

## Article

# The Addition of Biochar and Hyper-Thermal Inoculum Can Regulate the Fate of Heavy Metals Resistant Bacterial Communities during the Livestock Manure Composting

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**Abstract:** In the present investigation the effects of biochar and hyper-thermal inoculum on the heavy-metal-resistant bacteria (HMRB) during livestock manure composting were studied. An experiment was performed on composting livestock manure and wheat straw amended with biochar and hyper-thermal inoculum. Physicochemical properties, enzyme activity, heavy metals (HMs), and bacterial activities were monitored, and a comprehensive assessment was analyzed during the composting process. The results showed that the dominant phyla of Proteobacteria, Bacteroidota, Actinobacteriota, and Chloroflexi were enriched, but this was not the case with Firmicutes. The abundance of Galbibacter, Thermobifida, Sphaerobacter, and Actinomadura was significantly different in CT15 and BHCT15. In addition, this study showed that the selected factors are less correlated with HMRB compared with the CT group. Therefore, this study could provide new insights into the effect of biochar and hyper-thermal inoculum amendments on the fate of HMRB under HMs and high temperature stress during livestock manure composting.

**Keywords:** heavy metal resistance; biochar; hyper-thermal inoculum; livestock manure; composting; bacterial activities



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## 1. Introduction

Agricultural waste sharply increases every year with the rapid development of animal husbandry. The livestock manure contains organic contaminants and heavy metals (HMs), which may cause serious pollution to the environment if not properly treated [1]. In general, composting is the most efficient way to achieve organic matter recycling and alleviate environmental risks [2,3]. The bacterial bioactivity plays a momentous role in the bio-oxidation of composting, and the microorganisms exert different mechanisms to resist external stress factors such as HMs and high temperature (HT) [4,5]. Recently, the innovating hyper-thermal composting process with hyper-thermal inoculum amendments has attracted widespread attention, as it is superior in efficiency and quality compared with the conventional process [6]. Furthermore, biochar is considered an ideal additive for reducing the mobility and bioavailability of HMs. Therefore, more studies now investigate biochar and hyper-thermal inoculum amendments in the composting process [7].

Although many papers have extensively studied bacterial diversity during composting, knowledge regarding the effects of biochar and hyper-thermal inoculum addition on heavy-metal-resistant bacteria (HMRB) is limited [8]. The HT and concentration of HMs limit the expression and bioactivity of sensitive microbes, except HMRB, which can resist hyper-thermal environments and HM stress [9,10]. Many studies have reported that the addition of inorganic and organic additives can reduce potential environmental hazards to HMRB. Awasthi et al. [11] reported that an amendment of biochar mitigates HM mobility

and persistent toxicants to promote the metabolic activity of HMRB. Zhou et al. [12] showed that an application of biochar can provide a survival environment for HMRB and relevant enzymatic activities. Awasthi et al. [4] indicated that biochar-amended compost exhibited superior HMRB with driving physicochemical properties and toxic HMs. In addition, the combined amendment of biochar and bacterial inoculum can significantly regulate a functional bacteria consortium [13]. Meanwhile, hyper-thermal environment stress can change the structure of organic matter to mediate the passivation of HMs [14]. Liu et al. [15] reported that hyperthermophilic composting can accelerate the polycondensation reactions by intensifying the oxidation of humic acid to enhance quality and quantity. On the other hand, it has been reported that humic acid has superior metal binding capacities and can improve the abundance and diversity of HMRB [16,17]. Therefore, the application of hyper-thermal inoculum can influence HMRB by regulating the potential bacterial host. At present, there are still limitations to the abundance and diversity of HMRB under HM and HT pressure [18]. In particular, how biochar and hyper-thermal inoculum amendments affect HMRB succession and the correlations of HMRB with physicochemical parameters, enzyme activity, and HMs are unknown. Therefore, the specific performance of HMRB to HM and HT stress is worth exploring further.

Thus, the present study provides new insights into the effect of biochar and hyper-thermal inoculum amendments on the fate of HMRB under HM and HT stress during livestock manure composting. The objective of this study was to (1) investigate the impact of biochar and hyper-thermal inoculum on the relative abundance and diversity of HMRB, (2) demonstrate the predictive correlation between HMRB with physico-chemical parameters, enzyme activity, and HMs, and (3) explore the succession of an integrated bacterial consortium with a combined amendment of biochar and bacterial inoculum. Further, a novel and ecological hyperthermophilic composting approach with a biochar amendment was developed.

## 2. Materials and Methods

### 2.1. Compost Materials and Preparations

Livestock manure and wheat straw were obtained from local farmland in Heping District, Shenyang, China. The bamboo biochar and hyper-thermal inoculum were collected from Dongyuan Environmental Technology Co., Ltd., Shenyang, China, owned by the research team. The biochar was prepared from biomass feedstock via slow and dry pyrolysis at a temperature of 200–300 °C at atmospheric pressure for 4 h. Each pile consisted of a mixture of 35 kg of chicken manure, 85 kg of cow dung, and 20 kg of wheat straw. Afterwards, the raw material of each pile was homogenized for use as the main raw materials. Pile A was thoroughly mixed with 15 kg of saw dust as a control, while Pile B contained 7.5 kg of hyper-thermal inoculum, and Pile C contained 7.5 kg of hyper-thermal inoculum mixed with 7.5 kg of biochar, respectively indicated as CT, HCT, and BHCT. The end products of the hyper-thermal compost were used as the inoculum for the HTC and BHCT processes. The main physico-chemical properties of the raw materials, CT, HCT, and BHCT are shown in Table 1. The basic parameters of the biochar were the moisture ( $1.53 \pm 0.08\%$ ), the pH ( $8.69 \pm 0.05$ ), and the C/N ( $132.25 \pm 1.09$ ), and the parameters of the hyper-thermal inoculum were also the moisture ( $10.32 \pm 0.53\%$ ), the pH ( $8.15 \pm 0.06$ ), and the C/N ( $80.25 \pm 1.32$ ). The moisture content of the initial mixture for each pile was adjusted to nearly 55%. The compost was set into three  $800 \times 500 \times 500$  mm troughs. The continuous aeration rate was set to  $0.2 \text{ L}\cdot\text{kg}^{-1} \text{ dry matter}\cdot\text{min}^{-1}$ . The composting pile was turned up on Days 5 and 10 to ensure oxygen supply. An approximately 200 g sample was taken from four locations at a depth of 50–100 cm in three piles on Days 1, 3, 5, 7, 9, 11, 13, and 15. About 20 g of each homogenized sample was stored at  $-20$  °C for DNA extraction, and other samples were stored at 4 °C for corresponding analysis.

