Insights into Variable Effects of As (III) on the Fate of Antibiotics Resistance Genes and Microbial Community during Antibiotic Fermentation Dregs Co-composted with Swine Manure

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Abstract. Composting is used commonly for the treatment and cycle utilization of solid organic wastes like antibiotics fermentation dregs (AFDs) and swine manure. Heavy metal arsenic is prevalent in those two matters as well. While its effects on the fate of antibiotic resistance genes and succession of microbial communities are still unclearly. Therefore, the lab-scale composting experiments with two exposure levels of As were established in this study. The results showed that co-composting could reduce the biological activity and toxicity of As effectively, and the bioavailable As contents had a higher potential ecological effects on the dissemination of ARGs rather than the total amounts of As. Additionally, correlations analysis indicated that the succession of bacterial community and bioavailable heavy metals were all response for the ARGs propagation during As-amended co-composting process, indicating that the changes of ARGs were driven by the combined effects of various factors during the co-composting process.

1. Introduction
Large amounts of antibiotics fermentation dregs (AFDs) have been generated during the intensive production of antibiotics. AFDs, kinds of solid organic waste, are rich of varieties nutrients. They contain cellulose, protein and trace elements, also some potential contaminations such as residual antibiotics and heavy metals (copper (Cu), zinc (Zn), arsenic (As), etc) [1]. These solid wastes have posed serious environmental problems if not be disposed properly, such as land occupation and eutrophication of fresh water. Moreover, antibiotic residue in AFDs develop the microbial resistance and favor the dissemination of antibiotics resistance genes (ARGs) unavoidably which threaten the health of mankind [2]. Therefore, it is a fact that AFDs had become a reservoir of ARGs and antibiotic resistance bacteria (ARB).

Manure co-composting is an environmental approach for the stabilization of organic matter and remove of antibiotics before land application [3]. For swine manure, which is rich in bacteria, its co-composting with AFDs cannot only degrade large amounts of refractory organic matter effectively but remove antibiotics residue and kill the resistant bacteria as well. Therefore, co-composting is a good process to solve those environmental problems caused by transforming AFDs and manure into a safer and environmental friendly matter, which can be used for agriculture application to improve soil physical properties and recycle valuable nutrients [4]. However, one of the main limiting factors in the application of composts is heavy metal. 90% of heavy metals in livestock fodder could not be adsorbed or degraded directly by the organism, so the manure contain high concentrations of heavy metals through excreting by excreta [5]. The concomitant presence of heavy metals, other environment factors, resistance bacteria

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and resistance genes in the co-composting system may favor the selective function of multi-resistance bacteria and the dissemination of resistances into ecosystem. Therefore, heavy metals have represented a long-term selective pressure in the evolution of antibiotic resistance in bacteria isolated from some environmental mediums, especially like AFDs and animal feedings. As is commonly and typically detected in swine manure and AFDs, since it can modulate the growth performance and immune responses of broilers [6]. However to date, the extent to how heavy metals influence ARGs dynamics (including propagation or removal), and the correlations among the cooccurring ARGs and bacterial community during the AFDs co-composting system are seldom reported. Hence, considering the toxicity of As on the micro-ecological environment of composting, it is reasonable to evaluate their general and potential risk during the co-composting process.

In this work, a co-composting experiment (contained tylosin fermentation dregs and swine manure) were conducted to investigate the effects of As on the ARGs and bacterial communities. The research hopes to provide an in-depth theoretical basis into the effects of heavy metals which may contribute selective pressure on certain ARGs and microbial biodiversity during AFDs co-composting process, and help to evaluate the potential environmental risk of composting products.

2. Materials and Methods

2.1. Co-composting Experiments and Sample Collection

TFDs, swine manure and wood chips were mixed homogeneously to adjust the C/N to 25 and the water content to 60% for an optimal composting. Each co-composting treatment was conducted in triplicate using three identical polymethyl methacrylate rectangular containers, with volume of 49 L, and 25 mm² holes (5 mm×5 mm) on the top and bottom respectively to be well ventilated. Given the As residue contents in pig manure and AFDs, different As-amended levels of the co-composting were set as following: control composting treatment (CK) with only raw materials; adding sodium arsenite (As[III]) solution (1 g/L) to attain 5 mg As kg⁻¹ (As-L) and 100 mg As kg⁻¹ (As-H) respectively. The co-composting experiments were test for 40 days. Moisture was kept between 55%~60% during the co-composting process. Samples were taken from the top, middle, and bottom of each reactor unit respectively on days 1, 10, 22, 40, and mixed thoroughly to achieve high representativeness. Each sample was stored at 4℃.

