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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, 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

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 (Cerda 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

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

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

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

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

2. Materials and methods

2.1 Full-scale experimental setup for food waste composting

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

2.2 Sample collection and DNA extraction

Samples were collected at 0, 5, 10, 13, 24, 30, 41, and 50 days after start of the

composting, which allowed us to follow changes during various temperature stages

during the composting process. The sampling process followed a previously described

protocol by Liao et al. (2018) with some minor modifications. Briefly, to obtain

representative samples at each sampling time point, each pile was diagonally divided

into five domains, and each domain was sub-sampled (5000 g) from three different

depths from the top of the pile: 40-50 cm (top), 90-100 cm (middle) and 150-160 cm

(bottom). All sub-samples per sampled domain were pooled to obtain a final sample

(5 samples per replicate pile totaling 15 total samples per sampling time point),

homogenized and further divided into two 400 g aliquots of which one was stored in

liquid nitrogen for biological analyses and the other one was stored at 4 °C for

physicochemical analyses. This sampling approach was chosen to reduce the potential

bias caused by the heterogeneity of the original composting substrate in each replicate

pile. The ALFA-SEQ Advanced Soil Kit (mCHIP, Guangzhou, China) was used to

extract genomic DNA from freeze-dried samples (200 mg) according to the

manufacturer's instructions. DNA extraction was conducted three times per sample

and purified DNA samples were pooled for sequencing and genetic analysis. The

quality of DNA was quantified with NanoDrop ND-2000 (Thermo Fisher Scientific,

Wilmington, USA) spectrophotometric analysis and visualized on a 1% agarose gel

and normalized to equal concentrations before downstream qPCR processing.

2.3 Determination of physicochemical parameters during composting

We used previously described methods (Liao et al., 2018) to measure

physicochemical properties during the composting: pH, temperature (Temp), water

content (WC), total nitrogen content (TN), total carbon content (TC), total organic

carbon content (TOC), oil content (OIC), inorganic carbon content (IC) and ammonium (NH_4^+), sodium (NC) and nitrate (NO_3^-) concentrations. Briefly, TOC and IC were measured using an automatic TOC analyzer for liquid samples (Shimadzu TOC-L CPH, Kyoto, Japan). TN and TC were determined in an Elementar instrument (Vario MAX cube, Hanau, Germany) using dry combustion. OIC was measured using Soxhlet extractor method as described previously (Wang et al., 2017b). NC analysis was carried out with flame spectrophotometry. EC and pH were determined using a conductivity meter (Radiometer, model CDM210) and a pH meter (PB-10, Sartorius, Germany), respectively. NH_4^+ and NO_3^- were measured by a continuous-flow autoanalyser (FlowSys, Systea, Rome, Italy). WC was determined as the weight loss upon drying in an oven at 105 °C for 24 h. Daily monitoring of the composting temperature was measured with automatic thermometers placed at different depths of composting piles.

2.4 Measuring changes in ARG and MGE abundances with quantitative PCR (qPCR)

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

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

genes per 16S rRNA gene copy numbers.

2.5 Sequencing and bioinformatic analyses of the bacterial community composition during composting

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

Dominance, Chao1, Shannon and Simpson diversity indices. Differences between

microbial communities (beta-diversity) and principal coordinate analysis (PCoA)

were analyzed based on the weighted Unifrac distances. All sequences were deposited

in the National Center for Biotechnology Information Sequence Read Archive under

the accession number SRP156265.

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

Co-occurrence network analysis was used to explore pairwise correlations between

bacterial taxa and different ARGs and MGEs during FW composting. Several

previous reports have demonstrated that the non-random co-occurrence patterns could

provide indirect evidence for potential host bacteria of ARGs and MGEs (Liao et al.,

2018; Su et al., 2017b). Pairwise correlations were determined using Pearson and

Spearman correlations as described previously (Hu et al., 2017). Only relatively large

correlation coefficients ($\rho > 0.8$ and $P < 0.01$) detected with both methods (Pearson

and Spearman) were included into network analyses to minimize false-positive

correlations. Furthermore, the Benjamini-Hochberg procedure (q-value, $q < 0.01$) was

performed to adjust P-values of all correlations to control false-discovery rate. The

remaining significant interactions between ARGs, MGEs and bacteria were visualized

as a network in Cytoscape v3.4.0, and network statistics were analyzed with Network

Analyzer as undirected networks using default settings (Cline et al., 2007).