**Table 1.** The main physicochemical properties of the raw materials, CT, HCT, and BHCT.

Parameter	Composting Raw Materials						Initial Mixture		
	Chicken Manure	Cow Dung	Wheat Straw	Biochar	Saw Dust	Hyper-Thermal Inoculum	CT	HCT	BHCT
Weight (kg)	105 ± 1.51	255 ± 1.63	60 ± 1.01	7.5 ± 0.23	22.5 ± 0.32	15.0 ± 0.23	153.21 ± 1.34	155.19 ± 1.69	156.6 ± 1.76
Moisture (%)	67.85 ± 0.80	65.21 ± 0.93	7.64 ± 0.12	1.53 ± 0.08	8.63 ± 0.09	10.32 ± 0.53	56.61 ± 0.29	52.07 ± 0.68	53.72 ± 0.76
pH	6.79 ± 0.07	6.83 ± 0.05	7.23 ± 0.03	8.69 ± 0.05	8.45 ± 0.03	8.15 ± 0.06	7.69 ± 0.09	8.02 ± 0.11	7.92 ± 0.06
TOC (%)	55.31 ± 1.03	51.81 ± 0.87	62.35 ± 0.86	78.03 ± 0.56	58.76 ± 0.56	63.78 ± 0.91	52.72 ± 0.87	53.48 ± 0.76	64.39 ± 0.92
TN (%)	2.13 ± 0.02	1.62 ± 0.06	5.32 ± 0.06	0.59 ± 0.05	0.49 ± 0.02	0.79 ± 0.04	1.74 ± 0.02	1.65 ± 0.07	1.80 ± 0.09
C/N	25.97 ± 1.02	31.98 ± 1.08	11.72 ± 0.96	132.25 ± 1.09	121.21 ± 1.06	80.25 ± 1.32	30.03 ± 0.76	32.41 ± 0.84	35.77 ± 0.93

Note: Values indicate mean ± standard deviation based on the samples with 3 replications. TOC: total organic carbon; TN: total nitrogen; C/N ratio: carbon-to-nitrogen ratio.

## 2.2. Physicochemical Analysis and Enzyme Activities

Thermometers were used for the daily measure of the temperature of the environment and the piles. The pH value and electrical conductivity (EC) were detected with a pH meter and a conductivity meter, respectively [19,20]. The  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N concentrations were analyzed by the spectrophotometric method [6]. Total nitrogen (TN) and total organic carbon (TOC) concentrations were measured with an Elementar analyzer to calculate the C/N ratio [21]. Moisture content (MC) was determined following the method of Esperón et al. [22]. Germination index (GI) was determined as described by Sun et al. [23]. Total element concentrations of P, Cr, Pb, Cd, As, and the available heavy metals were determined using a method described previously [24]. The activities of protease, phosphatase, cellulase, urease, and arylsulfatase (ARS) were measured according to previous research [25].

## 2.3. Bacterial Community Detection

The total DNA was extracted using the E.Z.N.A.<sup>®</sup> soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), and the quality and concentration of the extracted DNA were determined with the NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The DNA was then sequenced with the Illumina Miseq platform. The primers 338F (ACTCCTA CGGGAG GCAGCAG) and 806R (GGACTACHV GGGTWT CTAAT) were used to amplify the V3–V4 hypervariable regions of the 16S rRNA gene, using the thermocycler PCR system (GeneAmp 9700, ABI, CA, USA) [5,26].

## 2.4. Statistical Analyses

Annotations and evaluations of operational taxonomic units were performed by rank abundance. The distribution and relative abundance of HMRB were determined by visualizations and ternary diagrams. A paired Fisher's exact test was conducted to evaluate divergence analysis. Principal component analysis (PCA) was conducted for beta diversity analysis, and redundancy analysis (RDA) was used to plot the relationship between heavy-metal-resistant bacteria and selected factors. The bioinformatic analysis and RDA were performed using the online Majorbio Cloud Platform ([www.majorbio.com](http://www.majorbio.com) accessed on 31 July 2021).

## 3. Results and Discussion

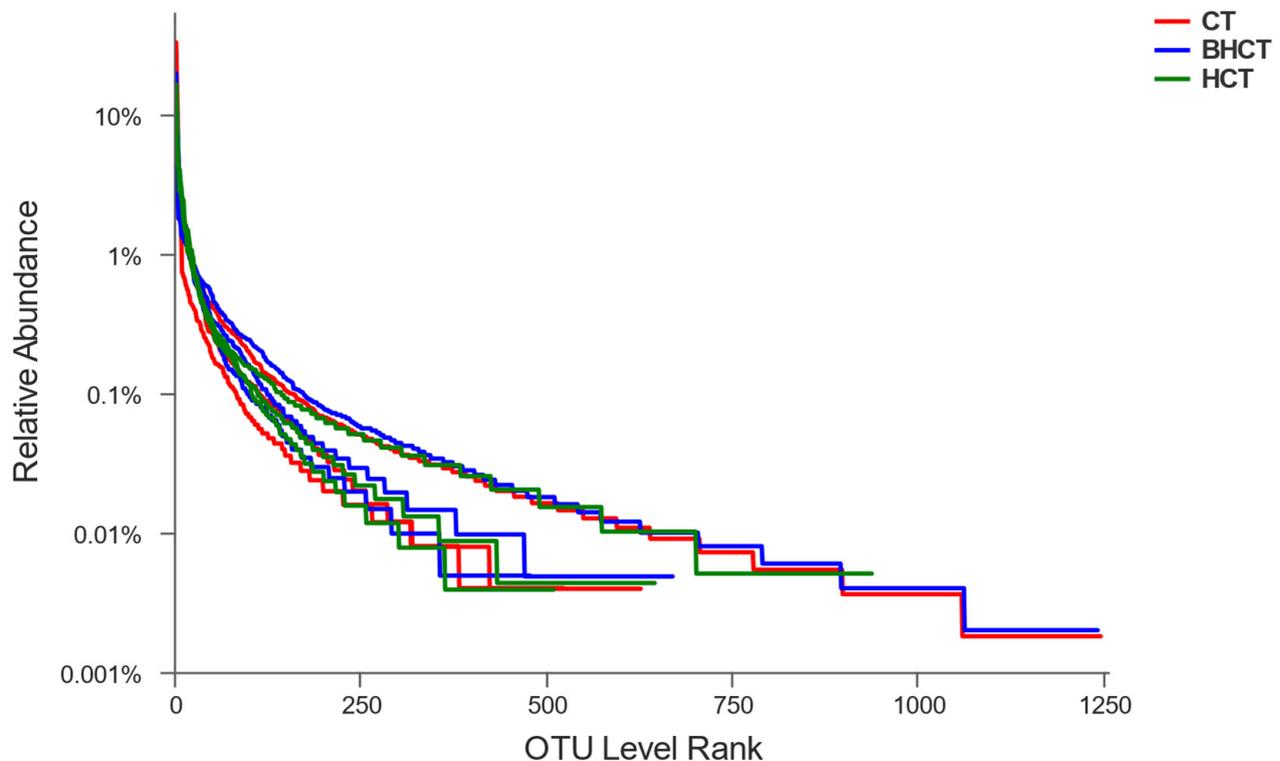
### 3.1. Species Assessment and Composition Analysis of Bacteria during Composting

#### 3.1.1. Annotation and Evaluation of Operational Taxonomic Units (OTUs)

The rank-abundance distribution curve of nine treatments at the OTU level are shown in Figure 1. In the rank-abundance distribution curve, each curve can be used to compare the evenness and richness of species by the width and smoothness. A wider curve reflects a richer species, and a flatter curve represents a higher evenness of species. As shown in Figure 1, the BHCT group was the widest and flattest in the transverse and longitudinal direction, which indicated that the BHCT group had more abundant and uniform bacterial communities than other treatments during different composting stages. It is suggested that

amendments of biochar and hyper-thermal inoculum can regulate the abundance of HMRB under HM and HT stress, enhancing bacterial reproduction [11,14].

### Rank-Abundance curves



**Figure 1.** Rank-abundance distribution curve at the OTU level.

#### 3.1.2. Relative Abundance of HMRB during Composting

The relative abundance of HMRB from the phylum to species level during composting is shown in Figure 2. The ternary phase diagrams explain the specific weight relationship in the phyla of the bacterial community among CT, BHCT, and HCT during three different composting stages (Figure 3), which vividly reflects the influence of the biochar and hyper-thermal inoculum on the HMRB. The circle indicates the bacterial communities at the phylum level, and the size of the circle represents the bacterial community abundance. As shown in Figures 2 and 3, the dominant phyla were Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Chloroflexi, consistent with the results of Awasthi et al. [27]. Among all phyla, Firmicutes abundance was 32.23%, 18.92%, and 55.58% in CT1, BHCT1, and HCT1 in the initial phase, respectively. Bacteroidota accounted for 18.54%, 24.41%, and 6.53%, and Bacteroidetes was identified with 7.39%, 12.30%, and 10.57%. In CT3, BHCT3, and HCT3, Firmicutes was observed with 87.86%, 60.94%, and 71.59%, Proteobacteria was observed with 4.48%, 8.93%, and 4.98%, and Actinobacteriota was identified with 4.79%, 12.53%, and 8.03%. Notably, in CT15, BHCT15, and HCT15, Chloroflexi was observed with 2.07%, 11.74%, and 2.13%. Additionally, obvious distinct compositions of heavy-metal-resistant bacteria were also identified at the class to species level Figure 2b–f. Among the class of CT3 and BHCT3, Bacilli affiliated with Firmicutes was observed with 83.41% and 55.90%, Bacteroidia accounted for 0.82% and 8.93%, Actinobacteria was identified with 4.36% and 12.29%, and Gammaproteobacteria abundance was 1.41% and 9.88%. Deinococci was observed with 2.31% and 2.95% in BHCT3 and HCT3. The order Bacillales had the lowest abundance, while Flavobacteriales were the most abundant in the thermophilic phase. Additionally, obvious distinct compositions of Thermomicrobiales were observed in BHCT15 and HCT15. Meanwhile, the families Weeksellaceae and Thermomicrobiaceae

had higher values in BHCT3 and BHCT15. Notably, genera *Muricauda*, *Moheibacter*, and *Sphaerobacter* were abundant in BHCT1, BHCT3, and BHCT15, respectively. Species of uncultured *Moheibacter* and unclassified *Sphaerobacter* were more dominant in BHCT3 and BHCT15.

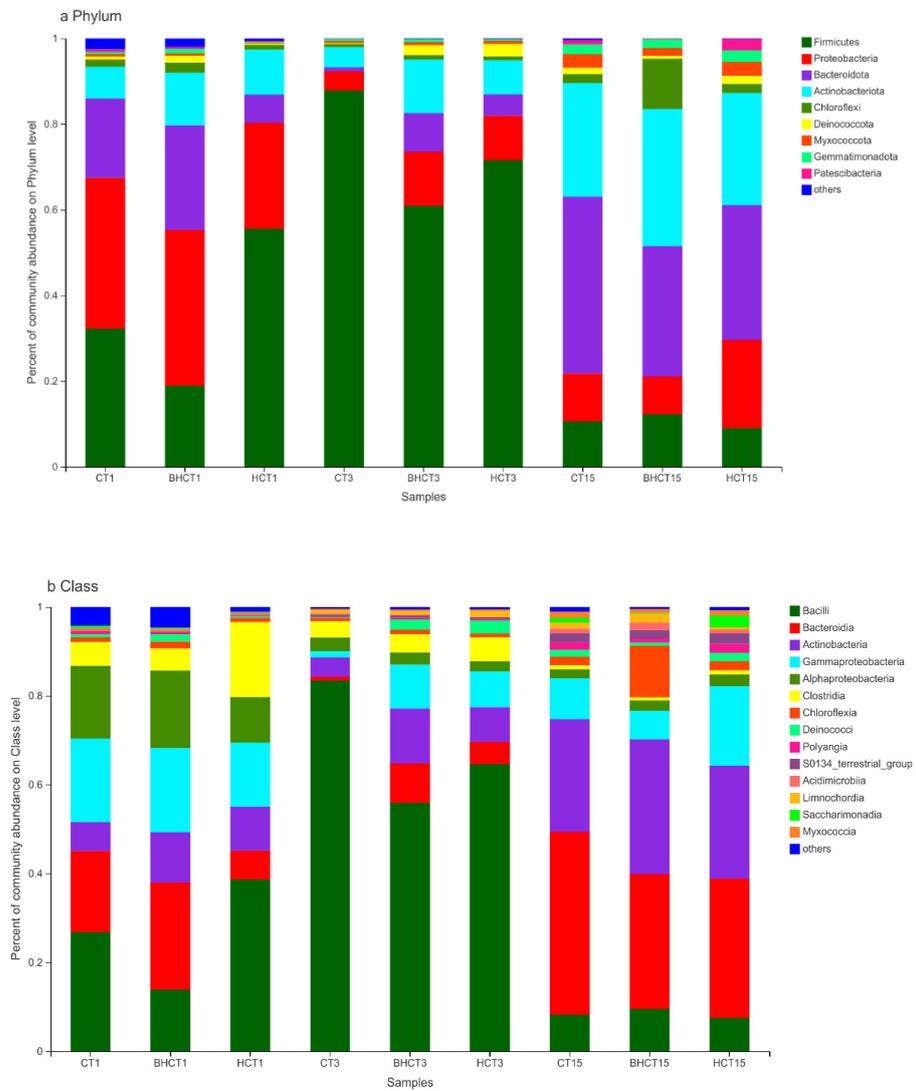


Figure 2. Cont.

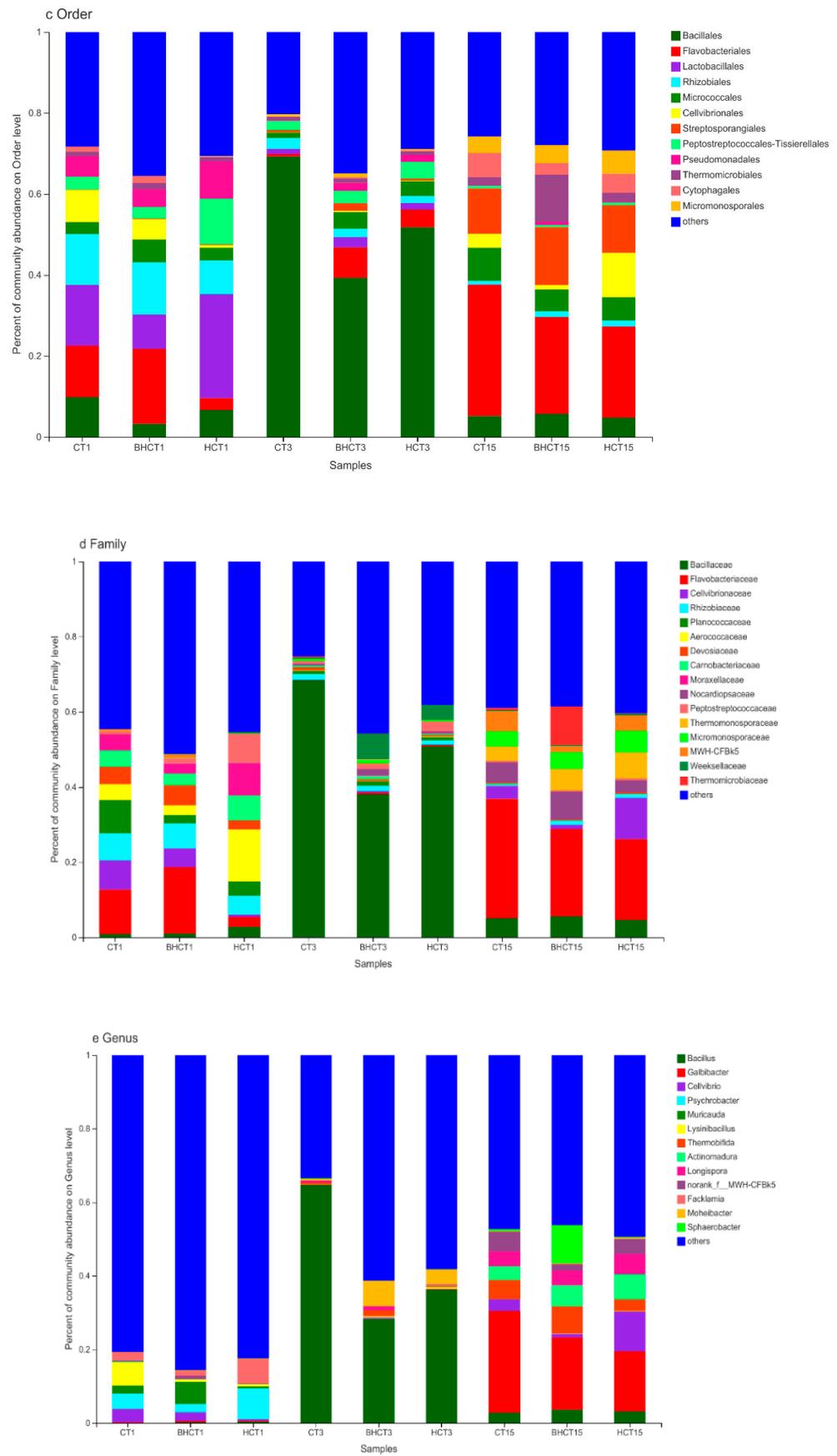
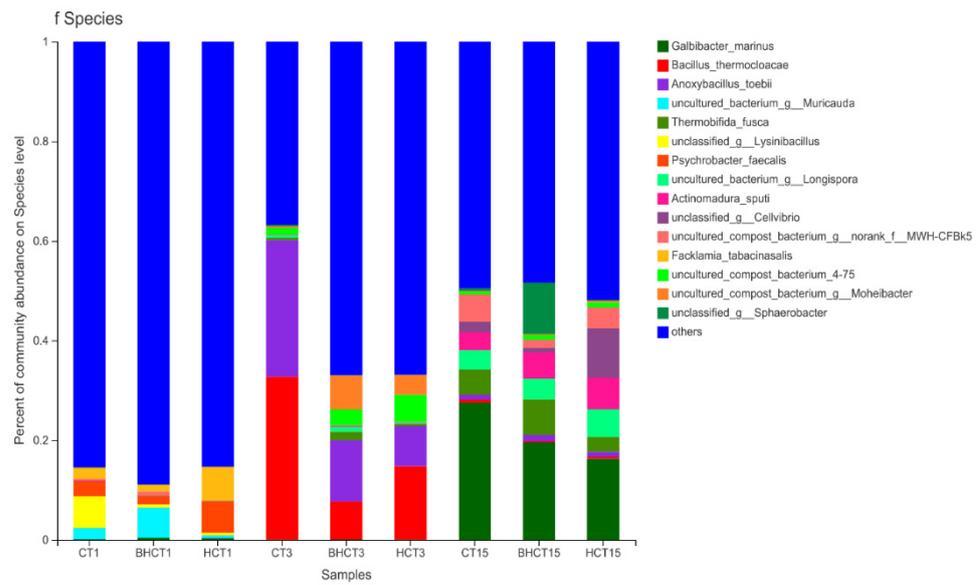
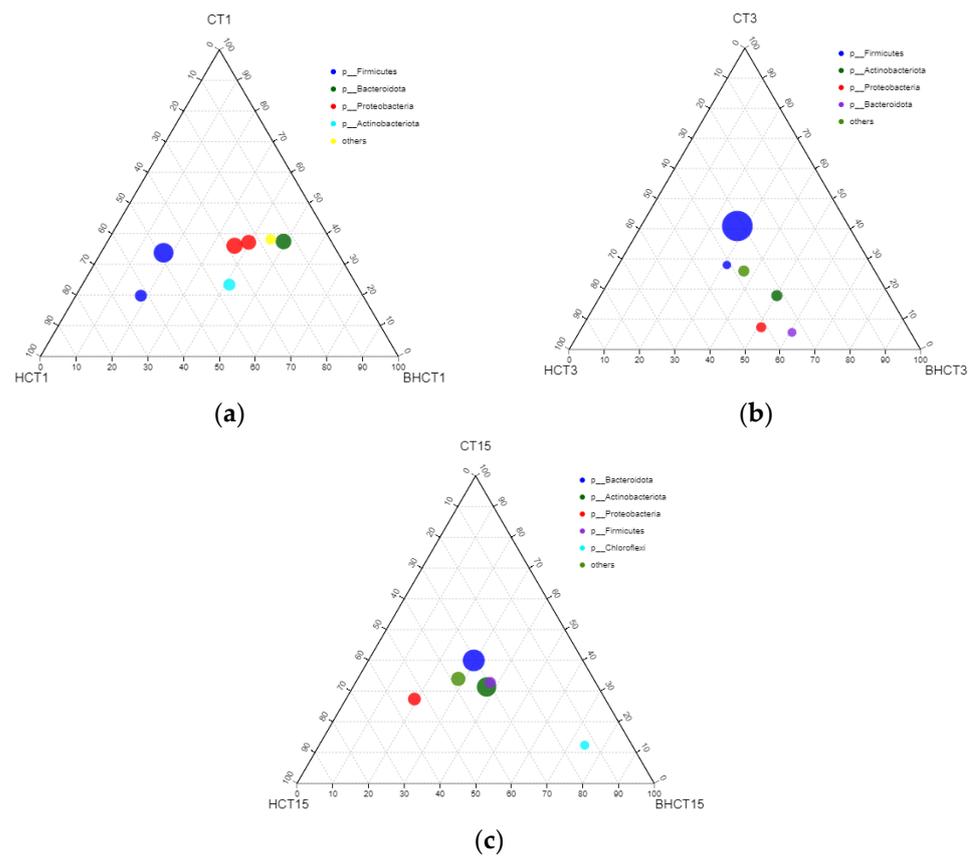


Figure 2. Cont.



**Figure 2.** The distribution histogram of the relative abundance of heavy-metal-resistant bacteria from the phylum to species level.



**Figure 3.** Ternary diagram at the phyla of the bacteria community among CT, BHCT, and HCT during three different composting stages: (a) Day 1, (b) Day 3, (c) Day 15.

As previous reported, Firmicutes and Actinobacteria largely caused the HMRB to disseminate into the environment, and Actinobacteria was the predominant HMRB phyla [28,29]. Awasthi et al. [11] pointed out that Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Chloroflexi were the most dominant HMRB-source phyla. In the present study, the reduction of identified Firmicutes might be explained by the removal of HMRB un-

der biochar and hyper-thermal inoculum amendments. The elevated relative abundance of Proteobacteria, Bacteroidota, Actinobacteriota, and Chloroflexi probably contributed to the action of biochar and hyper-thermal inoculum and effectively altered the habitat condition of the heavy metals and nutrients. Awasthi et al. [4] found that the relative abundance of bacterial communities changed with an increased biochar concentration, which suggests that biochar amendment can regulate potential bacterial hosts by changing the HMRB. Sui et al. [30] illustrated that the HMRB distribution is probably caused by bacterial communities altered by organic matter. The prominent characteristics of microbial community dynamics in hyperthermophilic composting, e.g., in Thermoactinomyces-aceae, were determined by the hyperthermophilic phase, changing the structure of dissolved organic matter, which can mediate the passivation of HMs [14,31]. This illustrates that the HMRB varied according to environmental conditions. Biochar and hyper-thermal inoculum amendments can regulate main bacterial communities to modify heavy metals by controlling modulations of the metabolic environment of microorganisms [14,28].

### 3.2. Diversity Analysis of HMRB

The distribution of HMRB compositions and Fisher's exact test bar plot on the genus level among all treatments is presented in Figure 4a–f. The addition of biochar and hyper-thermal inoculum exhibited a significant influence on the variation of HMRB. As shown in Figure 4, the bacterial community was dominated by the genera *Bacillus*, *Galbibacter*, *Cellovibrio*, *Thermobifida*, and *Actinomadura*. The results revealed that the abundance of *Bacillus* in BHCT3 accounted for 20% of the total abundance, which was lower than that in the CT3 treatment (46%). In CT15, BHCT15, and HCT15 in the maturation phase, *Galbibacter* (43%, 30%, and 25%), *Cellovibrio* (15%, 4.5%, and 50%), *Thermobifida* (28%, 40%, and 17%) and *Actinomadura* (23%, 34%, and 41%) became the dominant bacterial communities among all compost samples. The abundance of bacterial communities in each treatment was compared two-to-two by the Fisher's test to verify the difference. These results showed that the abundance of ubiquitous bacterial communities at the genus level in different treatments revealed significant differences ( $p < 0.05$ ). In particular, *Galbibacter*, *Thermobifida*, *Sphaerobacter*, and *Actinomadura* were dramatically different in CT15 and BHCT15 ( $p < 0.05$ ). In the same phase, the abundance of bacteria in the BHCT treatment was always more abundant than that of the CT treatment. Moreover, *Bacillus*, *Moheibacter*, and *Ammonibacillus* in CT3 and BHCT3 as well as *Confluentibacter*, *Muricauda*, *Lysinibacillus*, *Psychrobacter*, and *Corynebacterium* in CT1 and BHCT1 revealed significant differences ( $p < 0.05$ ).

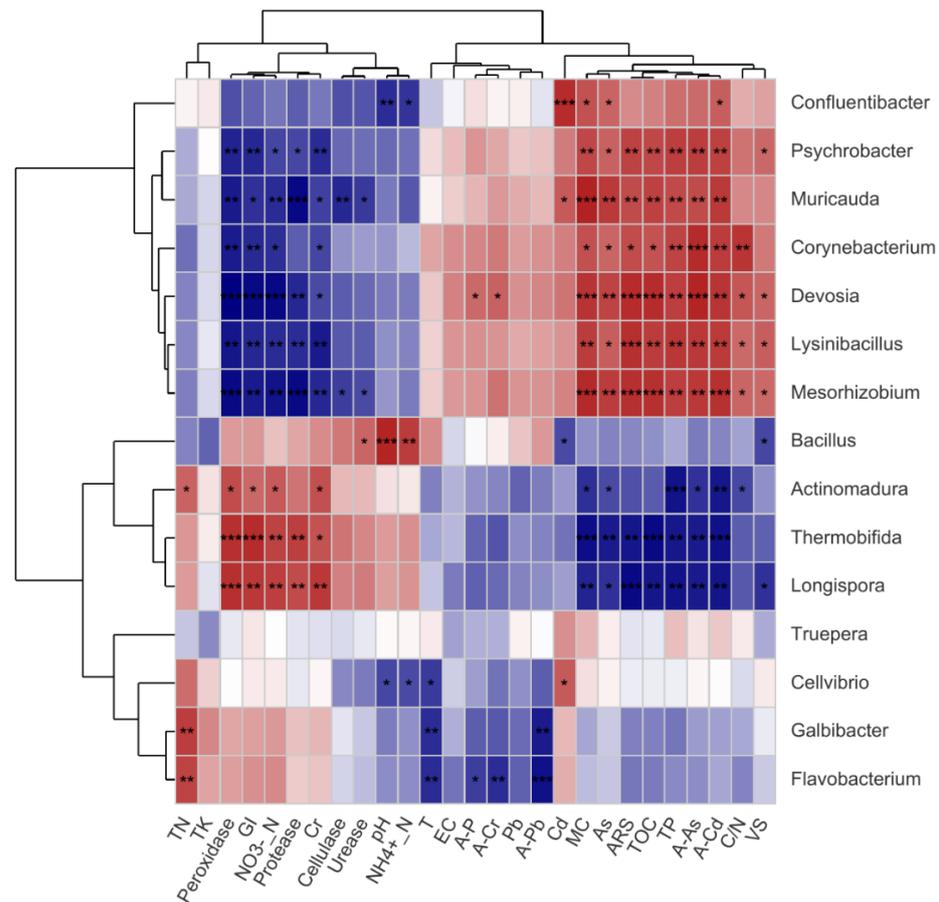
Overall, these dominant bacteria were considerably responsive to the regulation of HMRB and the abundance altered by the stress of biochar and hyper-thermal inoculum [31,32]. The fertilization of the compost significantly increased *Galbibacter* relative to most chemical characteristics, reducing the *Mizugakiibacter*, which was highly sensitive to Hg, Pb, Zn, and Cd [33,34]. Arsenic (As) methylation during manure composting was primarily attributed to *Sphaerobacter* (Chloroflexi), which are more active than other microbes [35]. Co-occurrence patterns revealed that *Actinomadura* is a significant heavy-metal-resistant gene subtype in soil contaminated with nickel iron, arsenic, copper, and zinc, and the nickel phytoextraction promoted the *Psychrobacter* bacterium [36,37]. The bacterial community profiles of *Thermobifida*, *Bacillus*, and *Corynebacterium* as potential HMRB hosts such as Cu, Zn, Cd, and Pb varied with the addition of biochar and thermo-tolerant bacterial inoculation [4,38]. The hyper-thermal inoculum generated distinctive bacterial communities. The predominant community was the thermophilic genus, which can produce abundant enzymes, such as *Actinomadura*, which is a heavy-metal-resistant gene subtypes and can produce highly thermostable xylanase [14]. The application of biochar altered the bacterial community and influenced heavy metal stress and microbial dynamics during composting, such as the dominant genus *Moheibacter* [39]. It is suggested that amendments of biochar and hyper-thermal inoculum probably change the metabolic environment and alter the formation and abundance of HMRB hosts, as biochar and hyper-



**Table 2.** The main physicochemical properties of CT, HCT, and BHCT in the initial and final stages.

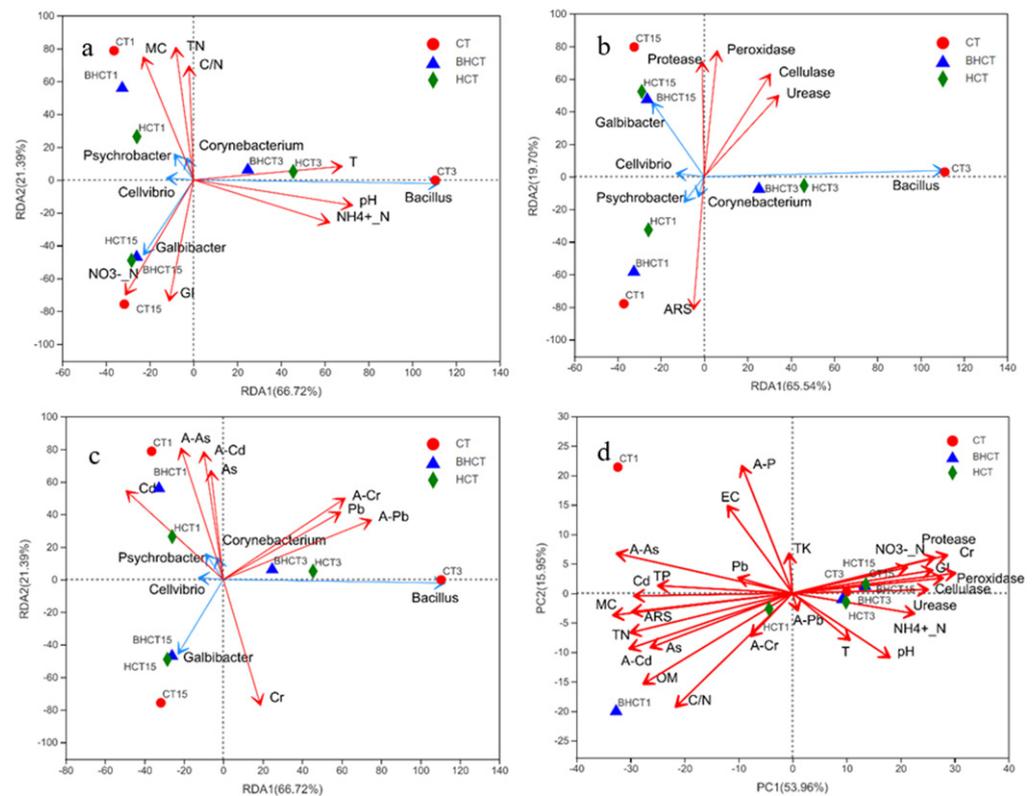
Parameter	CT (Initial Value)	BHCT (Initial Value)	HCT (Initial Value)	CT (Final Value)	BHCT (Final Value)	HCT (Final Value)
pH	6.79 ± 0.07	6.83 ± 0.05	7.23 ± 0.03	8.15 ± 0.06	8.02 ± 0.11	7.92 ± 0.06
TN	1.74 ± 0.02	1.65 ± 0.07	1.80 ± 0.09	0.95 ± 0.03	1.19 ± 0.05	0.98 ± 0.01
C/N	30.03 ± 0.76	32.41 ± 0.84	35.77 ± 0.93	24.37 ±	20.73 ±	21.41 ±
As (mg kg <sup>-1</sup> )	6.07 ± 0.04	6.68 ± 0.01	6.37 ± 0.01	4.33 ± 0.03	2.49 ± 0.04	3.61 ± 0.01
A-As (mg kg <sup>-1</sup> )	0.02	0.02	0.02	0.01	0.01	0.01
Pb (mg kg <sup>-1</sup> )	22.25 ± 0.05	21.92 ± 0.07	20.25 ± 0.05	21.03 ± 0.04	19.07 ± 0.02	20.11 ± 0.07
A-Pb (mg kg <sup>-1</sup> )	3.45 ± 0.01	3.35 ± 0.01	3.35 ± 0.02	1.25 ± 0.02	1.06 ± 0.03	1.27 ± 0.01
Cd (mg kg <sup>-1</sup> )	0.48 ± 0.01	0.48 ± 0.02	0.43 ± 0.01	0.40 ± 0.01	0.35 ± 0.01	0.44 ± 0.03
A-Cd (mg kg <sup>-1</sup> )	0.14	0.15	0.13	0.10	0.08	0.09
Cr (mg kg <sup>-1</sup> )	38.17 ± 0.27	37.11 ± 0.34	37.95 ± 0.25	51.41 ± 0.16	47.37 ± 0.1	52.69 ± 0.15
A-Cr (mg kg <sup>-1</sup> )	5.11 ± 0.05	5.41 ± 0.07	5.57 ± 0.03	4.19 ± 0.06	3.30 ± 0.05	3.98 ± 0.08

Note: Values indicate mean ± standard deviation based on the samples with 3 replications. TN: total nitrogen; C/N ratio: carbon-to-nitrogen ratio. A-P: available phosphorus; Cr: chromium; A-Cr: available chromium; Pb: lead; A-Pb: available lead; Cd: cadmium; A-Cd: available cadmium; As: arsenic; A-As: available arsenic.



**Figure 5.** Heat map of the 15 most abundant genera showing correlations between HMRB bacterial community and environmental factors, enzyme activity, and heavy metals. Vertical: bacterial information; horizontal: selected factors. The left and acroscopic cluster tree reflect species and factors, respectively. Different colors indicate different correlation coefficients (r). “\*”  $p < 0.05$ , “\*\*”  $p < 0.01$ , and “\*\*\*”  $p < 0.001$ . T: temperature; EC: electrical conductivity; GI: germination index; TOC: total organic carbon; TN: total nitrogen; C/N ratio: carbon-to-nitrogen ratio. TK: total potassium; TP: phosphorus; A-P: available phosphorus; Cr: chromium; A-Cr: available chromium; Pb: lead; A-Pb: available lead; Cd: cadmium; A-Cd: available cadmium; As: arsenic; A-As: available arsenic; ARS: arylsulfatase.

Combined with Figure 6, parameters among the environmental variables (e.g., pH, temperature, HM, and water-soluble carbon and nitrogen) and HMRB potential host bacteria registered significant correlations with bacterial community. *Bacillus* as a potential HMRB host was the dominant genus and was positively correlated with temperature during the composting process [4], and pH was positively related to *Bacillus* and *Galbibacter* [33]. Microbial inoculation enriched the abundance of the genera *Thermobifida*, *Bacillus*, and *Flavobacterium*, which served as or were related to potential HMRB hosts, enhancing the organic humification and ammonification [12]. Simultaneously, the bacterial community was primarily impacted by environmental elements. The close connection transformation might be attributed to the complex pathways of metabolic functions, which are influenced by cellular processes, genetic information, and the related environment condition [13]. In BHCT and HCT samples, biochar and hyper-thermal inoculum amendments can better resist excessively stressed environmental parameters. Overall, the exogenous additive of biochar and hyper-thermal inoculum affect the HMRB profile primarily due to self-adapting micro-environmental factors that indirectly affect the dominant functional bacterial community [4].



**Figure 6.** Redundancy analysis of HMRB bacterial community in relation to (a) environmental factors, (b) enzyme activity, and (c) heavy metals. (d) Principal coordinates analysis.

### 3.3.2. Correlation between the HMRB and Enzyme Activity

As shown in Figure 5, the relationship between HMRB and enzymes varied with biochar and hyper-thermal inoculum additions during the composting process. The distribution of enzymes was relatively concentrated and presented a strong correlation with the composting process stage, while the distribution without the addition of biochar or hyper-thermal inoculum was widely scattered. In addition, *Bacillus* shows a close relationship with cellulase and urease, and the relationship was obviously positive in the Day 3 group. *Galbibacter* is highly associated with peroxidase and protease, and this relationship was obviously positive in the Day 15 group. In contrast, *Corynebacterium* and *Psychrobacter* showed clear increasing trends with ARS in the Day 1 group.

It is well known that microbe inoculants can stimulate microbial activity to promote the composting process. The addition of biochar significantly decreased the heavy metal concentrations, increased the diversity of HM-resistant bacteria, and resulted in more synthesis and secretions of microbial enzymes due to increases in heavy-metal-resistant bacterial communities [13,40]. Zhu et al. [41] pointed out that applied biochar might improve hydrolytic enzyme generation by the increase of *Actinobacteria*. Biochar addition increases the abundance of *Bacillus* as HM-resistant bacteria and promotes the release of cellulase for organic matter decomposition, contributing to HM passivation, consistent with [42,43]. Harindintwali et al. [44] reported that the addition of microbial inoculants can generate various desired enzymes. Yu et al. [14] also pointed out that a thermus genus can produce hydrolytic enzymes and catalases as the predominant community during the hyperthermophilic composting. The application of biochar and hyper-thermal inoculum enhanced the activities of most functional enzymes, which benefits the evolution of functional enzymes in composting and weakens the relationship between enzyme activities and different treatments, hence strengthening the ability to resist heavy metal stress [4]. *Bacillus*, *Galbibacter*, *Corynebacterium*, and *Psychrobacter* are potential hosts for HMRB [4,33,36]. The correlations indicate that the application of biochar and hyper-thermal inoculum acting as an activator strongly regulates HMRB composition, ultimately improving enzyme activities for degradation process optimization.

### 3.3.3. Correlation between HMRB Communities and Heavy Metals

In Table 2, the heavy metal parameters of CT, HCT, and BHCT in the initial and final stages are shown. During the composting, heavy metals interacted with HMRB communities, revealing the effects of biochar and hyper-thermal inoculum application on the HMRB structure. The correlation between heavy metals and HMRB composition was studied via RDA. According to Figure 5, the sample points show a prominent dispersity. Those of BHCT and HCT are relatively concentrated, while those of CT are relatively dispersed. In addition, the *Bacillus* was positively related to Pb, A-Pb, and A-Cr. The *Galbibacter* genus showed a positive correlation with Cr, while the *Cellovibrio*, *Corynebacterium*, and *Psychrobacter* showed a positive correlation with A-As, As, A-Cd, and Cd. As previously reported, these dominant bacteria were usually considered major contributors to the bio-removal/persistence of HMRB, which can resist various extreme environments [45]. It is indicated that biochar and hyper-thermal inoculum amendments may weaken HM stress, which can induce the HM resistance gene and decrease the correlation with HMRB [46].

This is also consistent with the results of Figure 6. The high temperatures of BHCT and HCT were positively correlated with pH, but negatively correlated with the major HMRB distribution in the Day 3 group, suggesting that a higher pH could improve the passivation effect of HMs. It can be inferred that there are abundant HMRB and HT-resistant bacteria [47,48]. The hyper-thermal inoculum can increase the temperature and maintain a high degradation ability of organic matter, thereby enhancing the humic substances, the main driving parameter affecting the bioavailability of HMs [48,49]. Simultaneously, Figure 5 indicates a strong negative correlation between enzyme activities and heavy metals (except ARS), which further suggests that biochar and hyper-thermal inoculum amendments might activate enzyme activities by reducing the heavy metal values and accelerating compost maturity, while a higher humification degree can strengthen the passivation of HMs, similar to previous research [50]. The results indicate that biochar and hyper-thermal inoculum amendments altered HM toxicity and physicochemical properties, thereby changing the superior HMRB bacterial fate by regulating the potential bacterial hosts [51].

## 4. Conclusions

Biochar and hyper-thermal inoculum addition can regulate the abundance of HMRB under HM stress, enhancing bacterial reproduction. The prominent characteristics of microbial community dynamics in hyperthermophilic composting change the structure of dissolved organic matter, which can mediate the passivation of HMs. The bacterial

community profiles of *Thermobifida*, *Bacillus*, and *Corynebacterium* as potential HMRB hosts, such as Cu, Zn, Cd, and Pb, varied with the addition of biochar and thermotolerant bacterial inoculation. *Bacillus*, *Galbibacter*, *Corynebacterium*, and *Psychrobacter* as HMRB potential hosts, the correlations of which indicated that the application of biochar and hyper-thermal inoculum acting as an activator strongly regulated the HMRB composition, ultimately improved the enzyme activities in optimizing the degradation process. The strong negative correlation of enzyme activities with heavy metals, except ARS, further suggests that biochar and hyper-thermal inoculum amendments might activate enzyme activities by reducing heavy metal values. In addition, the dominant phyla of Proteobacteria, Bacteroidota, Actinobacteriota, and Chloroflexi were enriched, but this was not the case with Firmicutes. The genus of *Galbibacter*, *Thermobifida*, *Sphaerobacter*, and *Actinomadura* were dramatically different in CT15 and BHCT15. Therefore, the study can help to better understand the successions of HMRB under the biostimulation of compost amended with biochar and hyper-thermal inoculum.

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## References

1. Abd Elhafez, S.; Hamad, H.; Zaatout, A.; Malash, G. Management of agricultural waste for removal of heavy metals from aqueous solution: Adsorption behaviors, adsorption mechanisms, environmental protection and techno-economic analysis. *Environ. Sci. Pollut. Res.* **2017**, *24*, 1397–1415. [[CrossRef](#)] [[PubMed](#)]
2. Li, C.; Xie, S.; Wang, Y.; Pan, X.; Yu, G.; Zhang, Y. Simultaneous heavy metal immobilization and antibiotics removal during synergetic treatment of sewage sludge and pig manure. *Environ. Sci. Pollut. Res.* **2020**, *27*, 30323–30332. [[CrossRef](#)] [[PubMed](#)]
3. Niu, J.; Li, X. Effects of Microbial Inoculation with Different Indigenous *Bacillus* Species on Physicochemical Characteristics and Bacterial Succession during Short-Term Composting. *Fermentation* **2022**, *8*, 152. [[CrossRef](#)]
4. Awasthi, S.K.; Duan, Y.; Liu, T.; Zhang, Z.; Taherzadeh, M.J. Can biochar regulate the fate of heavy metals (Cu and Zn) resistant bacteria community during the poultry manure composting? *J. Hazard. Mater.* **2021**, *406*, 124593. [[CrossRef](#)]
5. Li, Z.; Fan, Y.; Huang, X.; Han, B. Microbial Diversity and Metabolites Dynamic of Light-Flavor Baijiu with Stacking Process. *Fermentation* **2022**, *8*, 67. [[CrossRef](#)]
6. Cui, P.; Liao, H.; Bai, Y.; Li, X.; Zhao, Q.; Chen, Z.; Yu, Z.; Yi, Z.; Zhou, S. Hyperthermophilic composting reduces nitrogen loss via inhibiting ammonifiers and enhancing nitrogenous humic substance formation. *Sci. Total Environ.* **2019**, *692*, 98–106. [[CrossRef](#)]
7. Ai, Y.-J.; Li, F.-P.; Gu, H.-H.; Chi, X.-J.; Yuan, X.-T.; Han, D.-Y. Combined effects of green manure returning and addition of sewage sludge compost on plant growth and microorganism communities in gold tailings. *Environ. Sci. Pollut. Res.* **2020**, *27*, 31686–31698. [[CrossRef](#)]
8. Cao, Y.; Wang, L.; Qian, Y.; Xu, Y.; Wu, H.; Zhang, J.; Huang, H.; Chang, Z. Contributions of thermotolerant bacteria to organic matter degradation under a hyperthermophilic pretreatment process during chicken manure composting. *BioResources* **2019**, *14*, 6747–6766. [[CrossRef](#)]
9. Guo, H.; Gu, J.; Wang, X.; Yu, J.; Nasir, M.; Peng, H.; Zhang, R.; Hu, T.; Wang, Q.; Ma, J. Responses of antibiotic and heavy metal resistance genes to bamboo charcoal and bamboo vinegar during aerobic composting. *Environ. Pollut.* **2019**, *252*, 1097–1105. [[CrossRef](#)]
10. Liao, H.; Lu, X.; Rensing, C.; Friman, V.P.; Geisen, S.; Chen, Z.; Yu, Z.; Wei, Z.; Zhou, S.; Zhu, Y. Hyperthermophilic Composting Accelerates the Removal of Antibiotic Resistance Genes and Mobile Genetic Elements in Sewage Sludge. *Environ. Sci. Technol.* **2018**, *52*, 266–276. [[CrossRef](#)]

11. Awasthi, S.K.; Liu, T.; Awasthi, M.K.; Zhang, Z. Evaluation of biochar amendment on heavy metal resistant bacteria abundance in biosolids compost. *Bioresour. Technol.* **2020**, *306*, 123114. [[CrossRef](#)] [[PubMed](#)]
12. Zhou, Y.; Awasthi, S.K.; Liu, T.; Verma, S.; Zhang, Z.; Pandey, A.; Varjani, S.; Li, R.; Taherzadeh, M.J.; Awasthi, M.K. Patterns of heavy metal resistant bacterial community succession influenced by biochar amendment during poultry manure composting. *J. Hazard. Mater.* **2021**, *420*, 126562. [[CrossRef](#)] [[PubMed](#)]
13. Duan, Y.; Awasthi, S.K.; Liu, T.; Verma, S.; Wang, Q.; Chen, H.; Ren, X.; Zhang, Z.; Awasthi, M.K. Positive impact of biochar alone and combined with bacterial consortium amendment on improvement of bacterial community during cow manure composting. *Bioresour. Technol.* **2019**, *280*, 79–87. [[CrossRef](#)] [[PubMed](#)]
14. Yu, Z.; Tang, J.; Liao, H.; Liu, X.; Zhou, P.; Chen, Z.; Rensing, C.; Zhou, S. The distinctive microbial community improves composting efficiency in a full-scale hyperthermophilic composting plant. *Bioresour. Technol.* **2018**, *265*, 146–154. [[CrossRef](#)]
15. Liu, X.; Hou, Y.; Li, Z.; Yu, Z.; Tang, J.; Wang, Y.; Zhou, S. Hyperthermophilic composting of sewage sludge accelerates humic acid formation: Elemental and spectroscopic evidence. *Waste Manag.* **2020**, *103*, 342–351. [[CrossRef](#)] [[PubMed](#)]
16. Jin, J.; Sun, K.; Yang, Y.; Wang, Z.; Han, L.; Wang, X.; Wu, F.; Xing, B. Comparison between soil- and biochar-derived humic acids: Composition, conformation, and phenanthrene sorption. *Environ. Sci. Technol.* **2018**, *52*, 1880–1888. [[CrossRef](#)] [[PubMed](#)]
17. Wei, Y.; Zhao, Y.; Zhao, X.; Gao, X.; Zheng, Y.; Zuo, H.; Wei, Z. Roles of different humin and heavy-metal resistant bacteria from composting on heavy metal removal. *Bioresour. Technol.* **2020**, *296*, 122375. [[CrossRef](#)]
18. Zhao, Y.; Zhao, Y.; Zhang, Z.; Wei, Y.; Wang, H.; Lu, Q.; Li, Y.; Wei, Z. Effect of thermo-tolerant actinomycetes inoculation on cellulose degradation and the formation of humic substances during composting. *Waste Manag.* **2017**, *68*, 64–73. [[CrossRef](#)]
19. Oustriere, N.; Marchand, L.; Rosette, G.; Friesl-Hanl, W.; Mench, M. Wood-derived-biochar combined with compost or iron grit for in situ stabilization of Cd, Pb, and Zn in a contaminated soil. *Environ. Sci. Pollut. Res.* **2017**, *24*, 7468–7481. [[CrossRef](#)]
20. Handa, S.; Sharma, N.; Pathania, S. Multiple parameter optimization for maximization of pectinase production by *Rhizopus* sp. C4 under solid state fermentation. *Fermentation* **2016**, *2*, 10. [[CrossRef](#)]
21. Zhu, L.-X.; Xiao, Q.; Shen, Y.-F.; Li, S.-Q. Effects of biochar and maize straw on the short-term carbon and nitrogen dynamics in a cultivated silty loam in China. *Environ. Sci. Pollut. Res.* **2017**, *24*, 1019–1029. [[CrossRef](#)] [[PubMed](#)]
22. Esperón, F.; Alberó, B.; Ugarte-Ruiz, M.; Domínguez, L.; Carballo, M.; Tadeo, J.L.; del Mar Delgado, M.; Moreno, M.Á.; de la Torre, A. Assessing the benefits of composting poultry manure in reducing antimicrobial residues, pathogenic bacteria, and antimicrobial resistance genes: A field-scale study. *Environ. Sci. Pollut. Res.* **2020**, *27*, 27738–27749. [[CrossRef](#)] [[PubMed](#)]
23. Sun, Z.-Y.; Zhang, J.; Zhong, X.-Z.; Tan, L.; Tang, Y.-Q.; Kida, K. Production of nitrate-rich compost from the solid fraction of dairy manure by a lab-scale composting system. *Waste Manag.* **2016**, *51*, 55–64. [[CrossRef](#)] [[PubMed](#)]
24. Zhong, X.; Chen, Z.; Li, Y.; Ding, K.; Liu, W.; Liu, Y.; Yuan, Y.; Zhang, M.; Baker, A.J.; Yang, W.; et al. Factors influencing heavy metal availability and risk assessment of soils at typical metal mines in Eastern China. *J. