Efficient reduction of antibiotic residues and associated resistance genes in tylosin antibiotic fermentation waste using hyperthermophilic composting

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ABSTRACT

Insufficient removal of antibiotics and antibiotic resistance genes (ARGs) from waste products can increase the risk of selection for antibiotic resistance in non-clinical environments. While composting is an efficient way to reduce ARGs, most conventional methods are ineffective at processing highly contaminated antibiotic fermentation waste. Here we explored the efficacy and underlying mechanisms of hyperthermophilic composting at removing tylosin antibiotic fermentation residues (TFR) and associated ARGs and mobile genetic elements (MGEs; plasmids, integrons and transposon). Hyperthermophilic composting removed 95.0% of TFR, 75.8% of ARGs and 98.5% of MGEs and this reduction mainly occurred after extended exposure to temperatures above 60 °C for at least 6 days. Based on sequencing and culture-dependent experiments, reduction in ARGs and MGEs was strongly associated with a decrease in the number of bacterial taxa that were initially associated with ARGs and MGEs. Moreover, we found 94.1% reduction in plasmid genes abundances (iscr1 and incq-oriV) that significantly correlated with reduced ARGs during the composting, which suggests that plasmids were the main carriers for ARGs. We verified this using direct culturing to show that ARGs were more often found in plasmids during the early phase of composting. Together these results suggest that hyperthermophilic composting is efficient at removing ARGs and associated resistance genes from antibiotic fermentation waste by decreasing the abundance of antibiotic resistance plasmids and associated host bacteria.

1. Introduction

Antibiotic fermentation residues are organic solid waste products created in the manufacturing process of antibiotics fermentation. They contain fermentation media, antibiotics residues, heavy metals (zinc and copper etc.) sludge and multiple different antibiotic resistance genes (ARGs) (Zhang et al., 2018a). Globally, millions of tons of antibiotic fermentation residues are produced every year, and traditionally, these waste products are disposed in landfills (Chen et al., 2017b). However, this practice can cause a serious threat to the environment through leaching of antibiotic pollutants into the natural environments including soils, groundwater and nearby waters (Chen et al., 2017a), where they could select for antibiotic resistant bacteria (Pepper et al., 2018). ARGs are thus considered emerging human-toxic pollutants that pose a major challenge to global public health through selection for multidrug resistant bacterial “superbugs” that are very difficult to treat (Povolo and Ackermann, 2019). Developing effective ways to treat waste that contains antibiotics and ARGs is important for controlling the development of antibiotic resistance in natural environments (Bondarzuk et al., 2016).

Treatment of antibiotic fermentation residues using composting methods has recently received more research interest (Wang et al., 2016; Zhang et al., 2018a). Besides making waste safer, composting end products contain high amounts of organic matter and mineral nutrients, which could allow it to be used as organic fertilizer. Despite attempts to assess the residual antibiotics and ARGs during the composting in laboratory conditions, traditional methods often fail to remove ARGs from the composting end products (Zhang et al., 2018a; Zhang et al.,...
2018c). For example, it was recently shown that 40 days of composting only removed 39% of initial tylosin antibiotic residues in swine manure (Zhang et al., 2018c). Moreover, Liu et al. (2018) recently reported that while the abundance of ARGs and mobile genetic elements (MGEs) initially declined during composting of gentamicin fermentation residues, their abundances rapidly recovered and even exceeded the initial concentrations during the later phases of composting. These failures could be attributed to several factors. For example, MGEs, such as plasmids, could mobilize ARGs allowing them to move between suitable bacterial hosts during the composting. Moreover, antibiotic fermentation waste often also includes antibiotic residues and heavy metals that could reinforce selection for multidrug resistance plasmids that often encode resistance genes for both antibiotics and heavy metals (Song et al., 2017). Here we tested if hyperthermophilic composting that uses relatively higher composting temperatures to traditional composting methods (Liao et al., 2018) could be an effective way to treat tylosin antibiotic fermentation waste.

Tylosin is one of the main macrolide antibiotics that is globally used in veterinary medicine and millions of tons of tylosin antibiotic fermentation residue (TFR) waste is generated every year. TFR waste typically contains a high amount of antibiotic residues and heavy metals (Zhang et al., 2018a) that could act strong selection pressures for the transmission and prevalence of ARGs in waste-impacted bacterial communities (Pal et al., 2015). In addition to macrolide resistance genes, antibiotic fermentation waste contains resistance genes to other antibiotics due to colocalization in multidrug resistance plasmids (González-Plaza, 2019). Our previous study focusing on composting of sewage sludge using hyperthermophilic composting (periodic temperatures reaching almost 90 °C) demonstrated efficient removal (89%) of ARGs potentially due to a reduction in the horizontal transfer of ARGs in bacterial communities (Liao et al., 2018). However, it is unclear if hyperthermophilic composting is efficient at removing antibiotic residues and ARGs in much more concentrated TFR waste. We also lack a deeper and causal understanding of underlying mechanisms behind ARG removal by hyperthermophilic composting, or which specific types of MGEs (plasmids, integrons or transposons) or host bacterial taxa are important for the maintenance of ARGs during composting. We hypothesized that, first, hyperthermophilic composting could be efficient at reducing ARGs by breaking down tylosin residues, which has been shown to occur faster at high temperatures (Yu et al., 2019). Second, high temperatures are likely to kill most of the nonthermophilic bacteria that carry ARGs leading to a reduction in ARG abundances. This process should also lead to a reduction in the abundance of MGEs, which could further decrease the horizontal transfer of ARGs between remaining surviving bacterial taxa. As a result, we expected to see changes in the composition and abundances of bacterial communities, ARGs and MGEs during the composting.