2.2. DNA Extraction, Quantitative PCR and 16S rRNA Gene High-throughput Sequencing Analysis

DNA was extracted from 0.3~0.5 g of each freeze-dried sample with a FastDNA SPIN Kit for Soil (MP Biomedical, France), and conducted with the instructions of the manufacturer. 5.0 M guanidinium isothiocyanate (Amesco) was added to remove the humic acid during the extraction process. The concentrations of DNA were measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and gel electrophoresis. Each sample was conducted in replicate to compensate the heterogeneity. Seven frequently detected tylosin resistance genes with different resistance mechanisms \textit{ermB, ermF, ermQ, mphA, mphC, mepA, mdtA} and two plasmid-borne As resistance genes \textit{(arsA, arsC)} were analyzed by qPCR. The 16S rRNA genes were also determined as the reference genes to minimize the variance in the relative abundances (RAs) of ARGs due to the differences in the background bacterial abundances and DNA extraction efficiency. The collected samples were used to detect the selected ARGs with high-capacity qPCR as followed our previous work [1]. RAs of the ARGs were calculated as: ARG copies/16S rRNA copies. Each gene was quantified in triplicate for each sample using a standard curve and a negative control.

The composition of bacterial communities during the co-composting system was examined by 16S rRNA high-throughput sequencing. Samples in the 1st, 10th and 40th day were collected. The V3~V4 region of 16S rRNA gene was amplified using the primers 341F/806R to target the bacteria for microbial community structure analysis. The details of 16S rRNA gene high-throughput sequencing analysis and data process were based on our previous study [1].
2.3. Data Analysis
Statistical calculations were analyzed using the SPSS 22.0 software and Origin 2017. The correlation tests and statistical differences were performed using the Pearson’s product-moment correlation coefficient. The significance was admitted at P<0.05. All of parameters are showed as average values of three replicates.

3. Results and Discussion

3.1. Variations of Metal Distribution Patterns
When considering the eco-risks of heavy metals, both total metal contents and its speciation should be analysed because the bioavailable parts of metal may exert selective pressure on the microorganism directly. Figure 1 (a) showed a 0.9~1.2-fold enrichment of total As with the co-composting proceed. The increment might due to the weight loss of com posts via respiration and mineralization of organic matters [7]. The bioavailability and ecotoxicity of heavy metals rely on the speciation which may change a lot in the composting process. According to the BCR sequencing extraction, the heavy metal could divide into the soluble/exchangeable, reducible, oxidizable and residual fractions. Among these fractions, the exchangeable and reducible ones were supposed to be the bioavailable and ecotoxicity forms, while the oxidizable and residual ones were stable and nonbioavailable. As shown in Figure. 1 (b), the bioavailable As (bio-As) accounted for a large proportion in the initial stage, then subsequently decreased and turned into oxidizable/residual fractions. In the As-amended treatment, the proportion of bioavailable As (bio-As, sum of oxidizable and residual fractions) declined from 62.4%~72% to 25.1%~25.6% after the composting completed. The increase in the humification of organic matters which formed during the composting process and could accelerate the conversion of water-soluble state of heavy metals might be responsible for the reducing in bioavailable metals and rise in unbioavailable fractions. Thus, composting process would facilitate the passivation of As and lower the potential risks of composts before the land application.

Figure 1. As speciation during the composting process. (a) total As contents; (b) the proportion in different fractions of As.

3.2. Variation in ARGs and MRGs during Co-composting
As for the relative gene abundances, total detected ARGs were decreased by 55.4%, 63.3% and 54.6% for treatment CK, As-L and As-H respectively, after the co-composting (Figure. 2 and Figure 3). Obvious differences in ARGs abundance were found between CK and the As-amended assays (P<0.05). RAs of mepA and mdtA kept rising, and dramatically increased by approximately 15.6~20.1 and 4.0~11.0-fold respectively throughout the entire composting. While, significantly negative correlations were observed among mepA, mdtA and bio-As contents, showing that As may exert a sustained pressure on these specific ARGs, though the proportion of bioavailable As reduced in the composting, and further suggesting the efflux pumps might work when stimulated by As. Another plausible explanation was as: the extracellular polymeric substances (EPS) formed during the composting process or stimulated by antibiotics and heavy metals could result in cell adhesion and biofilm formation. Therefore the bacteria would somehow favour a biofilm mode of growth and, therefore, develop an antibiotic-resistant phenotype.
Figure 2. Relative abundances of ARGs variation during TFDs co-composting process.

Figure 3. Changes in the relative abundances in As resistance genes during TFDs co-composting.
Under As stresses at relatively high dose, bacteria may obtain antibiotic resistance through (a) co-resistance; (b) cross-resistance and (c) co-regulation mechanism. Combined with the co-resistance analysis of resistance genes based on Pearson correlations (Table 1), significantly positive correlations ($P < 0.05$) were presented between ARGs and MRGs (like (i) $\text{asrC}, \text{ermF and mdtA}$; (ii) $\text{asrA}$ and $\text{mepA}$), indicating a potential co-existence of arsenic resistance genes and ARGs on the same cells. Besides the toxicity of heavy metals, most of the reductions in ARGs (like $\text{ermB}$, $\text{ermQ}$, and $\text{ermF}$) occurred in the thermophilic phase showed that temperature was one of the key factors affecting the removal of ARGs. This was similar with the previous study [8]. Therefore, more attention should be paid on the contents of bioavailable As or other heavy metals which may pose environmental risks to both human health and ecosystem.