2.7 Statistical analyses

Data were analyzed using repeated measures ANOVA (ARG and MGE density

dynamics), PCoA, redundancy analysis (RDA), PERMANOVA test, and Procrustes

test for correlation analysis between ARGs and bacterial community composition with

the vegan package v2.4-3 in R 3.3.2. Heat maps represent log-transformed relative abundances of ARGs and MGEs and were drawn with *ggplot2* package in R 3.3.2. Linear Discriminant Analysis Effect Size analysis (LEfSe) was used to determine differentially abundant taxa between different stages of composting following methods by Segata et al. (2011). Finally, a partial least squares path modeling (PLS-PM) was used to explore relationships between physicochemical composting properties (Temp, WC, pH, IC, TN, TC, TOC, NH_4^+, NO_3^-, OC, and NC), bacterial alpha-diversity (based on OTU numbers, phylogenetic diversity, Dominance, Chao1, Shannon and Simpson diversity indices), bacterial community composition (based on relative OTU abundances), MGEs (relative abundances), and ARGs (relative abundances). PLS-PM is a powerful statistical method to study interactive relationships among observed and latent variables (Wagg et al., 2014) and is widely applied to explain and predict relationships in multivariate data sets (Puech et al., 2015). Path coefficients (i.e. standardized partial regression coefficients) represent the direction and strength of the linear relationships between variables (direct effects). Indirect effects are the multiplied path coefficients between a predictor and a response variable, adding the product of all possible paths excluding the direct effect. Models with different structures were evaluated using the Goodness of Fit (GoF) statistic, a measure of their overall predictive power. The R package plspm (v 0.4.7) was used to construct the final PLS-PM model.

3. Results and Discussion

3.1 Tracing ARG and MGE abundances and diversity throughout composting

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

respectively (Figure S1). In contrast, the initial tobacco powder contained very low

amounts of ARGs (0.45% of ARGs observed in FW) and was not thus included in our

analysis of initial ARG and MGE composition. The tetracycline and macrolide

resistance genes were the most dominant genes accounting for 84.3% of total ARGs

while the transposon *Tn916* gene was the most dominant MGE in the initial FW

accounting for 94.2% of all MGEs (Figure S1). We also detected all targeted 27

ARGs and 5 MGEs that were initially present in the FW samples in the end of the

composting (Figure S2). Surprisingly, the total abundances of all ARGs and MGEs

significantly increased during the 50 days of FW composting ($P < 0.01$, Figure 1a). In

particular, the abundances of *tetL*, *sul2*, *strA*, *ermB*, *ISCR1* and *intI1* were enriched by

9.5, 10.7, 10.7, 377.2, 69.4, and 48.0 times, respectively (Figure 1c). This result is in

line with previous studies showing that abundances of *tetL*, *sul2*, *ermB* can be found

in reasonably high abundances after composting (Qian et al., 2016; Zhang et al., 2016).

One explanation could be that these ARGs were located in bacteria that were tolerant

to high temperatures. Alternatively, ARGs could have been located in MGEs that

could have been able to relocate to thermophilic bacteria during composting. We

found support for the both hypotheses in terms of predictable changes in bacterial

community composition and positive correlation between the abundances of plasmid

(*ISCR1*) and mobile integron (*intI1*) genes with the increase in ARGs during

composting (Figure S3, $P < 0.001$). Together these results suggest that traditional

composting is not efficient enough to remove ARGs and MGEs from FW

(mechanisms discussed in more detail later). Instead, we observed that FW contained

a wide diversity and abundance of ARGs and MGEs that even increased during

composting. Our results thus suggest that FW could be an important but often

neglected source of ARGs which could also potentially serve as a transfer route for

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

To investigate the dynamics of ARGs during composting, we focused on the link between the abundances of bacteria, ARGs and MGEs during composting in more detail. We found that total bacterial abundances based on 16S rRNA gene copy numbers increased during composting (Figure 1a). This is in line with previous results (Wang et al., 2017b) and can be explained by the increased amount of nutrients that become available for microbial growth during composting (Adhikari et al., 2009). Crucially, the increase in the total bacteria abundances was relatively higher than the increase in ARGs, and as a result, the relative abundance of ARGs per number of bacterial cells decreased during the composting (Figure 1b and d, $P < 0.01$). Particularly, tetracycline, macrolide, aminoglycoside and sulfonamide resistance genes showed clear decreases in their relative abundance, while a significant but less drastic decrease in the relative abundance of MGEs was observed (Figure 1b and d, $P < 0.01$). Together, these results suggest that even though the absolute abundances of ARGs and MGEs increased during composting, their relative abundances decreased and fewer bacteria carried ARGs and MGEs at the end of composting. This could have been due to because composting conditions favored non-ARG carrying bacteria, because carrying ARGs incurred fitness costs reducing bacterial competitive ability (Durão et al., 2018) or because bacteria that were less susceptible to MGEs became 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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dynamics during 50 days of composting (Figure 2c). More specifically, the relative abundance of Acidobacteria, Proteobacteria, and Bacteroidetes decreased from 21.59% to 7.12%, from 7.70% to 0.57%, and from 2.46% to 0.024%, respectively. Correspondingly, Firmicutes (principally consisting of the family Staphylococcaceae and Bacillaceae) increased from 67.95% to 91.90%. The majority of Bacillaceae form heat-resistant spores that provide a competitive advantage over thermolabile bacteria at elevated temperatures during composting (Liao et al., 2018; Zhang et al., 2015). Interestingly, the increase in Staphylococcaceae abundance from 0.8% to 22.0% towards the end of composting correlated positively with the increase in total ARG and MGE abundances (Figure S7, $P < 0.01$). Staphylococcaceae includes several food-related, often antibiotic resistant pathogens that often naturally carry various ARGs and MGEs (Ravcheev and Rodionov, 2011). Specifically, the foodborne bacterial genera *Jeotgalicoccus* and *Staphylococcus* (Deák, 2011) (both within Staphylococcaceae) increased in abundance during composting and replaced various initially dominant genera in the FW: *Bacillus* (22.43%), *Oceanobacillus* (21.95%), and *Corynebacterium* (12.92%) (Figures 2d and Figure S8). It has been shown previously that incubation at 80 °C for 60 min is required to completely kill *Staphylococcus aureus* (Yang et al., 2008). Therefore, it is likely that the maximum temperature of 65 °C was not high enough to eradicate these bacteria. This is in line with previous studies showing that most foodborne pathogens such as *Staphylococcus* spp. and *Enterococcus* spp. persist during composting (Awasthi et al., 2018). Together, these results suggest that composting can predictably change the composition of bacterial communities potentially favouring bacteria that can resist periodically high temperatures, and hence, be also responsible for carrying and disseminating ARGs and MGEs during FW composting.

376

Figure 2

378

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

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

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

genetic material (Zhang et al., 2011). In support of this, we found that for example the

genus *Acinetobacter* was significantly associated with 22 different resistance genes,

while *Sphingobacterium* and *Lactobacillus* had significant associations with 20 and 9

different resistance genes, respectively. Certain *Acinetobacter* spp. are

multidrug-resistant and act as major infection agents in debilitated patients (Towner,

2009). Similarly, *Lactococcus lactis* has been shown to carry genes conferring

resistance to tetracycline, erythromycin, and vancomycin (Mathur and Singh, 2005).

We also found that *Acinetobacter*, *Alcaligenes*, and *Ignatzschineria* correlated with

three MGEs (*IncQ*, *intI2*, and *Tn916*) and multiple ARGs (Figure S12), suggesting

that these bacterial genera might act as hubs for horizontal transfer of ARGs. Our

results thus suggest that ARGs could have been maintained as multi-drug resistance

plasmids or transposons in bacterial community and be potentially transferred by a

certain key group of bacteria. However, we have to note that our DNA-based

approach cannot fully address whether microorganisms were viable. Therefore,

cultivation-based approaches are needed to confirm that ARG-carrying

microorganisms in the compost are active. Furthermore, subsequent genome

sequencing would allow to detect the exact number and diversity of ARGs within

individual species and if ARGs are located in the bacterial chromosome or plasmids.

We next explored the dynamics of ARG and/or MGE-associated bacteria during

composting. In the initial FW material, *Bacillus* (22.4%), *Oceanobacillus* (21.9%),

Corynebacterium (12.9%), *Saccharomonospora* (6.8%), and *Ignatzschineria* (5.7%)

were the main ARG/MGE-associated bacteria (67.0% of total 16S rRNA gene

sequences). The abundance of these genera gradually decreased to 23.3% during 50

days of composting (Figure 3b), and at the same time, the relative abundance of the

genera (*Jeotgalicoccus*, *Staphylococcus*, and *Sporosarcina*) increased by 123.9, 11.6,

and 6.3 times, respectively. This gives more support to the idea that ARG and

MGE-associated taxa increased during the composting likely due to the high

composting temperature (Burch et al., 2017; Su et al., 2015). Although the maximum

temperature during composting reached up to 65 °C (>55 °C for approximately 7 days,

Figure S13), most bacterial genera still persisted and some genera even increased in

their relative abundance over time (Figure 3b). While this shift could have been

driven by horizontal gene transfer between heat-susceptible and tolerant bacteria in

the beginning of the experiment, it is also possible that ARGs and MGEs survived at

elevated composting temperatures as a cell-free DNA and were picked up by other

bacteria later during composting. For example, a previous laboratory study has shown

that cell-free DNA is stable even at temperatures up to 70 °C (Zhang and Wu, 2005)

and that cell-free DNA or plasmids originating from lysed bacterial cells at high

temperature can contain ARGs and/or MGEs (Nielsen et al., 2007). Together these

results suggest that FW contains several bacterial taxa that may disseminate ARGs to

other bacterial hosts through horizontal gene transfer and due to their survival at high

temperatures. In addition to plasmids, alternative carriers of ARGs or MGEs, such as

bacteriophages (Lekunberri et al., 2017; Wang et al., 2018), which were not

investigated here, should be included in future studies.

Figure 3

Table 1

3.4 Determining the relative importance of bacterial community composition and

diversity, MGEs and physicochemical properties for ARG abundances during

composting

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

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, 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 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

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

Figure 4

4. Conclusions

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

533

534 **Appendix A. Supplementary data**

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

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Figure captions

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

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

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, 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, NO_3^--N: nitrate concentration. Relative abundance data was used

723 to analyze PLS-PM.

724

Table 1 Spearman's correlations between the relative abundance of ARGs and

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

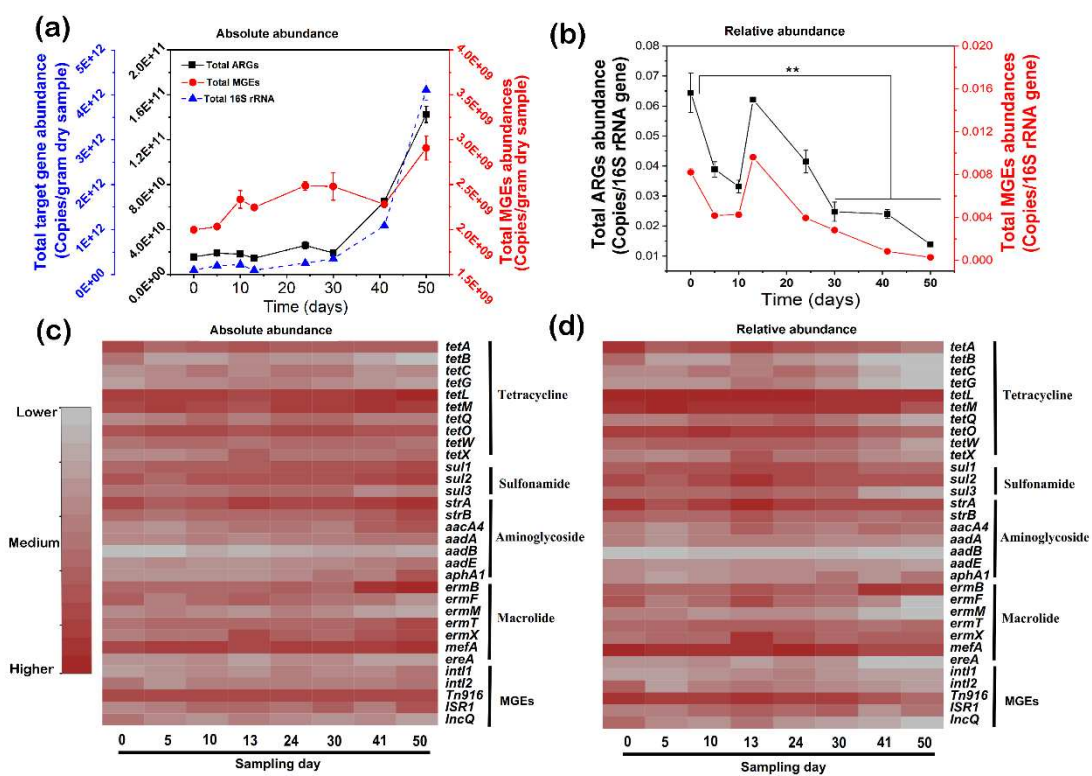**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).
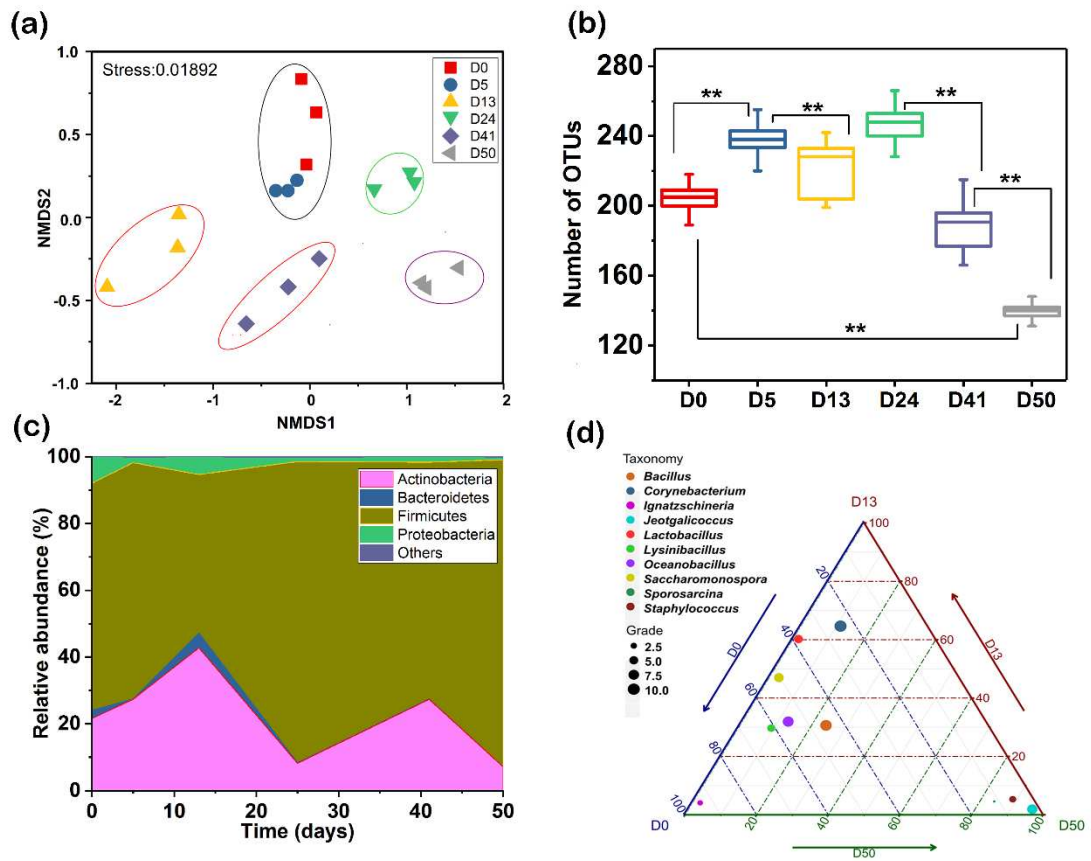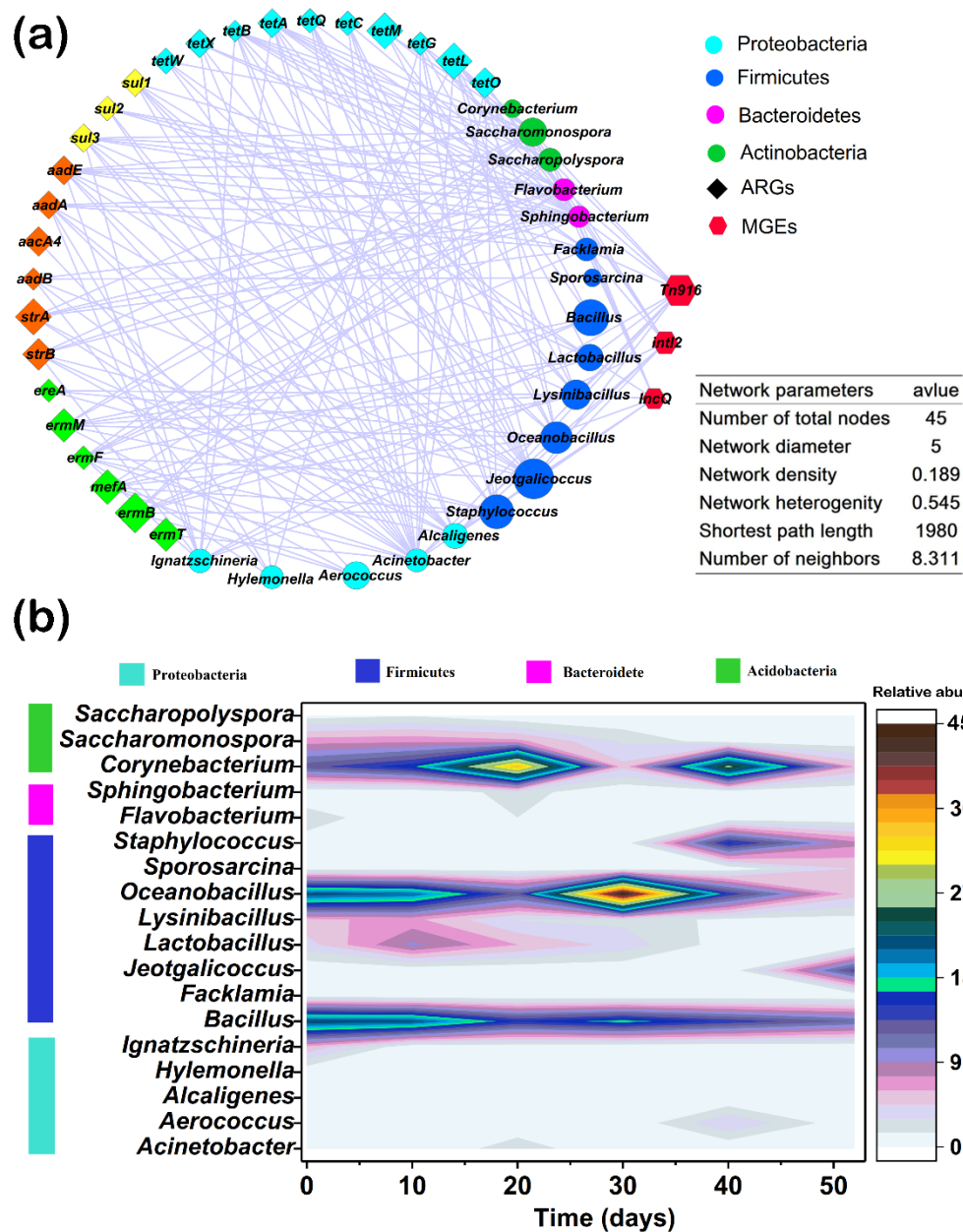