To study these questions, we conducted a replicated full-scale hyperthermophilic composting experiment of TFR waste, where we compared the dynamics of tylosin residues, heavy metals, ARGs and MGEs and changes in bacterial community composition during early (4–13 days) and late (18–31 days) phases of hyperthermophilic composting. We used temporal sampling followed by quantification of the abundance of tylosin residues and bio-active heavy metals, and quantitative PCR to determine the abundances of 27 ARGs and three types of MGEs (plasmids, integrons and transposon). Furthermore, we applied 16S rRNA gene amplicon sequencing to determine the composition of the ARG and MGE-associated bacterial communities and used direct culture assays to validate the presence and location of ARGs in chromosomes and plasmids in the beginning and at the end of the composting experiment. The specific objectives of our study were (1) to investigate the feasibility of hyperthermophilic composting in removing tylosin residues and associated ARGs and MGEs at an industrial scale, (2) to understand the underlying mechanisms behind ARG removal and (3) to establish potential links between abiotic (composting properties, tylosin residues, heavy metals) and biotic (bacterial community composition) factors affecting ARG and MGE abundances during hyperthermophilic composting.

2. Materials and methods

2.1. Full-scale experimental setup for hyperthermophilic composting

Hyperthermophilic composting experiments were conducted in a full-scale aerobic composting plant located in Henan district, Ningxia, China as described by Liao et al. (2018). The hyperthermophilic composting material (approximately 21 tons) consisted a mixture of TFR waste (70% water content) and rice straw husk (15% water content, provided by a local farm) in a ratio of 4:1 (w/w). TFR waste was obtained from a local biological pharmaceutical factory (Ningxia, China). The main characteristics of the raw materials used for composting are shown in Table S1. Both raw waste materials were mixed thoroughly resulting in final moisture content of approximately 55% before loading into a fermentation compartment with the following dimensions: 2.0 m height, 8.0 m length, and 4.0 m width. A forced ventilation system at the bottom of the compartment was used to ensure aerobic conditions. To mix the compost substrate well and to reduce pile-edge effects, a mechanical turning of the hyperthermophilic composting material was performed every seven days using pile-specific forklifts. Fermentation temperature was daily monitored with automatic thermometers placed at different depths of the hyperthermophilic composting piles and three replicate piles were used for the composting experiment, which was run for 31 days.

2.2. Sample collection and physicochemical analysis

To investigate the effect of time on the removal of ARGs during composting, samples were collected at the beginning (D0) and after 4 (D4), 7 (D7), 13 (D13), 18 (D18), 25 (D25) and 31 (D31) days since the start of composting. This temporal sampling data was divided into early and late phases of composting based on temperature differences as reported earlier (An et al., 2012). Briefly, the early and late phases of composting were split by samples before (D4 to D7) and after (D18 to D31) day 13, which was considered as the ‘middle point’ based on the maximum temperature reached during the composting.

Samples were collected using a previously described protocol (Liao et al., 2018). To obtain a uniform sampling distribution and representative samples at each time point, each pile was diagonally divided into 5 domains and each domain was sub-sampled (5000 g) from upper, central and lower regions of the composting pile. After sampling, each sample was mixed well and divided into two parts of which one was shock-frozen in liquid nitrogen for biological analyses and the other kept at 4 °C for physicochemical analysis. The physicochemical properties including pH, temperature (Temp), water content (WC), electrical conductivity (EC), total nitrogen content (TN), total carbon content (TC), total organic carbon content (TOC), ammonium (NH4+), and nitrate (NO3−) concentrations were measured as described previously (Liao et al., 2018). DTPA-extractable heavy metals (nickel (Ni2+), copper (Cu2+), cobalt (Co2+), zinc (Zn2+), and plumbum (Pb2+)) were defined as bio-available heavy metals and analyzed as described previously (Guo et al., 2018). More detailed measurement protocol for determining bio-availability of heavy metals is included in the supplementary file.

2.3. LC-MS/MS analysis of tylosin content

Changes in tylosin residue quantities were determined using a previously described Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method with some modifications (Zhang et al., 2018a). Briefly, tylosin was extracted from 1.0 g composting samples using 5 mL 90% acetonitrile aqueous solution (v/v, pH 4.0) as follows. The tubes were vortexed for 5 min and then sonicated in an ultrasonic bath for 30 min.
Subsequently, the mixture was centrifuged at 14,000 g for 10 min and the supernatant filtered through a 0.45 μm filter. Samples were cleaned-up by solid phase extraction (SPE) cartridges (HLB, 6 cc/500 mg, Waters, USA) and SPE eluents were concentrated until dry under a gentle nitrogen flow and then dissolved in 1.0 mL of methanol. The final samples were filtered through a 0.22 μm membrane filter (Milllex, Millipore Corp., Billerica, MA), transferred to 1.5 mL amber vials, and stored at −20 °C before the LC-MS/MS analysis. Quantity of tyllosin was measured by liquid chromatography combined with ion trap mass spectrometer system (XEVO-TQS, Waters, USA) as described previously (Zhang et al., 2018a). The tyllosin standard was obtained from Dr Ehrenstorfer GmbH (Germany). The average recovery rates of the blank samples spiked with 10–100 μg/kg of tyllosin were 93.6% and the detection limit of the tyllosin was 1.5 μg/kg. All composting samples were analyzed in triplicate to reduce measurement errors.

2.4. DNA extraction and quantitative PCR (qPCR) for determining abundances of genes linked with antibiotic resistance and mobile genetic elements

Total genomic DNA was extracted using a Fast DNA spin kit (MP Biomedicals, Cleveland, OH, USA) from freeze-dried composting samples according to the manufacturer’s instructions. In addition to quantifying tyllosin macrolide antibiotic resistance gene abundances, three other common ARGs found in organic waste (Liao et al., 2018; Zheng et al., 2019) were also measured including resistance genes to tetracycline, aminoglycoside and sulfonamide antibiotics. We chose several variants of each type of ARGs including 10 tetracycline resistance genes (tetA, tetB, tetC, tetG, tetL, tetM, tetQ, tetO, tetW, and tetX), 7 macrolide resistance genes (ermB, ermF, ermM, ermT, ermX, mefA, and ermA), 7 aminoglycoside resistance genes (aacA-4, aadA, aadB, aadE, aphA1, strA, and strB) and 3 sulfonamide resistance genes (sul1, sul2, and sul3). From here on, all tetracycline, macrolide, aminoglycoside and sulfonamide resistance genes are abbreviated as Tet, Mac, Amin, and Sul, respectively. To investigate potential changes in the abundance of different types of MGEs, we chose the following commonly observed MGEs based on previously published studies (Ma et al., 2017): 2 integrons (intI1 and intI2), 2 conjugative plasmids (ISCR1 and IncQ-oriV, abbreviated as IncQ) and one transposon (Tn916/1545, abbreviated as Tn916). All information about primers, annealing temperatures, reaction conditions and amplification used for all target genes were validated in a previous study (Liao et al., 2018) and are listed in the supplementary materials (Table S2 including information about positive and negative controls and standard curves). The absolute abundances of target genes are presented as copy numbers per gram of sample. The relative abundance of different types of ARGs (Tet, Mac, Amin, and Sul) and MGEs (integrons, plasmids, and transposon) are presented as the proportion of all detected target genes.

2.5. High-throughput sequencing and bioinformatics analysis exploring bacterial community diversity and composition

The changes in bacterial community composition and diversity during hyperthermophilic composting were determined using 16S rRNA gene amplicon sequencing on an Illumina HiSeq 2500 platform (Guangdong Magigene Biotechnology Co. Ltd, Guangzhou, China). The V4 region of the bacterial and archaeal 16S rDNA gene was amplified using the primers 515F (5′-GTGCCACGCMGCCGCGGTAA-3′) and 806R (5′-GACTACHVGGGTWTCA-3′) (Caporaso et al., 2012). Raw Illumina sequence data was quality filtered using a pipeline coupling Trimomatic (version 0.33) and QiIME (1.8.0) (Caporaso et al., 2010). Primer sequences and low-quality reads that contained ambiguous nucleotides, mismatches in primer regions, or had a length shorter than 100 bp were removed to obtain better sequence read data. Operational taxonomic units (OTUs) were defined at 97% sequence similarity level using Uclust (Edgar, 2010) and taxonomic assignment of OTUs was performed using a Ribosome Database Project Classifier provided by the Greengenes 13.8 16S rRNA gene database with 80% sequence similarity threshold as described previously (McDonald et al., 2012). Differences in the diversity (beta-diversity) and composition between microbial communities (PCoA principal coordinate analysis) were analyzed using weighted UniFrac metric distances. Within microbial community diversity (alpha-diversity) was quantified using Chao1 and Shannon diversity indexes. All sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive under the accession number PRJNA551919.

2.6. Isolation and identification of antibiotic resistant bacteria

Culture-based methods were used to identify potential ARG hosts at different phases of composting. Isolation and identification cultivable antibiotic resistant bacteria was performed as described by Zhang et al. (2018b). Samples from D4 to D7 and D25 to D31 were selected as representative samples of the early and late phases of composting. Briefly, 10 g of mixed sample was suspended into 90 mL phosphate-buffer by shaking at 200 rpm for 30 min. The sample was then serially diluted to different concentrations and 100 μL of all dilutions spread on Luria-Bertani (LB) agar plates containing four antibiotics: tetracycline, erythromycin, gentamicin and sulfadiazine at final concentrations of 16 mg/L, 10 mg/L, 16 mg/L, 512 mg/L, respectively (Ren et al., 2018; Yang et al., 2017). After 48 h incubation at 30 °C, the number of colony forming units (CFU) was determined for each type of agar plates. Based on colony color and morphology, a total of 29 cultivable antibiotic resistant strains (including 10, 9, 8 and 2 isolates resistant to tetracycline, erythromycin, gentamicin, and sulfadiazine, respectively) were isolated from the early phase composting samples. A total of 21 culturable antibiotic resistant strains (including 6, 5, 6 and 4 isolates resistant to tetracycline, erythromycin, gentamicin, and sulfadiazine resistant isolates, respectively) were isolated from late phase composting samples. To identify bacterial colonies, we extracted the genomic DNA from all isolates using Bacteria DNA Kit (Tiangen, Beijing, China) and amplified the 16S rRNA genes using the primers 27F (5′-AGAGTTTGATCTCCTGGCTCAG-3′) and 1492R (5′-GGTACCTTGTAGACTACCT-3′).

