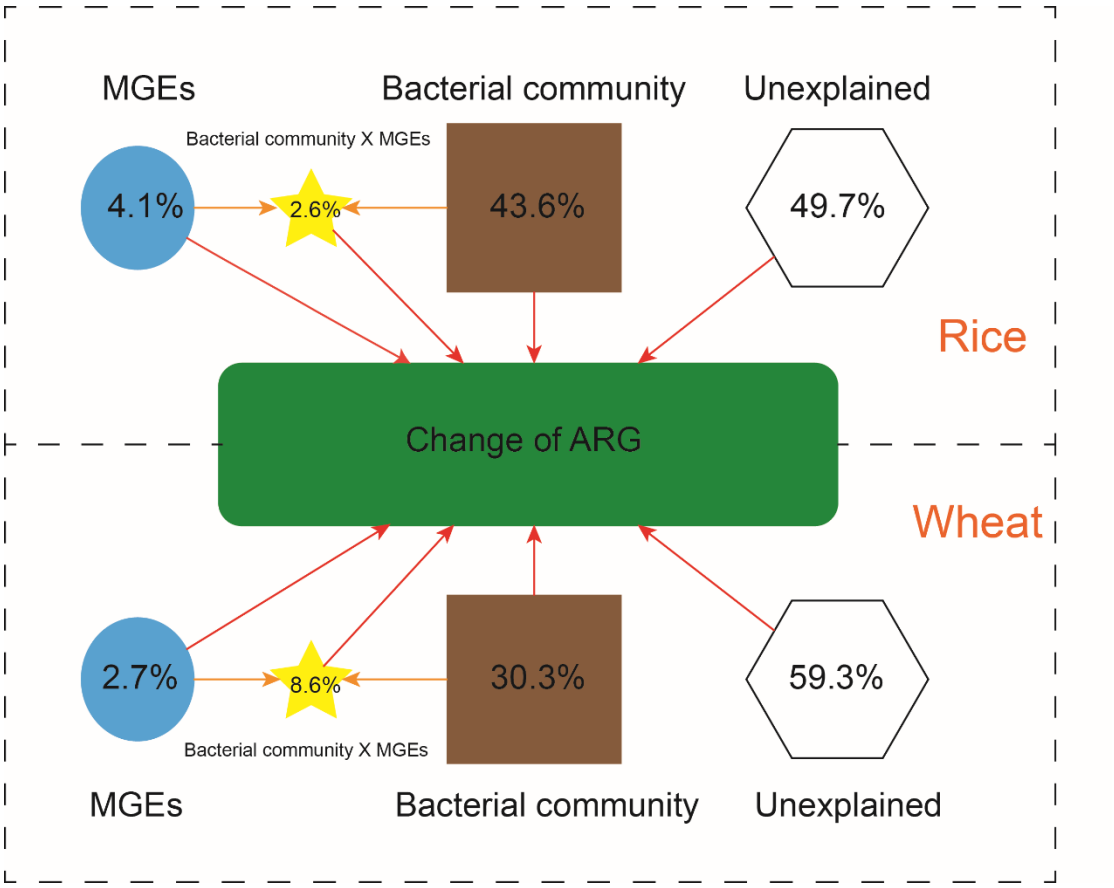
Figure 6


MGEs
Bacterial community
Unexplained
Bacterial community X MGEs
4.1%
2.6%
43.6%
49.7%
Rice
Change of ARG
Wheat
2.7%
8.6%
30.3%
59.3%
Bacterial community X MGEs
MGEs
Bacterial community
Unexplained