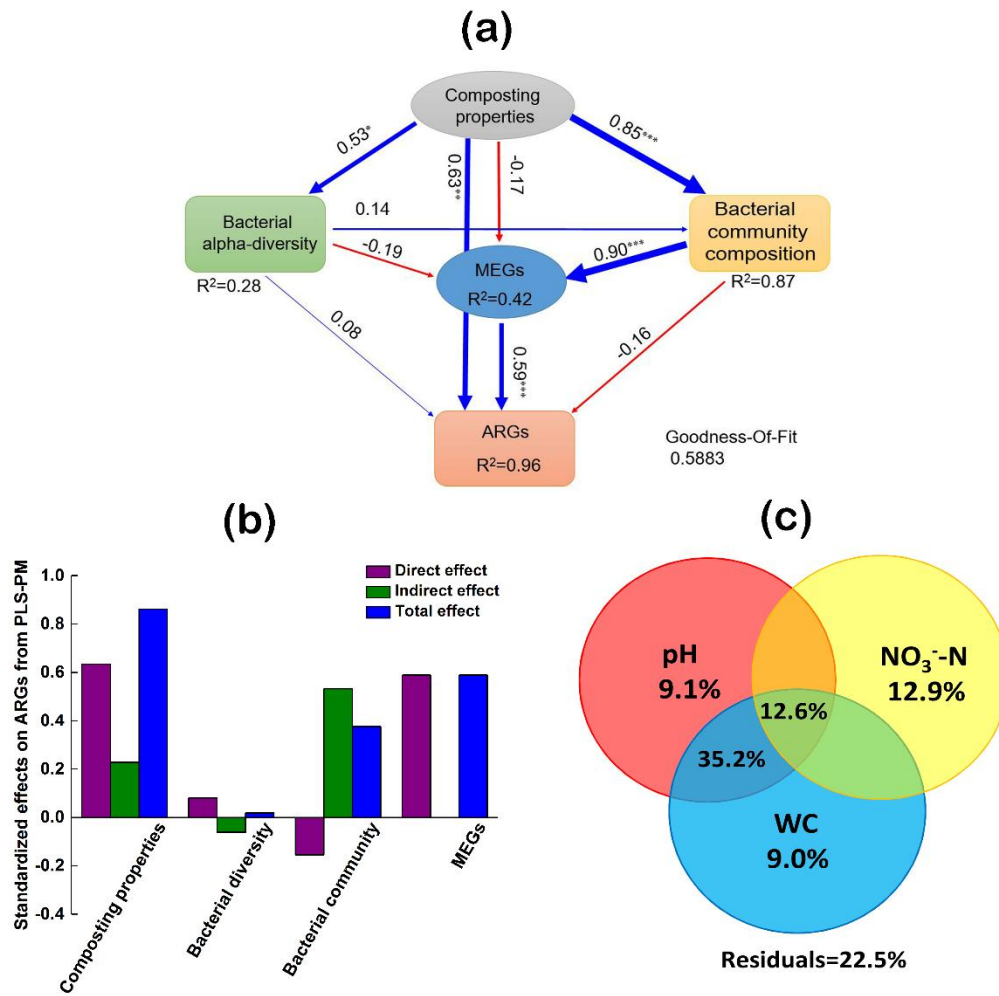
Figure 1

Figure 2

Figure 3

Figure 4

Supplementary material for on-line publication only