2.7. Extraction of plasmid and genomic DNA from antibiotic resistant bacteria

To detect if ARGs were located on chromosomes or plasmids in isolated antibiotic resistant bacteria, genomic and plasmid DNA were extracted using bacteria and plasmid DNA Kit (Tiangen, Beijing, China) following manufacturer’s protocol. Residual linear chromosomal DNA fragments were removed using plasmid-safe ATP-dependent DNase (Epicentre, Madison, WI, USA) treatment for 24 h to 48 h at 37 °C following a previous method (Kothari et al., 2019). The presence of chromosomal DNA was tested by PCR using 16S rRNA universal primers (BAC338F, 5′-ACTTCTACGCGGAGGCAGC-3′, and BAC805R, 5′-GAGCAGTACCTATGCTTCTC-3′). If a 16S rRNA genes PCR product was visible on a 1% agarose gel, another overnight digestion reaction was performed until the product could no longer be visualized. The DNase was inactivated at 70 °C for 30 min. The chromosomal DNA-free plasmid and genomic DNA extracted from isolates were used to detect the ARGs and MGEs using specific primers using PCR (Table S2). Strains isolated from composting pile replicates at the same phases of composting were merged to analyze the data of ARGs and MGEs.

2.8. Co-occurrence network analysis exploring different bacterial taxa, ARGs and MGEs

Co-occurrence network analysis was used to explore pairwise correlations between bacterial taxa (based on genus level with abundance > 0.1%) and different ARGs and MGEs during composting. Pearson and Spearman correlations were extracted using PAST software
v3.04 as described previously (Liu et al., 2019). Only relatively large correlation coefficients ($\rho > 0.8$ and $P < 0.01$) detected with both methods (Pearson and Spearman) were included in network analyses to minimize false-positive correlations. Furthermore, Benjamin Hochberg multiple tests ($q$-value, $q < 0.01$) were used to adjust $P$-values to reduce false-discovery rates. Co-occurrence networks were visualized using Gephi v0.9.2.

2.9. Statistical analyses

Analysis of variance (ANOVA) and linear regression was used to test for differences in the abundances of ARGs and MGEs and the diversity of the bacterial community between different phases of composting (initial TFR vs. early and late phases) using OriginPro 9.4 (OriginLab Corporation, Northampton, MA). Partial Least Squares Path Modeling (PLS-PM) was used to study relationships between physicochemical composting properties (WC, TC, TN, EC, TOC, C/N, and NO$_3^-$), the amount of tylosin residues, bio-availability of heavy metals (Ni$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$), bacterial community composition (based on OTUs abundance composition value) and absolute abundances of MGEs and ARGs. PLS-PM is a powerful statistical method to study relationships among observed and latent variables (Wagg et al., 2014), where path coefficients (i.e. standardized partial regression coefficients) represent the direction and strength of 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 of a given model (Cui et al., 2016; Wagg et al., 2014). PLS-PM was also chosen instead of structural equation modeling because it is more appropriate for data sets with small sample sizes and less sensitive to the sampling distribution of PLS weights. The R package plsplm (v 0.4.7) was used to construct the final PLS-PM model. Canonical correspondence analysis (CCA), Adonis test and Procrustes analysis were performed in R 3.5.1 using the vegan package v2.4-3 and labdsv v1.8. Linear discriminant analysis effect size analysis (LEfSe) was used to determine differentially abundant taxa between different stages of composting using the Galaxy web application (Segata et al., 2011). All data are presented as mean values ± 1 standard error.

3. Results

3.1. Hyperthermophilic composting is effective at removing tylosin residues and ARGs

The initial composting TFR waste contained high levels of tylosin residues (85.0 mg/kg) and bio-available heavy metals (987.4 mg/kg). The hyperthermophilic composting method was effective at removing 95.0% of tylosin residues and 88.9% of bio-available heavy metals in 31 days (Fig. 1a). The maximum composting temperature increased to about 80 °C after 13 days (Fig. 1b) which coincided with the reduction of both tylosin residues and bio-available heavy metals (Fig. 1a–b). To further understand how tylosin residues and heavy metals influenced ARG and MGE abundances during composting, we explored their relationships using correlation analysis. We found no significant correlation between the amount of tylosin residues and total ARGs abundances ($P > 0.05$, Fig. 1c). However, the amount of tylosin residues correlated positively with total MGE abundances and especially with ISCR1 and IncQ plasmid gene abundances ($P < 0.001$, Fig. 1d). Similarly, all heavy metals (Ni$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$) significantly correlated with MGE abundances (Spearman, $P < 0.05$). In contrast, the majority of heavy metals (Ni$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$) did not significantly correlate with total ARMs abundances (Spearman, $P > 0.05$, Table 1). However, all detected heavy metals correlated significantly with plasmid abundances (ISCR1 and IncQ) and Ni$^{2+}$ and Zn$^{2+}$ also with integron abundances (intI1 and intI2; Spearman, $P < 0.05$, Table 1). Together these results suggest that the reduction in tylosin residues and bio-available heavy metals was linked with elevated composting temperature and reduction in the abundance of MGEs during hyperthermophilic composting.

