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**Horizontal gene transfer and shifts in linked bacterial community composition are associated with maintenance of antibiotic resistance genes during food waste composting**

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1 **Horizontal gene transfer and shifts in linked bacterial community composition**  
2 **are associated with maintenance of antibiotic resistance genes during food waste**  
3 **composting**

4  
5 **Abstract**

6 About 1.3 billion tons of food waste (FW) is annually produced at a global scale. A  
7 major fraction of FW is deposited into landfills thereby contributing to environmental  
8 pollution and emission of greenhouse gasses. While increasing amounts of FW are  
9 recycled more sustainably into fertilizers in industrial-scale composting, very little is  
10 known about the antibiotic resistance genes (ARGs) present in FW and how their  
11 abundance is affected by composting. To study this, we quantified the diversity and  
12 abundance of ARGs, mobile genetic elements (MGEs) and bacterial communities in  
13 the beginning, during and at the end of the FW composting. All targeted 27 ARGs and  
14 5 MGEs were detected in every sample suggesting that composted FW remains a  
15 reservoir of ARGs and MGEs. While the composting drastically changed the  
16 abundance, composition and diversity of bacterial communities, an increase in total  
17 ARG and MGE abundances was observed. Changes in ARGs were linked with shifts  
18 in the composition of bacterial communities as revealed by a Procrustes analysis ( $P <$   
19  $0.01$ ). Crucially, even though the high composting temperatures reduced the  
20 abundance and diversity of initially ARG-associated bacterial taxa, ARG abundances  
21 were maintained in other associated bacterial taxa. This was likely driven by  
22 horizontal gene transfer and physicochemical composting properties as revealed by a  
23 clear positive correlation between ARGs, MGEs, pH,  $\text{NO}_3^-$  and moisture. Together  
24 our findings suggest that traditional composting is not efficient at removing ARGs  
25 and MGEs from FW. More effective composting strategies are thus needed to

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26 minimize ARG release from composted FW into agricultural environments.

27 **Keywords:** Antibiotic resistance genes, Municipal solid waste, Mobile genetic  
28 elements, Bacterial community composition, Composting physicochemical  
29 parameters

30

## 31 **1. Introduction**

32 A significant portion of food ends up as unused waste resulting in more than 1.3  
33 billion tons of food waste (FW) annually at the global scale (Gustavsson et al., 2011).

34 In China and in the U.S. about 90% of FW ends up in landfills (Breunig et al., 2017;  
35 Yong et al., 2015). However, landfilling FW is unsustainable leading to increased

36 greenhouse gas emissions and prevents the required land from being used for other  
37 purposes (Zhang et al., 2014). Composting FW into organic fertilizers provides an

38 environmentally friendly alternative to FW landfilling and is increasingly used around  
39 the world (Cerdeira et al., 2018; Li et al., 2013; Wang et al., 2017b). However, there

40 exists only little information about composting of FW. One potential risk could be  
41 human-associated pathogens and bacteria-associated antibiotic resistance genes that

42 could potentially get enriched during the composting process (ADD REF). Antibiotic  
43 resistance is recognized as a major threat to public health worldwide and antibiotic

44 resistant bacteria and antibiotic resistance genes (ARGs) are widely found in the  
45 natural environments, wastewater, manure, and sewage sludge (Lekunberri et al.,

46 2017; Ma et al., 2015; Su et al., 2017b). Previous studies have shown that ARGs are  
47 found in various foods such as pork, beef, raw fruits and fresh vegetables (Rolain,

48 2013; Ruimy et al., 2010). However, the identity and composition of ARGs and  
49 MGEs in FW remains unknown. Many antibiotic-resistant microbes, including both

50 foodborne pathogenic and commensal bacteria, are also found in food chains ranging

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51 from manufacturing to commercial products (Wang et al., 2012) and numerous studies  
52 support the link between the use of antibiotics and enrichment of ARGs during  
53 agricultural production. Food waste could thus serve as an important source of ARGs  
54 and MGEs similar to other types of organic wastes such as sewage sludge (Su et al.,  
55 2017a) and animal manure (Wang et al., 2017a). Hence, it is important to understand  
56 how FW composting impacts the abundance, mobility and diversity of ARGs to  
57 minimize the potential spread of ARGs to agricultural environments along the final  
58 composting products.

59         Agricultural application of composted organic waste introduces high loads of  
60 bacteria that often carry ARGs into the soil (Xie et al., 2016). In the soil, ARGs  
61 propagate along with their bacterial hosts and can disseminate via horizontal gene  
62 transfer between different bacterial species, including many human pathogenic  
63 bacteria (Forsberg et al., 2014). Mobile genetic elements (MGEs), such as plasmids,  
64 integrons and transposons are often linked with ARGs and their horizontal  
65 dissemination (Bengtsson-Palme et al., 2018; Gillings, 2017) and they often confer  
66 resistance to multiple antibiotics (Pehrsson et al., 2016). If ARGs enter humans via  
67 the food chain, the efficiency of antibiotics could be reduced. While the diversity and  
68 abundance of ARGs and MGEs has been studied largely in the process of manure and  
69 sewage sludge composting (Ma et al., 2015; Su et al., 2017b; Wu et al., 2017), there  
70 are no studies investigating ARG and MGE dynamics during FW composting. Even  
71 though most food products do not normally contain antibiotics, the emergence of  
72 abundant ARGs have been found in some food products such as meat and dairy  
73 products (Wang et al., 2012). It is thus important to understand the abundance and  
74 distribution of ARGs and MGEs in FW, if these genes are mobile or associated with  
75 certain bacterial hosts, and crucially, whether composting can be used to remove or

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76 considerably reduce their abundance resulting in safe composting end product (Liao et  
77 al., 2018).

78 FW has many specific physical and chemical characteristics that separates it  
79 from other organic wastes including high organic matter content, high salt, oil and  
80 protein content and low pH (Cerdeira et al., 2018). It is known that these properties have  
81 a strong effect for the transmission of ARGs (Bengtsson-Palme et al., 2018). For  
82 example, Liu *et al.* found that salinity can improve the removal of antibiotic resistance  
83 genes in wastewater treatment bioreactors (Liu et al., 2018). However, it is unclear  
84 how the specific physicochemical properties of FW affect the dynamics of ARGs  
85 during composting. Composting is a complex fermentation process that induces  
86 dynamic changes in biotic components such as the microbial community structure and  
87 abiotic factors including physicochemical properties of compost (Su et al., 2015;  
88 Zhang et al., 2016). These could potentially drive the abundance and composition of  
89 ARGs and MGEs directly or have indirect effects on their bacterial hosts. For  
90 example, composting temperature has been shown to play an important role in  
91 removing ARGs via effects on MGEs and on the bacterial community composition  
92 (Liao et al., 2018).

93 Here we investigated the impact of FW composting on the abundance of ARGs,  
94 MGEs and soil bacterial community composition, diversity and densities at an  
95 industrial scale. The objectives of our study were: (1) to investigate the abundance  
96 and diversity of ARGs and MGEs during FW composting; (2) to determine potential  
97 bacterial carriers of ARGs and MGEs (3) to understand the relative importance of  
98 various biotic and abiotic factors (composting properties, bacterial community  
99 diversity, bacterial community composition and MGE abundances) on the dynamics  
100 and abundance of ARGs during FW composting. To this end, we used temporal

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101 sampling followed by quantitative PCR (qPCR) to determine the abundances of 27  
102 ARGs and 5 MGEs in a full-scale FW composting experiment. Furthermore, we  
103 applied Illumina Hiseq sequencing of the bacterial 16S rRNA genes to determine the  
104 composition of the ARG and MGE-associated bacterial communities.

## 105 **2. Materials and methods**

### 106 **2.1 Full-scale experimental setup for food waste composting**

107 FW composting experiments were conducted in a full-scale aerobic composting plant  
108 located in Jinshui district, Zhengzhou, China. The aerobic composting process has  
109 previously been described by [Liao et al. \(2018\)](#) and the same methods were used in  
110 this experiment with some modifications. Briefly, raw composting materials consisted  
111 of deoiled FW (with 78% water content, provided by Xinmi food waste processing  
112 factory) and tobacco powder (with 20% water content, provided by Zhengzhou  
113 cigarette factory). Tobacco powder was used as a composting amendment as it is an  
114 abundant and accessible waste produced locally near the composting plant.  
115 Approximately 80 tons of composting material was created by mixing FW and  
116 tobacco powder in a ratio of 3:1 (v/v), respectively. The final composting material had  
117 approximately 58% water content. The composting mixture was loaded into three  
118 independent replicate piles (8 m length, 6 m width, and 3 m height) at 2.2 m bulk  
119 height. Forced aeration was supplied from the bottom to the top of the pile according  
120 to aeration needs during different phases of composting ([Liao et al., 2018](#)). To mix the  
121 compost substrate well and to reduce pile-edge effects, mechanical turning of the  
122 composting material was performed every seven days using pile-specific forklifts to  
123 prevent cross-contamination between replicate piles.

124

### 125 **2.2 Sample collection and DNA extraction**

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126 Samples were collected at 0, 5, 10, 13, 24, 30, 41, and 50 days after start of the  
127 composting, which allowed us to follow changes during various temperature stages  
128 during the composting process. The sampling process followed a previously described  
129 protocol by [Liao et al. \(2018\)](#) with some minor modifications. Briefly, to obtain  
130 representative samples at each sampling time point, each pile was diagonally divided  
131 into five domains, and each domain was sub-sampled (5000 g) from three different  
132 depths from the top of the pile: 40-50 cm (top), 90-100 cm (middle) and 150-160 cm  
133 (bottom). All sub-samples per sampled domain were pooled to obtain a final sample  
134 (5 samples per replicate pile totaling 15 total samples per sampling time point),  
135 homogenized and further divided into two 400 g aliquots of which one was stored in  
136 liquid nitrogen for biological analyses and the other one was stored at 4 °C for  
137 physicochemical analyses. This sampling approach was chosen to reduce the potential  
138 bias caused by the heterogeneity of the original composting substrate in each replicate  
139 pile. The ALFA-SEQ Advanced Soil Kit (mCHIP, Guangzhou, China) was used to  
140 extract genomic DNA from freeze-dried samples (200 mg) according to the  
141 manufacturer's instructions. DNA extraction was conducted three times per sample  
142 and purified DNA samples were pooled for sequencing and genetic analysis. The  
143 quality of DNA was quantified with NanoDrop ND-2000 (Thermo Fisher Scientific,  
144 Wilmington, USA) spectrophotometric analysis and visualized on a 1% agarose gel  
145 and normalized to equal concentrations before downstream qPCR processing.

146

### 147 **2.3 Determination of physicochemical parameters during composting**

148 We used previously described methods ([Liao et al., 2018](#)) to measure  
149 physicochemical properties during the composting: pH, temperature (Temp), water  
150 content (WC), total nitrogen content (TN), total carbon content (TC), total organic

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151 carbon content (TOC), oil content (OIC), inorganic carbon content (IC) and  
152 ammonium ( $\text{NH}_4^+$ ), sodium (NC) and nitrate ( $\text{NO}_3^-$ ) concentrations. Briefly, TOC and  
153 IC were measured using an automatic TOC analyzer for liquid samples (Shimadzu  
154 TOC-L CPH, Kyoto, Japan). TN and TC were determined in an Elementar instrument  
155 (Vario MAX cube, Hanau, Germany) using dry combustion. OIC was measured using  
156 Soxhlet extractor method as described previously (Wang et al., 2017b). NC analysis  
157 was carried out with flame spectrophotometry. EC and pH were determined using a  
158 conductivity meter (Radiometer, model CDM210) and a pH meter (PB-10, Sartorius,  
159 Germany), respectively.  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were measured by a continuous-flow  
160 autoanalyser (FlowSys, Systea, Rome, Italy). WC was determined as the weight loss  
161 upon drying in an oven at 105 °C for 24 h. Daily monitoring of the composting  
162 temperature was measured with automatic thermometers placed at different depths of  
163 composting piles.

164

#### 165 **2.4 Measuring changes in ARG and MGE abundances with quantitative PCR** 166 **(qPCR)**

167 It has previously been shown that tetracycline, macrolide, aminoglycoside and  
168 sulfonamide resistance genes are the most common ARGs in organic waste (Su et al.,  
169 2015; Wang et al., 2017a). Therefore, we decided to study the abundance of these  
170 ARGs during FW composting by choosing ten tetracycline resistance genes (*tetA*, *tetB*,  
171 *tetC*, *tetG*, *tetL*, *tetM*, *tetQ*, *tetO*, *tetW*, and *tetX*), seven macrolide resistance genes  
172 (*ermB*, *ermF*, *ermM*, *ermT*, *ermX*, *mefA*, and *ereA*), seven aminoglycoside resistance  
173 genes (*aacA4*, *aadA*, *aadB*, *aadE*, *aphA1*, *strA*, and *strB*), and three sulfonamide  
174 resistance genes (*sul1*, *sul2*, and *sul3*) totaling 27 different ARGs. To investigate  
175 potential changes in the abundance of mobile genetic elements (MGEs), we measured

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176 abundances of two integrase genes (*intI1*, *intI2*), two plasmid genes (*ISCR1*, *IncQ*)  
177 and one transposon (*Tn916/1545*, abbreviated as *Tn916*) gene that have often been  
178 connected with the movement of ARGs in the environment (Ma et al., 2017; Zhang et  
179 al., 2016). The bacterial abundance was measured as 16S rRNA gene copy numbers  
180 using SYBR-Green qPCR. All information about primers, annealing temperatures and  
181 amplification sizes used for all target genes are listed in Table S1. The qPCR reactions  
182 were carried out in a LightCycler 96 System (Roche, Mannheim, Germany) using  
183 96-well plates. After amplification, a melting curve analysis with a temperature  
184 gradient ranging from 0.1 °C/s to 70 °C and to 95 °C was performed to confirm that  
185 only specific products were amplified. Standards were created using plasmids  
186 carrying target genes with TA cloning and extracted using a TIAN pure Mini Plasmid  
187 kit (Tiangen, Beijing, China). Concentrations of the standard plasmids (ng/μL) were  
188 determined using Nanodrop ND-2000 (Thermo Fisher Scientific, Wilmington, USA)  
189 to calculate copy number concentrations (copies/mL). Each qPCR reaction contained  
190 10 μL GoTaq qPCR Master Mix (Promega, Madison, USA), 1.5 μL of each forward  
191 and reverse primers (4 mmol/L), 1 μL of template genomic DNA (approximately 10  
192 ng), and 6 μL of nuclease-free water. Amplification conditions were 95 °C for 2 min,  
193 followed by 40 cycles of denaturation at 95 °C for 30s per cycle, annealing for 30 to  
194 45s according to the amplicon length at the primer-specific annealing temperature  
195 (detail in Table S1), and extension for 30s at 72 °C. The amplification efficiencies of  
196 all qPCR products ranged from 90% to 110% with linear coefficient ( $R^2$ ) values above  
197 0.99 for all standard curves. Each reaction was run in triplicate alongside negative  
198 controls including DNA-free water instead of template genomic DNA. Absolute  
199 abundances of target genes are presented as gene copy numbers per gram of dry  
200 compost sample, while the relative abundances of target genes are shown as target

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201 genes per 16S rRNA gene copy numbers.

202

## 203 **2.5 Sequencing and bioinformatic analyses of the bacterial community** 204 **composition during composting**

205 We used 16S rRNA amplicon sequencing to determine changes in bacterial  
206 community diversity and composition during composting. The V4 region of the  
207 bacterial 16S rRNA gene was amplified using 515F  
208 (GTGCCAGCMGCCGCGGTAA)/806R (GGACTACHVGGGTWTCTAAT) primers  
209 and sequenced with the Illumina Hiseq 2500 platform and paired-end sequencing (2 ×  
210 150 bp) (Biddle et al., 2008). DNA libraries were prepared with an Illumina Hiseq  
211 Nextera library preparation kit following the manufacturer's protocol. The reverse  
212 primer contained a unique barcode for each sample and DNA was amplified in  
213 triplicate before sequencing. The amplification was initiated at 94 °C for 5 min and  
214 followed by 31 amplification rounds (94 °C for 30s, 52 °C for 30s, 72 °C for 45s and  
215 72 °C for 10 min). Raw Illumina sequence data was processed with a pipeline  
216 coupling Trimmomatic (v 0.33) and QIIME (v 1.9.1) (Caporaso et al., 2010). Briefly,  
217 the raw sequences with low-quality reads that contained ambiguous nucleotides,  
218 mismatches in primer regions, or a length shorter than 100 bp were removed (An et al.,  
219 2018). Sequences were clustered into operational taxonomic units (OTUs) with  
220 UCLUST (version) at 97% sequence similarity (Edgar, 2010). Taxonomic OTU  
221 assignment was performed up to an 80% threshold using a Ribosome Database  
222 Project Classifier as described previously using the Greengenes database (McDonald  
223 et al., 2012). The normalization of the quality-curated sequences was conducted by  
224 subsampling to 10,759 sequences from each sample data set. Alpha-diversity was  
225 estimated using OTU richness based on the number of OTUs, phylogenetic diversity,

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226 Dominance, Chao1, Shannon and Simpson diversity indices. Differences between  
227 microbial communities (beta-diversity) and principal coordinate analysis (PCoA)  
228 were analyzed based on the weighted Unifrac distances. All sequences were deposited  
229 in the National Center for Biotechnology Information Sequence Read Archive under  
230 the accession number SRP156265.

231

## 232 **2.6 Analyzing and visualizing bacterial, ARG and MGE co-occurrence networks**

233 Co-occurrence network analysis was used to explore pairwise correlations between  
234 bacterial taxa and different ARGs and MGEs during FW composting. Several  
235 previous reports have demonstrated that the non-random co-occurrence patterns could  
236 provide indirect evidence for potential host bacteria of ARGs and MGEs (Liao et al.,  
237 2018; Su et al., 2017b). Pairwise correlations were determined using Pearson and  
238 Spearman correlations as described previously (Hu et al., 2017). Only relatively large  
239 correlation coefficients ( $\rho > 0.8$  and  $P < 0.01$ ) detected with both methods (Pearson  
240 and Spearman) were included into network analyses to minimize false-positive  
241 correlations. Furthermore, the Benjamini-Hochberg procedure (q-value,  $q < 0.01$ ) was  
242 performed to adjust  $P$ -values of all correlations to control false-discovery rate. The  
243 remaining significant interactions between ARGs, MGEs and bacteria were visualized  
244 as a network in Cytoscape v3.4.0, and network statistics were analyzed with Network  
245 Analyzer as undirected networks using default settings (Cline et al., 2007).

246

## 247 **2.7 Statistical analyses**

248 Data were analyzed using repeated measures ANOVA (ARG and MGE density  
249 dynamics), PCoA, redundancy analysis (RDA), PERMANOVA test, and Procrustes  
250 test for correlation analysis between ARGs and bacterial community composition with

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251 the vegan package v2.4-3 in R 3.3.2. Heat maps represent log-transformed relative  
252 abundances of ARGs and MGEs and were drawn with *ggplot2* package in R 3.3.2.  
253 Linear Discriminant Analysis Effect Size analysis (LEfSe) was used to determine  
254 differentially abundant taxa between different stages of composting following  
255 methods by [Segata et al. \(2011\)](#). Finally, a partial least squares path modeling  
256 (PLS-PM) was used to explore relationships between physicochemical composting  
257 properties (Temp, WC, pH, IC, TN, TC, TOC, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, OC, and NC), bacterial  
258 alpha-diversity (based on OTU numbers, phylogenetic diversity, Dominance, Chao1,  
259 Shannon and Simpson diversity indices), bacterial community composition (based on  
260 relative OTU abundances), MGEs (relative abundances), and ARGs (relative  
261 abundances). PLS-PM is a powerful statistical method to study interactive  
262 relationships among observed and latent variables ([Wagg et al., 2014](#)) and is widely  
263 applied to explain and predict relationships in multivariate data sets ([Puech et al.,](#)  
264 [2015](#)). Path coefficients (i.e. standardized partial regression coefficients) represent the  
265 direction and strength of the linear relationships between variables (direct effects).  
266 Indirect effects are the multiplied path coefficients between a predictor and a response  
267 variable, adding the product of all possible paths excluding the direct effect. Models  
268 with different structures were evaluated using the Goodness of Fit (GoF) statistic, a  
269 measure of their overall predictive power. The R package *pls* (v 0.4.7) was used to  
270 construct the final PLS-PM model.

271

## 272 **3. Results and Discussion**

### 273 **3.1 Tracing ARG and MGE abundances and diversity throughout composting**

274 The total concentrations of ARGs and MGEs in the initial raw FW were  
275 approximately  $1.5 \times 10^{10}$  and  $2.0 \times 10^9$  gene copies per gram (dry weight) of FW,

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276 respectively (Figure S1). In contrast, the initial tobacco powder contained very low  
277 amounts of ARGs (0.45% of ARGs observed in FW) and was not thus included in our  
278 analysis of initial ARG and MGE composition. The tetracycline and macrolide  
279 resistance genes were the most dominant genes accounting for 84.3% of total ARGs  
280 while the transposon *Tn916* gene was the most dominant MGE in the initial FW  
281 accounting for 94.2% of all MGEs (Figure S1). We also detected all targeted 27  
282 ARGs and 5 MGEs that were initially present in the FW samples in the end of the  
283 composting (Figure S2). Surprisingly, the total abundances of all ARGs and MGEs  
284 significantly increased during the 50 days of FW composting ( $P < 0.01$ , Figure 1a). In  
285 particular, the abundances of *tetL*, *sul2*, *strA*, *ermB*, *ISCR1* and *intI1* were enriched by  
286 9.5, 10.7, 10.7, 377.2, 69.4, and 48.0 times, respectively (Figure 1c). This result is in  
287 line with previous studies showing that abundances of *tetL*, *sul2*, *ermB* can be found  
288 in reasonably high abundances after composting (Qian et al., 2016; Zhang et al., 2016).  
289 One explanation could be that these ARGs were located in bacteria that were tolerant  
290 to high temperatures. Alternatively, ARGs could have been located in MGEs that  
291 could have been able to relocate to thermophilic bacteria during composting. We  
292 found support for the both hypotheses in terms of predictable changes in bacterial  
293 community composition and positive correlation between the abundances of plasmid  
294 (*ISCR1*) and mobile integron (*intI1*) genes with the increase in ARGs during  
295 composting (Figure S3,  $P < 0.001$ ). Together these results suggest that traditional  
296 composting is not efficient enough to remove ARGs and MGEs from FW  
297 (mechanisms discussed in more detail later). Instead, we observed that FW contained  
298 a wide diversity and abundance of ARGs and MGEs that even increased during  
299 composting. Our results thus suggest that FW could be an important but often  
300 neglected source of ARGs which could also potentially serve as a transfer route for

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301 ARGs between agricultural environments, food manufacturing processes and  
302 consumers (Berendonk et al., 2015; Verraes et al., 2013; Wang et al., 2012). Great  
303 caution should thus be taken in using FW as composting raw material. However, we  
304 also want to note that only one type of FW was investigated in this study, and hence,  
305 other FW types should be examined in future studies to explore the generality of our  
306 results.

307 To investigate the dynamics of ARGs during composting, we focused on the link  
308 between the abundances of bacteria, ARGs and MGEs during composting in more  
309 detail. We found that total bacterial abundances based on 16S rRNA gene copy  
310 numbers increased during composting (Figure 1a). This is in line with previous results  
311 (Wang et al., 2017b) and can be explained by the increased amount of nutrients that  
312 become available for microbial growth during composting (Adhikari et al., 2009).  
313 Crucially, the increase in the total bacteria abundances was relatively higher than the  
314 increase in ARGs, and as a result, the relative abundance of ARGs per number of  
315 bacterial cells decreased during the composting (Figure 1b and d,  $P < 0.01$ ).  
316 Particularly, tetracycline, macrolide, aminoglycoside and sulfonamide resistance  
317 genes showed clear decreases in their relative abundance, while a significant but less  
318 drastic decrease in the relative abundance of MGEs was observed (Figure 1b and d,  $P$   
319  $< 0.01$ ). Together, these results suggest that even though the absolute abundances of  
320 ARGs and MGEs increased during composting, their relative abundances decreased  
321 and fewer bacteria carried ARGs and MGEs at the end of composting. This could  
322 have been due to because composting conditions favored non-ARG carrying bacteria,  
323 because carrying ARGs incurred fitness costs reducing bacterial competitive ability  
324 (Durão et al., 2018) or because bacteria that were less susceptible to MGEs became  
325 more abundant (Youngquist et al., 2016). While all these hypotheses should be tested

---

326 in the future experiments to better understand the mobilization of ARGs during  
327 composting, our study suggest that concentrating both absolute and relative ARG  
328 abundances is important for predicting the risk of ARG movement across different  
329 environmental compartments.

330

331 **Figure 1**

332

### 333 **3.2 Changes in the bacterial community composition during FW composting**

334 The composting process significantly influenced the composition of bacterial  
335 communities as shown in the non-metric multidimensional scaling plot based on  
336 weighted Unifrac distances (PERMANOVA test,  $R^2=0.8571$ ,  $P < 0.01$ , [Figure 2a](#)).

337 The bacterial community composition showed also a distinct clustering at different  
338 phases of composting based on Unweighted Pair-group Method with Arithmetic Mean  
339 (UPGMA) clustering ([Figure S4](#)). This result was further confirmed in a PCoA based  
340 on the weighted UniFrac distances ([Figure S5](#)) and LEfSe ([Figure S6](#)). These results  
341 are similar to previous studies and demonstrate that distinct changes in the bacterial  
342 community occur throughout composting ([Su et al., 2015](#); [Zainudin et al., 2017](#)) and  
343 this is probably due to changes in temperature, physicochemical composting  
344 properties and a natural succession of microbial communities. Alpha-diversity of the  
345 bacterial community varied significantly during composting ([Table S2](#)). In particular,  
346 OTU richness slightly increased until 24 days then decreased significantly compared  
347 to the initial FW ([Figure 2b](#),  $P < 0.01$ ). This was likely due to a removal of  
348 thermolabile bacteria by the high composting temperature (approximately 65 °C) and  
349 an increased abundance of thermotolerant bacteria. In support of this, the composition  
350 of bacterial communities measured at the phylum level displayed clear temporal

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351 dynamics during 50 days of composting (Figure 2c). More specifically, the relative  
352 abundance of Acidobacteria, Proteobacteria, and Bacteroidetes decreased from 21.59%  
353 to 7.12%, from 7.70% to 0.57%, and from 2.46% to 0.024%, respectively.  
354 Correspondingly, Firmicutes (principally consisting of the family Staphylococcaceae  
355 and Bacillaceae) increased from 67.95% to 91.90%. The majority of Bacillaceae form  
356 heat-resistant spores that provide a competitive advantage over thermolabile bacteria  
357 at elevated temperatures during composting (Liao et al., 2018; Zhang et al., 2015).  
358 Interestingly, the increase in Staphylococcaceae abundance from 0.8% to 22.0%  
359 towards the end of composting correlated positively with the increase in total ARG  
360 and MGE abundances (Figure S7,  $P < 0.01$ ). Staphylococcaceae includes several  
361 food-related, often antibiotic resistant pathogens that often naturally carry various  
362 ARGs and MGEs (Ravcheev and Rodionov, 2011). Specifically, the foodborne  
363 bacterial genera *Jeotgalicoccus* and *Staphylococcus* (Deák, 2011) (both within  
364 Staphylococcaceae) increased in abundance during composting and replaced various  
365 initially dominant genera in the FW: *Bacillus* (22.43%), *Oceanobacillus* (21.95%),  
366 and *Corynebacterium* (12.92%) (Figures 2d and Figure S8). It has been shown  
367 previously that incubation at 80 °C for 60 min is required to completely kill  
368 *Staphylococcus aureus* (Yang et al., 2008). Therefore, it is likely that the maximum  
369 temperature of 65 °C was not high enough to eradicate these bacteria. This is in line  
370 with previous studies showing that most foodborne pathogens such as *Staphylococcus*  
371 spp. and *Enterococcus* spp. persist during composting (Awasthi et al., 2018). Together,  
372 these results suggest that composting can predictably change the composition of  
373 bacterial communities potentially favouring bacteria that can resist periodically high  
374 temperatures, and hence, be also responsible for carrying and disseminating ARGs  
375 and MGEs during FW composting.

---

376

377 **Figure 2**

378

379 **3.3 Relationships between ARGs and MGEs with different taxa in bacterial**  
380 **communities**

381 A Procrustes analysis based on bacterial OTUs and ARG abundances showed that  
382 ARGs significantly correlated with the bacterial community composition (Figure S9,  
383  $M^2= 0.5675$ ,  $R = 0.7407$ ,  $P < 0.0001$ , 9999 permutations). Similarly, the relative and  
384 total MGE abundances correlated positively with total ARG abundances ( $P < 0.001$ ,  
385 Figure S10a) and this finding was further confirmed by nonrandom co-occurrence  
386 patterns between individual ARGs and MGEs (Table 1 and Figure S10b). These  
387 results indicate that MGEs likely played an important role in the dissemination of  
388 ARGs during FW composting, which is consistent with previous findings on ARG  
389 movement in general (Bengtsson-Palme et al., 2018; Gillings, 2017; Zhu et al., 2017).  
390 The relationships between individual ARGs, MGEs and abundances of different  
391 bacterial taxa were further explored using correlation-based network analyses. We  
392 found that most ARGs and MGEs (87%) correlated positively ( $P < 0.01$ ) with 18  
393 bacterial genera. All these ARG-associated genera belonged to four phyla: Firmicutes,  
394 Acidobacteria, Proteobacteria, and Bacteroidetes (Figure 3a). Consistent with  
395 previous finding (Liao et al., 2018), more than 65% of ARG- and MGE-associated  
396 bacteria belonged to Proteobacteria and Firmicutes (Figure S11), which were the  
397 dominant phyla in the initial FW material. Together these results further suggest that  
398 MGEs and ARGs are strongly linked with certain bacterial taxa in FW.

399 Many bacteria frequently carry multiple ARGs and/or MGEs at the same time in  
400 their plasmids, which are considered as the main vehicle for horizontal transfer of

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401 genetic material (Zhang et al., 2011). In support of this, we found that for example the  
402 genus *Acinetobacter* was significantly associated with 22 different resistance genes,  
403 while *Sphingobacterium* and *Lactobacillus* had significant associations with 20 and 9  
404 different resistance genes, respectively. Certain *Acinetobacter* spp. are  
405 multidrug-resistant and act as major infection agents in debilitated patients (Towner,  
406 2009). Similarly, *Lactococcus lactis* has been shown to carry genes conferring  
407 resistance to tetracycline, erythromycin, and vancomycin (Mathur and Singh, 2005).  
408 We also found that *Acinetobacter*, *Alcaligenes*, and *Ignatzschineria* correlated with  
409 three MGEs (*IncQ*, *intI2*, and *Tn916*) and multiple ARGs (Figure S12), suggesting  
410 that these bacterial genera might act as hubs for horizontal transfer of ARGs. Our  
411 results thus suggest that ARGs could have been maintained as multi-drug resistance  
412 plasmids or transposons in bacterial community and be potentially transferred by a  
413 certain key group of bacteria. However, we have to note that our DNA-based  
414 approach cannot fully address whether microorganisms were viable. Therefore,  
415 cultivation-based approaches are needed to confirm that ARG-carrying  
416 microorganisms in the compost are active. Furthermore, subsequent genome  
417 sequencing would allow to detect the exact number and diversity of ARGs within  
418 individual species and if ARGs are located in the bacterial chromosome or plasmids.

419 We next explored the dynamics of ARG and/or MGE-associated bacteria during  
420 composting. In the initial FW material, *Bacillus* (22.4%), *Oceanobacillus* (21.9%),  
421 *Corynebacterium* (12.9%), *Saccharomonospora* (6.8%), and *Ignatzschineria* (5.7%)  
422 were the main ARG/MGE-associated bacteria (67.0% of total 16S rRNA gene  
423 sequences). The abundance of these genera gradually decreased to 23.3% during 50  
424 days of composting (Figure 3b), and at the same time, the relative abundance of the  
425 genera (*Jeotgalicoccus*, *Staphylococcus*, and *Sporosarcina*) increased by 123.9, 11.6,

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426 and 6.3 times, respectively. This gives more support to the idea that ARG and  
427 MGE-associated taxa increased during the composting likely due to the high  
428 composting temperature (Burch et al., 2017; Su et al., 2015). Although the maximum  
429 temperature during composting reached up to 65 °C (>55 °C for approximately 7 days,  
430 Figure S13), most bacterial genera still persisted and some genera even increased in  
431 their relative abundance over time (Figure 3b). While this shift could have been  
432 driven by horizontal gene transfer between heat-susceptible and tolerant bacteria in  
433 the beginning of the experiment, it is also possible that ARGs and MGEs survived at  
434 elevated composting temperatures as a cell-free DNA and were picked up by other  
435 bacteria later during composting. For example, a previous laboratory study has shown  
436 that cell-free DNA is stable even at temperatures up to 70 °C (Zhang and Wu, 2005)  
437 and that cell-free DNA or plasmids originating from lysed bacterial cells at high  
438 temperature can contain ARGs and/or MGEs (Nielsen et al., 2007). Together these  
439 results suggest that FW contains several bacterial taxa that may disseminate ARGs to  
440 other bacterial hosts through horizontal gene transfer and due to their survival at high  
441 temperatures. In addition to plasmids, alternative carriers of ARGs or MGEs, such as  
442 bacteriophages (Lekunberri et al., 2017; Wang et al., 2018), which were not  
443 investigated here, should be included in future studies.

444

445 **Figure 3**

446 **Table 1**

447

448 **3.4 Determining the relative importance of bacterial community composition and**  
449 **diversity, MGEs and physicochemical properties for ARG abundances during**  
450 **composting**

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451 The results from the RDA analysis showed that selected variables (including  
452 physicochemical composting properties, bacterial community composition and MGE  
453 abundances) could explain 74% of the total variance of ARG abundance dynamics  
454 during composting (Figure S14). To further study how ARGs were affected by biotic  
455 and abiotic factors during composting, we built a Partial Least Squares Path Model  
456 (PLS-PM) describing direct and indirect relationships between different variables. We  
457 found that both physicochemical composting properties and MGE abundances had  
458 equally strong direct positive effects on ARG abundances, while bacterial community  
459 diversity or composition had no statistically significant effect (Figure 4). This result is  
460 in contrast with a previous finding showing that the bacterial community composition  
461 is the main factor driving changes in ARG abundances in a mariculture sediment (Han  
462 et al., 2017). However, it is consistent with another study, where environmental  
463 factors rather than bacterial community composition were more important in driving  
464 changes in ARG abundances during composting (Liao et al., 2018). Composting  
465 properties also had direct positive effects on bacterial community composition and  
466 alpha-diversity, and indirect positive effects on MGE abundances via bacterial  
467 community composition. MGEs have recently been proposed to have a more  
468 important role in the spread of ARGs than the microbial community composition or  
469 microbial diversity (Ma et al., 2017; Wu et al., 2017). One possible reason may be  
470 that MGEs are very successful at mobilizing ARGs in environmental microbial  
471 communities via horizontal gene transfer (Wang et al., 2017a). Additionally,  
472 resistance genes are often clustered for example in multi-drug resistance plasmids.  
473 Together with the PLS-PM analysis (Figure 4b), our results provide more support to  
474 the idea that changes in ARG abundances were likely driven by horizontal gene  
475 transfer (MGEs), which itself was strongly affected by the bacterial community

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476 composition. While previous research has connected the microbial community  
477 structure with predictable changes in ARG abundances in various environments (Su et  
478 al., 2015; Wu et al., 2017), our results suggest that also physicochemical composting  
479 properties can directly and indirectly change ARG abundances during FW composting.  
480 This could be specifically explained by certain properties, such as temperature and pH,  
481 that can have direct positive effects on ARG- and MGE-associated bacterial growth  
482 during composting (Awasthi et al., 2018). For example, high temperature will  
483 decompose polymeric substances present in the FW increasing nutrient availability  
484 (Li et al., 2013).

485 To better understand the specific composting properties influencing ARG  
486 abundances, we used variance partitioning to analyze our data in more detail (VPA).  
487 We found that pH,  $\text{NO}_3^-$  concentration and water content (WC) alone explained 9.1%,  
488 12.9%, and 9.1% of total variation of ARG abundances, respectively (Figure 4c).  
489 Interestingly, interactive effects between pH and WC and pH, WC and  $\text{NO}_3^-$   
490 explained 35.2 % and 12.6% of the total variation of ARG abundances, respectively  
491 (Figure 4c). The initially low pH (4.4) remained below 5.5 during the first 30 days of  
492 composting (Figure S13). This likely resulted from the acidification of FW that  
493 contained organic acids and some low-molecular-weight volatiles produced by  
494 microbes (Yu and Huang, 2009), which could have prevented the growth of  
495 indigenous bacteria (Awasthi et al., 2018) and constrained the horizontal transfer of  
496 ARGs via less frequent encounter rates during composting (Ma et al., 2015). In  
497 addition, moisture content played an important role alone and interactively with the  
498 pH. Moisture content is known to influence microbial activity, free airspace,  
499 temperature and aeration during composting (Awasthi et al., 2018) and could thus  
500 affect the connectivity and MGE-induced horizontal transfer of ARGs between

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501 bacterial sub-populations. However, further work is needed to better understand how  
502 physicochemical properties and horizontal gene transfer might interactively drive  
503 ARG dynamics during composting.

504

505 **Figure 4**

506

#### 507 **4. Conclusions**

508 Our study shows that FW is an important reservoir of ARGs and MGEs and that  
509 traditional composting is inefficient in removing the ARGs despite a clear decrease  
510 in relative abundances of ARGs. Moreover, our study supports the idea that  
511 horizontal gene transfer and physicochemical composting properties, such as  
512 temperature, pH and moisture, are important for disseminating and maintaining  
513 ARGs during FW composting. As a result, even though high composting  
514 temperatures reduced the number of initial ARG-associated bacterial taxa, ARGs  
515 were likely maintained in other bacterial taxa potentially due to horizontal gene  
516 transfer as indicated by strong positive correlations between ARGs and MGEs. These  
517 findings suggest that new composting methods are needed for removal of ARGs  
518 during composting to reduce the risk of disseminating ARGs to agricultural  
519 environments.

520

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533

#### 534 **Appendix A. Supplementary data**

535 Supplementary data related to this article can be found online.

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682 **Figure captions**

683 **Figure 1. The dynamics of total ARG, MGE and bacterial abundances (16S rRNA gene**  
684 **copies) during FW composting.** Panel (a-b): Changes in target gene (ARGs, MGEs and 16S  
685 rRNA) abundances during composting presented as the sum of target genes based on absolute (a)  
686 and relative gene abundances (b). Panel (c-d): Heat maps showing changes in the density of  
687 individual ARG and MGE gene abundances based on absolute (c) and relative abundance (d)  
688 during FW composting. Bars denote for mean  $\pm$  standard error. Significance levels are indicated by  
689 \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ) levels.

690

691 **Figure 2. Changes in bacterial community composition and diversity during FW composting.**  
692 Panel (a): Overall distribution pattern of OTU-based bacterial community dissimilarity during  
693 composting (non-metric multidimensional scaling (NMDS); ordination derived from  
694 weighted-UniFrac distances). Symbols with different colors denote for different sampling days.  
695 Panel (b): Changes in observed OTU number during composting. An asterisk (\*) and two asterisks

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696 (\*\*) indicate significant differences at 0.05 and 0.01 significance levels, respectively. Panel (c):  
697 Relative abundance of different bacterial phyla during composting. (d): Ternary plot depicting the  
698 distribution of bacterial taxa (at genus level, relative abundance > 1%) at different stages of  
699 composting (D0 presents the control (day 0), D13 the thermophilic phase (day 13) and D50 the  
700 maturation phase (day 50))

701

702 **Figure 3. Co-occurrence network showing positive associations between ARGs, MGEs and**  
703 **different bacterial taxa.** Panel (a): Nodes coded with different colors and shapes represent  
704 different ARGs, MGEs and bacterial phyla, and the edges correspond to strong and significant  
705 correlations between nodes ( $P < 0.01$ ). Node sizes represent the relative abundances of ARGs,  
706 MGEs, and bacterial phyla during FW composting. Panel (b): Distribution profiles showing the  
707 relative abundance of ARG- and MGE-associated bacteria at genus level during FW composting.  
708 The legend on the right side of the panel denote for relative abundances (%) of total 16S rRNA  
709 gene sequences for each presented bacterial taxon.

710

711 **Figure 4. Partial least squares path model (PLS-PM) showing direct and indirect effects of**  
712 **different factors on ARG abundances.** Panel (a): PLS-PM describing the relationships between  
713 composting properties, bacterial diversity, bacterial community composition (at phylum level) and  
714 mobile genetic elements (MGEs) on ARG abundances. Arrow widths describe the magnitude of  
715 the path coefficients, and blue and red colors indicate for positive and negative effects,  
716 respectively. Path coefficients and coefficients of determination ( $R^2$ ) were calculated after 999  
717 bootstrap replicates and significance levels are indicated by \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ) and \*\*\* ( $P$   
718  $< 0.001$ ) levels. (b): Standardized effects (direct and indirect effects) derived from the partial least  
719 squares path models. (c): Variation partitioning analysis (VPA) comparing the effects of different  
720 composting properties including pH,  $\text{NO}_3^-$ , and WC on the ARGs abundances. The explanatory  
721 factor with values less than 0.01 (explained < 1% of total ARGs variations) was removed from  
722 VPA results. WC: water content,  $\text{NO}_3^-$ -N: nitrate concentration. Relative abundance data was used  
723 to analyze PLS-PM.

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724

725 **Table 1 Spearman's correlations between the relative abundance of ARGs and**  
726 **MGEs during food waste composting**

727

**Table 1 Spearman's correlations between the relative abundance of ARGs and MGEs during food waste composting**

ARGs	Relative abundance of MGEs					
	<i>intI1</i>	<i>intI2</i>	<i>Tn916</i>	<i>ISR1</i>	<i>IncQ</i>	<i>Total MGEs</i>
<i>tetA</i>	0.414*	0.918**	0.885**	0.285	0.887**	0.901**
<i>tetB</i>	0.339	0.842**	0.888**	0.214	0.921**	0.888**
<i>tetC</i>	0.795**	0.554**	0.42*	0.589**	0.338	0.448*
<i>tetG</i>	0.797**	0.503*	0.645**	0.619**	0.55**	0.666**
<i>tetL</i>	0.204	0.643**	0.735**	0.055	0.806	0.73**
<i>tetM</i>	-0.296	0.17	0.341	-0.456*	0.517**	0.345
<i>tetQ</i>	0.807**	0.531**	0.432*	0.593**	0.344	0.465*
<i>tetO</i>	0.582**	0.69**	0.698**	0.255	0.675**	0.723**
<i>tetW</i>	0.505*	0.497*	0.659**	0.274	0.624**	0.675**
<i>tetX</i>	0.609**	0.558**	0.593**	0.456*	0.505**	0.59**
<i>sul1</i>	0.85**	0.561**	0.538**	0.651**	0.45*	0.572**
<i>sul2</i>	0.577**	0.884**	0.663**	0.51*	0.601**	0.705**
<i>sul3</i>	0.428*	0.823**	0.813**	0.376	0.781**	0.81**
<i>strA</i>	0.439*	0.77**	0.593**	0.289	0.529**	0.635**
<i>strB</i>	0.517**	0.744**	0.843**	0.37	0.815**	0.861**
<i>aacA4</i>	0.256	0.238	0.058	0.056	-0.034	0.090
<i>aadA</i>	0.453*	0.905**	0.771**	0.33	0.728**	0.808**
<i>aadB</i>	0.658**	0.53**	0.396	0.716**	0.227	0.429*
<i>aadE</i>	0.322	0.843**	0.846**	0.158	0.906**	0.843**
<i>aphA1</i>	0.237	0.147	-0.155	0.37	-0.154	-0.127
<i>ermB</i>	-0.269	-0.209	-0.283	-0.421*	-0.268	-0.268
<i>ermF</i>	0.497*	0.917**	0.871**	0.468*	0.812**	0.887**
<i>ermM</i>	0.11	0.492*	0.643**	-0.095	0.79**	0.62**
<i>ermT</i>	0.657**	0.579**	0.63**	0.524**	0.542**	0.663**
<i>ermX</i>	0.486*	0.035	-0.09	0.338	-0.215	-0.055
<i>mefA</i>	0.298	0.72**	0.677**	0.025	0.763**	0.682**
<i>ereA</i>	0.547**	0.61**	0.414*	0.261	0.44*	0.449*
Total ARGs	0.28	0.792**	0.895**	0.106	0.938**	0.904**

\*\*Correlation is significant at the 0.01 level (2-tailed). \*Correlation is significant at the 0.05 level (2-tailed).

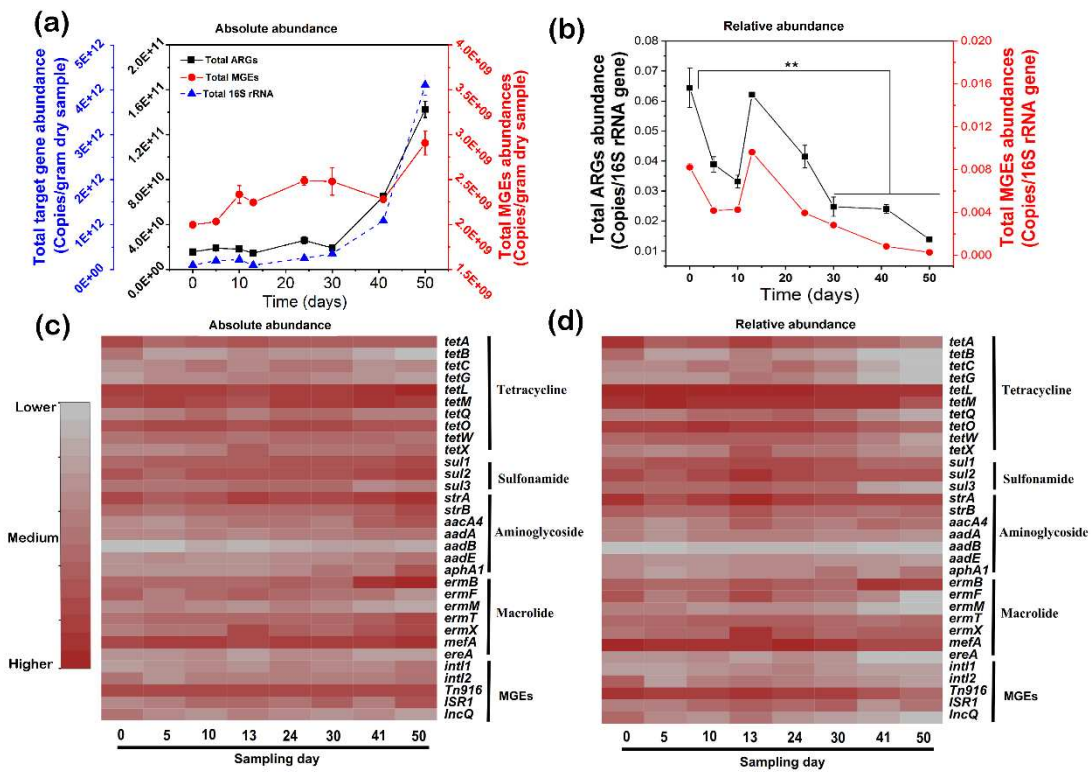


Figure 1

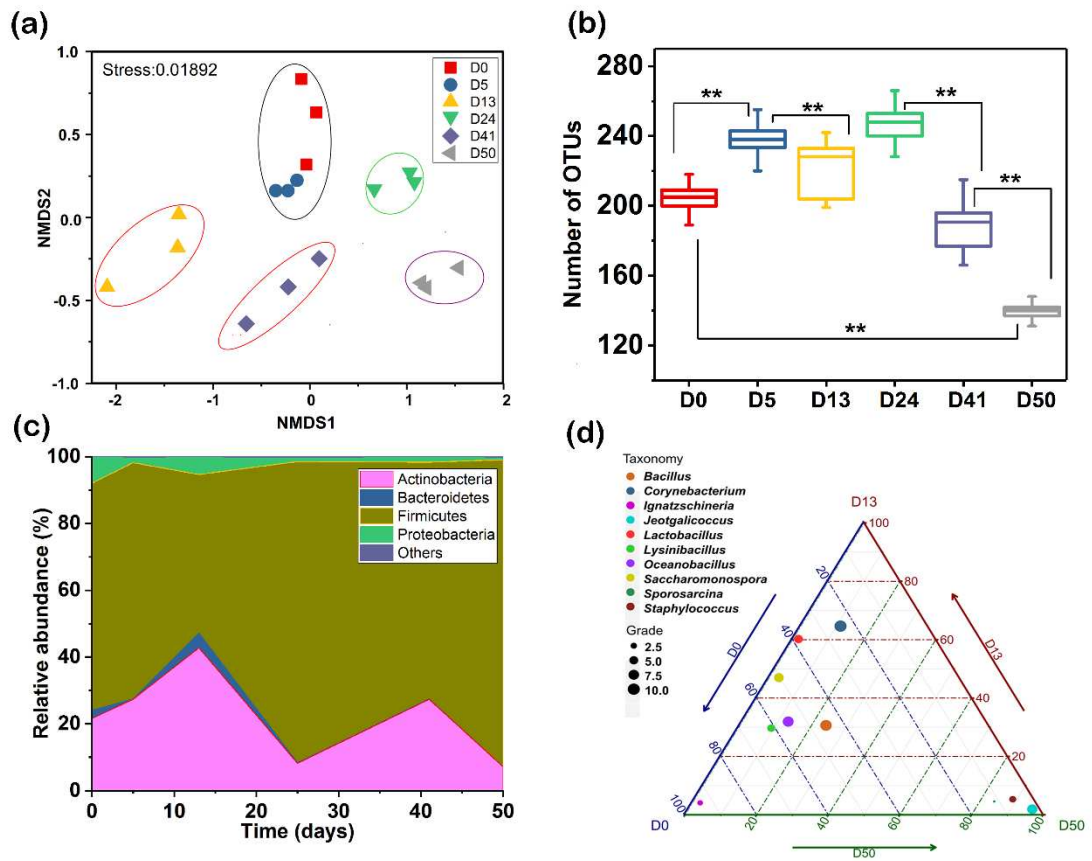


Figure 2

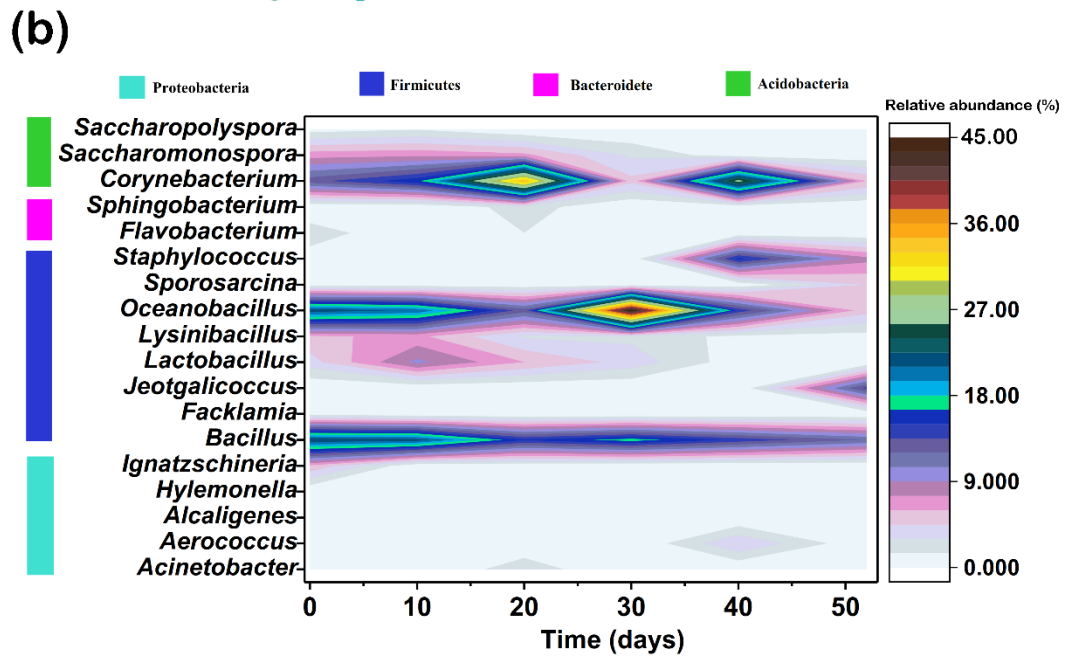
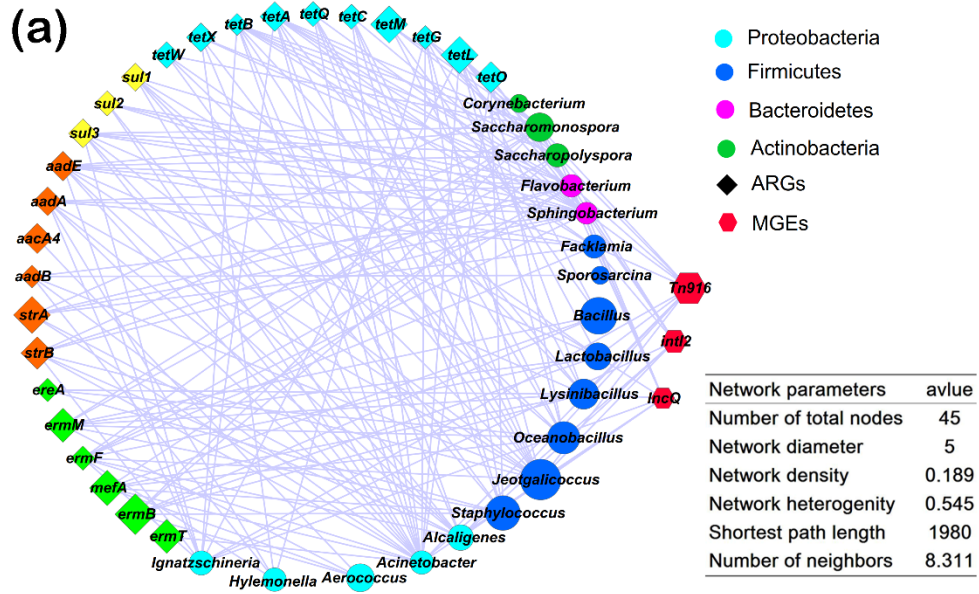


Figure 3

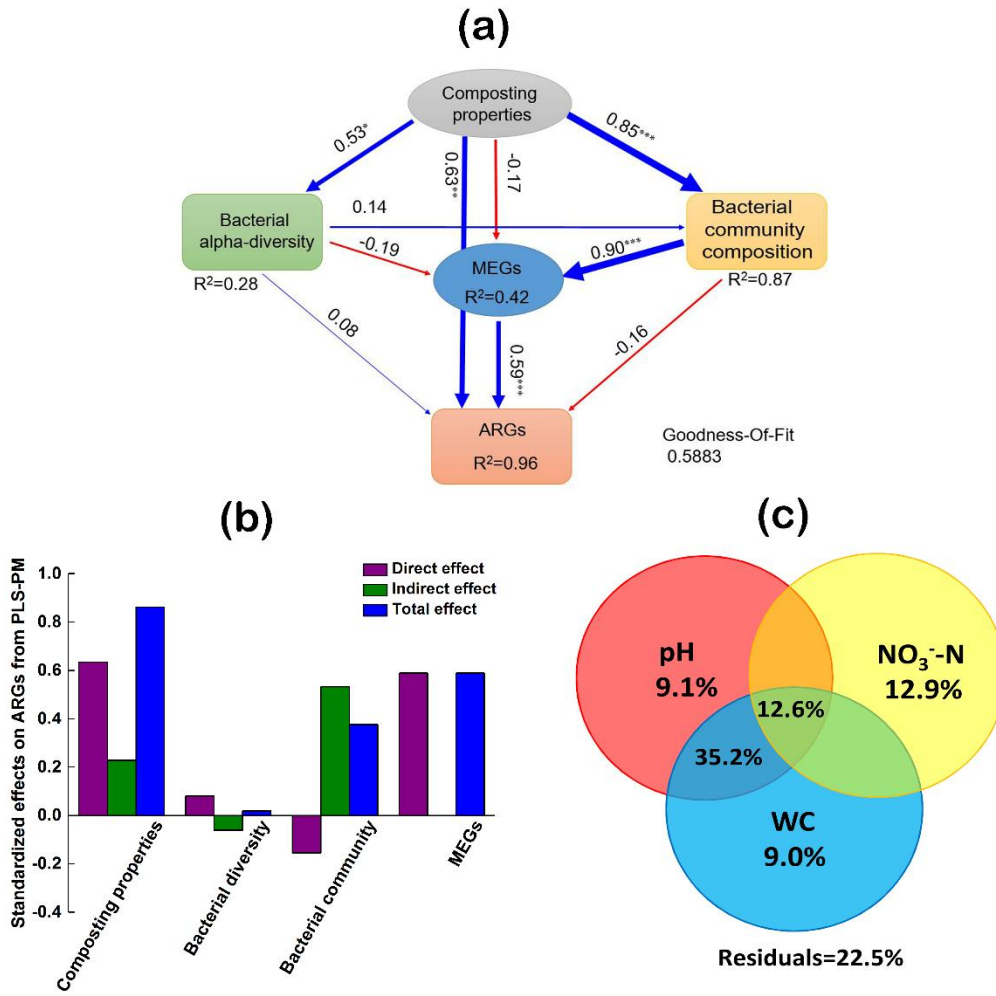


Figure 4

**Supplementary material for on-line publication only**

[Click here to download Supplementary material for on-line publication only: Supplementary files\\_1201.docx](#)