3.3. Changes of Bacterial Communities
Microbes are the main carriers of ARGs, so the succession of microbial community may lead to the variations in ARGs as well. The compositions of bacterial communities showed diverse temporal variations at both phylum and genus levels (Figure. 4). As for phyla level, *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* were predominant during the whole co-composting process. RAs of *Proteobacteria* increased from 66.5% to 91.2% in CK group, while decreased in As-H (from 43.1% to 31.0%) group. *Proteobacteria* were the most common bacteria in soil which may contain many...
pathogenic bacterium. Meanwhile, *Proteobacteria* and *Actinobacteria* were also reported the important hosts of ARGs. *Pseudomonas*, *Bordetella* and *Bacillus* were the most abundant genus, followed by the genus of *Klebsiella*, *Corynebacterium* and *Bergeyella*. *Pseudomonas* and *Bordetella*, which belong to the *Proteobacteria* phylum, were enriched in CK treatment but significantly declined in metal treatments subsequently. The decrease in the abundance of these bacteria may have been associated with the toxicity of heavy metals and changing of circumstances during the composting process. *Bacillus*, the primary contributor to the variations in *Firmicutes*, kept raising from mesophilic to maturation stages in all the treatments (ranging from 0.94% to 9.90%). These results were different from the previous research, and might be caused by the different materials used in our co-composting and the presence of heavy metal as well. The final abundances of *Klebsiella* were 0.68% and 2.60% for the control and As-amended groups, respectively. The results suggested that *Klebsiella*, typical human pathogenic bacterium, might induce tolerance of resistance to some environment changings (like heavy metals). In addition, the *Corynebacterium* and *Acinetobacter*, which also belonged potential human pathogens, were the dominant genus during this composting process, and presented similar variations in all the treatments. Since *Actinobacteria* had been reported to be the microbial groups probably carrying or disseminating ARGs, and been often detected with multi-resistance and self-resistance, it could be referred that the high ratio of *Actinobacteria* in As-amended treatment aggravated the fate of ARGs during the experiments.

**Figure 4.** Changes in the relative abundances in ARGs during TFDs co-composting process.

Previous work had confirmed that heavy metals might play key role in shaping the bacterial communities structure through co-selection/co-occurrence which would directly or indirectly affect the changes of resistance genes [9]. It is worth noting that bacteria could respond to environment changes by co-regulation mechanism which relies on a two-component system and takes effect when stimulated by heavy metals, then promoted bacteria to obtain antibiotic resistance and propagated ARGs among bacterial community [10]. Hence, some potential host bacteria of ARGs might become dominant population under the pressure of metals which was consisted with former discussion in this study.

**Table 1.** Pearson correlation analysis among ARGs and metal resistance genes (MRGs).

|        | ermB      | ermF      | ermQ      | mepA      | mphA      | mdtA      | asrA      | asrC      | bio-As |
|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|--------|
| ermB   | 1         |           |           |           |           |           |           |           |        |
| ermF   | 0.014     | 1         |           |           |           |           |           |           |        |
| ermQ   | 0.750*    | -0.130    | 1         |           |           |           |           |           |        |
| mepA   | -0.881**  | 0.396     | -0.732*   | 1         |           |           |           |           |        |
| mphA   | -0.215    | 0.352     | -0.469    | 0.588     | 1         |           |           |           |        |
| mdtA   | 0.314     | 0.751*    | -0.630    | 0.756*    | 0.474    | 1         |           |           |        |
| asrA   | -0.810**  | -0.048    | -0.237    | 0.589*    | 0.527    | 0.126    | 1         |           |        |
| asrC   | 0.281     | 0.671*    | -0.156    | -0.049    | 0.114    | 0.579*    | -0.218    | 1         |        |
| bio-As | 0.994*    | -0.658    | 0.936     | -0.980*   | 0.011    | -0.910   | 0.725     | 0.960**   | 1      |

* Significant at P< 0.05; ** Significant at P< 0.01
4. Conclusion
Using the combination of qPCR and high-throughput sequencing of bacterial 16S rRNA, the results of this study indicated that bioavailable metals contents had a higher potential ecological effects on the dissemination of ARGs rather than the contents of heavy metals. In addition, the bacterial community shift and bioavailable heavy metals were responsible for the ARGs variation profiles during this metal-treated co-composting process. In summary, composting is an effectively method to decrease the content of bioavailable metals and lower the environmental risk of ARGs in TFDs composts, which may be due to the selective pressure from heavy metals was reduced. To lower the potential ecological risks, specific management strategies should be proposed to control the dissemination of antibiotic resistance caused by heavy metals during the co-composting process.

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