3.2. The effect of hyperthermophilic composting on the abundance and diversity of ARGs and MGEs

All targeted genes, including 27 ARGs and 5 MGEs, were detected in the initial tylosin residue waste and all the samples collected during the composting (Fig. S1). The mean ARG and MGE abundances in the initial compost were approximately $5.9 \times 10^{12}$ and $2.2 \times 10^{12}$ copies per gram of compost (dry weight), respectively (Fig. 2a). Because the initial rice husk contained only very low amounts of ARGs (0.25% of the mean amount of TFR), it was not included in further analyses. To study these patterns in more detail, we compared changes in ARGs and MGEs during the early and late phases of composting. We could not detect statistically significant reductions in ARGs and MGEs during the early phase of composting relative to the initial TFR waste (all $P > 0.05$). However, 75.8% and 98.5% reduction in the total abundances of ARGs and MGEs were observed between the early and late phases of composting (Fig. 2a–b, $P < 0.01$). Even though the absolute ARG abundances decreased significantly, the proportion and diversity of different types of ARGs did not change drastically (Fig. 2c); while some temporal dynamics were observed in the proportion of all ARGs, the Mac and Tet type ARGs were the most dominant accounting for 49% to 80% of all ARGs during the composting (Fig. 2c). In contrast, the proportion of MGEs changed more drastically during the composting. Specifically, the percentage of plasmid genes decreased from 98% to 9%, while integron and transposon genes became the dominant MGEs during the composting accounting for 91% of all MGEs (Fig. 2d). Furthermore, we found a strong correlation between MGEs, specifically ISCR1 plasmid gene, and total ARGs abundances ($R^2 = 0.7$, $P < 0.0001$), which implies that ARGs were likely carried in plasmids (Fig. 52).

3.3. Changes in the bacterial community composition during hyperthermophilic composting

We next compared changes in bacterial community composition and diversity between initial TFR waste and early and late phases of hyperthermophilic composting. Based on the PCoA analysis (weighted UniFrac distances), no difference was found between initial and early phase composting samples (Adonis test, $P > 0.05$, Fig. S3a). However, early and late composting phase samples showed distinct clustering indicative of a difference in bacterial community composition (Adonis test, $P = 0.01$, Fig. S3a). Similarly, total bacterial abundances (observed OTUs), community richness (Chao1 index) and community diversity (Shannon index) differed between early and late phases of composting ($P < 0.01$, Fig. S3b–d), while no differences were found between initial and early phase samples ($P > 0.05$). Early and late phase composting samples clustered distinctly also at phylum and genus levels (unsupervised hierarchical clustering based on the relative abundance of most prevalent taxa (> 1% in any given sample)). Specifically, early phase communities had typically high relative abundances of Proteobacteria, Chloroflexi, OP11 and Thermus phyla and Psychrobacter, T78, and Methanosarcina, Ignotaschineria genera, whereas late phase samples were enriched with Firmicute and Actinobacteria phyla and Geogenia, Yaniella, Alcaligenes, Pseudomonas, Staphylococcus, Bacillus genera (Fig. 3). These bacterial community composition differences were further confirmed using linear discriminant analysis effect size analysis (Fig. S4). Together, these results suggest that changes in ARG and MGE abundances were linked with a reduction in bacterial abundances and changes in the diversity and taxonomic composition of composting communities.
3.4. Correlation between ARG, MGE and bacterial taxa abundances

Based on procrustes analysis, changes in resistomes (based on all ARGs and MGEs) were significantly correlated with bacterial community composition at genus level ($P = 0.0017$, $M^2 = 0.5537$, $R = 0.6681$, 999 permutations, Fig. S5a). Similarly, changes in MGEs (based on all types of MGEs) and bacterial community composition were significantly correlated ($P = 0.0185$, $M^2 = 0.6940$, $R = 0.5531$, 999 permutations, Fig. S5b). As expected, all types of ARGs (tetracycline, sulfonamide, aminoglycoside and macrolide gene) and MGEs (plasmids, integrons and transposon) were significantly correlated with their associated bacterial community (all $P < 0.05$, 999 permutations, Fig. S6).

The co-occurrence patterns between ARGs, MGEs and bacterial taxa abundances were further compared using correlation-based co-occurrence network analysis. The networks showed a clear shift between early and late phases of composting mainly due to differences in bacterial diversity and community composition (Fig. 4). The co-occurrence network constructed at early phase of composting was larger and more connected compared to the late phase network (Fig. 4). Also, a larger number of nodes and edges were included in early versus late composting phase network and several network indices such as network diameter, network density, network modularity, average path length, and average degree were greater for early compared to late phase network. Based on a previous study (Li et al., 2015), we hypothesized that non-random co-occurrence patterns between ARGs and microbial taxa abundances were significantly correlated with bacterial community composition.

### Table 1

**Correlation analysis between the amount of bio-available heavy metals, individual MGEs and total MGEs and ARGs during hyperthermophilic composting.**

<table>
<thead>
<tr>
<th>MGEs type</th>
<th>Ni$^{2+}$</th>
<th>Cu$^{2+}$</th>
<th>Co$^{2+}$</th>
<th>Zn$^{2+}$</th>
<th>Pb$^{2+}$</th>
<th>T-metals</th>
</tr>
</thead>
<tbody>
<tr>
<td>indI</td>
<td>Integron</td>
<td>$-0.68^{**}$</td>
<td>0.33</td>
<td>$-0.53^{*}$</td>
<td>0.55***</td>
<td>0.38</td>
</tr>
<tr>
<td>indI2</td>
<td>Integron</td>
<td>0.57***</td>
<td>$-0.24$</td>
<td>0.41</td>
<td>$-0.66^{**}$</td>
<td>$-0.02$</td>
</tr>
<tr>
<td>Tn916</td>
<td>Transposon</td>
<td>$-0.6^{**}$</td>
<td>0.63***</td>
<td>$-0.62^{**}$</td>
<td>$-0.49^{*}$</td>
<td>0.81***</td>
</tr>
<tr>
<td>ISCR1</td>
<td>Plasmid</td>
<td>0.77***</td>
<td>$-0.63^{**}$</td>
<td>0.68***</td>
<td>0.86***</td>
<td>$-0.48^{*}$</td>
</tr>
<tr>
<td>IncQ</td>
<td>Plasmid</td>
<td>0.78***</td>
<td>$-0.62^{**}$</td>
<td>0.71***</td>
<td>0.86***</td>
<td>$-0.52^{*}$</td>
</tr>
<tr>
<td>T-MGEs</td>
<td>/</td>
<td>0.77***</td>
<td>$-0.64^{**}$</td>
<td>0.69***</td>
<td>0.86***</td>
<td>$-0.5^{*}$</td>
</tr>
<tr>
<td>T-ARGs</td>
<td>/</td>
<td>$-0.30$</td>
<td>0.20</td>
<td>$-0.27$</td>
<td>0.49</td>
<td>$-0.05$</td>
</tr>
</tbody>
</table>

Notes: Spearman’s rank order correlation analysis was used based on absolute target gene abundances with following significances:

- $^{*}$ Significant at $P < 0.05$.
- $^{**}$ Significant at $P < 0.01$.
- $^{***}$ Significant at $P < 0.001$.

T-metals, T-MGEs and T-ARGs denote for total concentration of bio-available heavy metals, total abundance of MGEs and total abundance of ARGs, respectively.
taxa could be used to identify potential ARG hosts. By following this analysis, we identified 22 candidate bacterial genera as potential ARG and MGE hosts at the early phase of composting (Fig. 4a). Similarly, 11 potential bacterial genera were non-randomly associated with ARGs and MGEs during the late phase of composting (Fig. 4b). Crucially, the taxonomic composition of the potential host taxa differed between early and late phases of composting. The *Psychrobacter*, *Morganella*, and *T78* were the dominant potential hosts at the early phase of composting and in the initial TFR waste accounting for 49.7% of the total 16S rRNA gene sequences. However, after 31 days of composting the abundance of these taxa gradually decreased to 0.52% (Fig. S7) and were no longer significantly associated with ARGs and MGEs. *Staphylococcus* and *Bacillus* were associated with ARGs only at the early phase of composting. Moreover, two potential ARG hosts, *Lysobacter* and *Georgenia*, were associated with ARGs only at the late phase of composting. Together, these results suggest that associations between ARGs, MGEs, and their potential host bacterial taxa changed during the composting.

### 3.5. Isolation of potential ARG host bacteria and identifying the location of ARGs in chromosomes and plasmids

The number of cultivable antibiotic resistant strains conferring resistance to tetracycline, erythromycin, gentamicin, and sulfadiazine were significantly higher during the early versus late phase of composting ($9.1 \times 10^5$ vs $1.3 \times 10^7$ CFU/gram dry sample, $P < 0.05$, Fig. S8). The antibiotic resistant strains isolated from the early phase of composting belonged to 6 genera (*Alcaligenes*, *Bacillus*, *Staphylococcus*, *Saccharopolyspora*, *Paenibacillus*, and *Vagococcus*; Table S3). Only 2 genera (*Alcaligenes* and *Staphylococcus*) were found in the late phase of composting (Table S4). In line with our previous analyses, we detected more ARGs and MGEs (61 vs 23 genes in total; average of 2.1 and 1.0 target genes per isolated strain) during early compared to the late phase of composting (Fig. 5a). Interestingly, ARGs were on average located more often on plasmids than on chromosomes with early phase samples (Fig. 5a). However, the location of ARGs and MGEs was highly variable at the finer taxonomic level and in some cases a higher abundance of target genes was observed on plasmids compared to chromosomes even with late composting phase isolates (Fig. 5b). These results are line with our sequencing results demonstrating that cultivable isolates carried fewer ARGs and MGEs at the end of the hyperthermophilic composting. Furthermore, the high prevalence of antibiotic resistance genes during the early phase of composting was likely linked with a relatively high number of plasmids that might have carried multiple antibiotic resistance genes.

### 3.6. Comparing the relative contributions of abiotic and biotic factors on ARG and MGE abundances during early and late phases of composting

A total of 81.2% variance of ARG abundances could be explained by composting properties (WC, TC, TN, EC, TOC, C/N, and $\text{NO}_3^-$), the concentration of tylosin residues and bio-available heavy metals, bacterial community composition (based on OTUs) and MGE abundances (CCA analysis, Fig. S9). To further study how ARGs were affected by abiotic and biotic factors at different phases of composting, we constructed a Partial Least Squares Path Model (PLS-PM) describing direct and indirect relationships between biotic and abiotic factors. We found that tylosin residue and heavy metal concentrations and other abiotic composting properties had no statistically significant effect on ARG abundances during the early phase of composting (Fig. 5a). However, tylosin residue and heavy metal concentrations had strong direct effects on the abundance of MGEs and bacterial community composition during the early phase of hyperthermophilic composting. MGE abundances had a strong direct positive effect on ARG abundances at the early phase of composting. However, this effect became much weaker during the late phase of composting (Fig. 5a). In contrast, bacterial community composition affected ARG abundances during both the early and late phases.
of composting (Fig. 6a–b). Association between bacterial community composition and MGEs were positively correlated only during the early phase of composting, which suggests that this relationship was lost during the late phase of composting. Together these results suggest that tylosin residues and heavy metal concentrations were not directly linked with ARG abundances, but instead, had highly significant effects on MGE abundances, which were strongly linked with ARGs and changes in bacterial community composition (Fig. 6b).

4. Discussion

4.1. Hyperthermophilic composting efficiently reduces the amount of tylosin residues and bio-active heavy metals and associated antibiotic resistance genes

In this study, we explored the efficiency of hyperthermophilic composting at treating antibiotic fermentation waste in a full-scale industrial composting experiment. High abundances of diverse ARGs (27 ARGs and 5 MGEs with $8.1 \times 10^{12}$ gene copies per gram) were found in the initial TFR waste. These ARG abundances are 1–2 orders of magnitude higher than previously reported in other kinds of waste such as food waste (Liao et al., 2019), sewage sludge (Liao et al., 2018) and animal manure (Munir and Xagoraraki, 2011).

This is likely attributed to a strong selection pressure for bacteria to become antibiotic-resistant in order to survive in antibiotic and heavy metal-rich environment (Baker-Austin et al., 2006). Despite the initially high ARG concentrations, hyperthermophilic composting was effective at reducing the amount of ARGs (75%), MGEs (98%), tylosin residues (95%) and bioactive heavy metals (89%) in 31 days. This was likely due to degradation of antibiotic residues at high temperatures (Yu et al., 2019) and change in the bioavailability of heavy metals into unavailable form during the composting (Chen et al., 2019; Zhou et al., 2018).

No clear difference in ARG and MGE abundances was found between initial and early phase composting samples when the composting temperature did not differ much from ambient temperature (mean of 23 °C). However, changes became pronounced during the late phase of composting when the temperatures reached ~60 °C (Fig. 1b). This suggests that removal of ARGs happened during the late phase of composting due to prolonged exposure to high temperatures. Although most ARGs decreased during the composting, few ARGs such as tetL and sul1 increased towards the end. This is in line with previous studies (Zhang et al., 2016), suggesting a potential enrichment of these ARGs in thermophilic bacteria that can survive high composting temperatures. However, this hypothesis needs to be tested experimentally in the future.

Presence of heavy metals in composting waste can induce selection for metal resistance genes. Because metal resistance genes are often located in multidrug-resistance plasmids, high heavy metal...
concentrations can indirectly co-select for ARGs in soils and in animal guts (Ding et al., 2019; Zhao et al., 2018). In line with this, we observed that Zn²⁺ concentration (927 mg/kg) was significantly linked with total ARGs abundances during the composting - an association that has previously been reported in dairy farms (Zhou et al., 2016). Notably, we found that most heavy metals were more strongly associated with MGE abundances, which suggests that they mainly affected the mobilization of ARGs via MGEs (Hu et al., 2017). Importantly, also the concentrations of bio-available heavy metals decreased during composting (89%), which was strongly correlated with the reduction in the abundance of plasmids. Together these results suggest that hyperthermophilic composting was efficient at removing ARGs by reducing the concentrations of both antibiotics and heavy metals and the strength of selection for ARGs and MGEs.

![Network analysis exploring candidate bacterial hosts (genus level) associated with ARGs and MGEs based on gene co-occurrence analysis during early (a) and late phases (b) of hyperthermophilic composting (only strong and highly significant correlations based on both Spearman and Pearson's correlation coefficient were included to the analysis ρ > 0.8, P < 0.01). The different colors represent different modules and the node sizes and edge widths are proportional to the correlation coefficient values.](image)

![The total number of ARGs and MGEs detected on chromosomes or plasmids of all culturable isolates from early and late phase composting samples (a). Panel (b) shows the number of resistance genes on plasmids and chromosomes at the taxa level.](image)
4.2. The potential mechanisms underlying the removal of ARGs and MGEs during hyperthermophilic composting

We found three interrelated mechanisms for ARG removal in our experiment: reduction in the strength of selection for ARGs and MGEs, reduced horizontal transfer of ARGs via MGEs and loss of suitable hosts for ARGs. First, the reduction in tylosin and heavy metal concentrations (bioavailable phase) was positively linked with a reduction in total MGE abundances (specifically, ISCR1 and IncQ plasmid genes). While the relationship between tylosin and heavy metal concentrations and ARG abundances was non-significant, the total MGE and ARG abundances were significantly positively correlated (Fig. S2). This suggests that hyperthermophilic composting weakened the strength of selection for ARGs and MGEs by reducing the concentrations of tylosin and heavy metals in the compost substrate. The mechanistic explanation for this might be that ARGs and MGEs often impose severe costs for host bacteria in terms of reduced growth and competitive ability (Björkman and Andersson, 2000). As a result, a reduction in antibiotic and metal concentrations likely decreased the relative benefit of ARGs and favored bacteria that did not carry costly ARGs and MGEs (Baker-Austin et al., 2006). The relative contribution of various types of MGEs (integrons, plasmids and transposon) varied during composting, suggesting that different types of MGEs could have been responsible for the dissemination and prevalence of ARGs at different phases of composting. Specifically, ISCR1 and IncQ plasmid genes were maintained at high levels during the early phase of composting while they almost completely vanished during the late phase of composting. Plasmids, in particular, are known to incur large fitness costs for bacteria (San Millan and MacLean, 2017) because they often carry multiple ARGs and heavy metal resistance genes (Gullberg et al., 2014) leading to a high metabolic burden (Andersson and Hughes, 2010). Reduction in the frequency of plasmids likely reduced the horizontal gene transfer of ARGs between different host bacteria. For example, horizontal gene transfer mediated by a conjugative plasmid RP4 that carries multiple antibiotic resistance genes has been shown to maintain antibiotic resistance in the presence of heavy metals and non-antibiotic pollutions (Klumper et al., 2017; Lu et al., 2018). In support for this, we found that larger number of resistance genes were located on plasmids during the early versus late phase of composting when the selective pressure by antibiotics and heavy metals was stronger (Fig. 5). These results suggest that the elimination of ISCR1/IncQ plasmid genes played a key role in reducing ARG abundances during hyperthermophilic composting.

In addition to affecting the strength of selection and the relative abundance of MEGs, hyperthermophilic composting could have affected the ARG abundances by changing the bacterial communities. We observed a clear decrease in bacterial abundances and community diversity during the experiment, which is consistent with previous composting studies (Zhang et al., 2018c). The high composting temperature was likely detrimental for many bacteria during the composting, which could have also led to a loss of associated resistance genes. The
reduction in bacterial densities could have also indirectly reduced ARG abundances by lowering the horizontal transfer of ARGs via less frequent bacterial encounter rates (Liao et al., 2018). In line with this, we observed clear correlations between bacterial abundances and community composition and the prevalence of ARGs and MGEs based on our sequencing data. Furthermore, less abundant and less diverse community of antibiotic resistant bacteria could be isolated and cultured from the late compared to early phase composting samples (Table S3-S4).

We also found that some taxa were non-randomly linked with changes in ARG and MGE abundances. For example, the Chloroflexi and Proteobacteria taxa, which are often linked with ARG carriage (Wu et al., 2017), had high relative abundances during the early but low abundances during the late phase of composting. To study this in more detail, we performed a network analysis exploring associations between ARGs and different bacterial taxa. We found that early and late phase composting networks were very different, which was most likely driven by a reduction and loss of several bacterial taxa (Li et al., 2015). Importantly, ARGs were associated with different bacterial taxa during early and late phases of composting. For instance, the Psychrobacter, Morganella, and T78 were the most potential ARG-associated hosts in the initial TFR waste and during the early phase of composting. In contrast, Alcaligenes, Bacillus, and Staphylococcus were the most likely ARG-associated hosts during the late phase of composting. Specifically, we identified Georgenia as a candidate bacterial host genus for the sal1 resistance gene. This taxon could only be isolated at the late phase of composting when it had 38 times higher abundance compared to the initial TFR waste. Together these results suggest that associations between ARGs and their potential host bacterial taxa changed during composting and that certain antibiotic resistant bacterial taxa might be difficult to eradicate even with hyperthermophilic composting.

Finally, we explored complex relationships between abiotic and biotic drivers on ARG removal using partial least squares path modeling. We found that variation in ARGs abundances was mainly explained by MGE abundances during the early phase of composting. In contrast, bacterial community composition had a significant and strong influence on MGE and ARG abundances throughout composting. The tylosin residue and bio-available heavy metal concentrations indirectly affected ARGs through direct effects on MGEs. Interestingly, the direct effect of bacterial community composition on ARG abundances became stronger during the late phase of composting. This finding is supported by the above results showing that most ARGs observed at the end of the experiment were located on bacterial chromosomes instead of plasmids. In the future, it will be important to study if hyperthermophilic composting favors certain type of plasmids and if it can directly select for de novo antibiotic resistance via rapid bacterial evolution.

4.3. Conclusions

In conclusion, our study shows that hyperthermophilic composting is efficient at removing tylosin antibiotic residues, heavy metals and associated ARGs and MGEs from tylosin antibiotic fermentation waste. Mechanistically, this was driven by a reduction in the abundance of plasmid genes (ISCR1 and IncQ-oriV) during the late phase of composting. These plasmid genes were highly correlated with the abundance of ARGs and thus likely acted as carriers of the resistance genes. Co-occurrence network analysis and culture-dependent experiment further revealed that the potential hosts for ARGs were effectively reduced during the composting, which likely reduced opportunities for horizontal gene transfer of ARGs. Together these results suggest that hyperthermophilic composting can be a successful strategy for treating highly concentrated antibiotic fermentation waste at industrial scale by having negative effects on bacterial hosts associated with multidrug-resistance plasmids.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105203.

References


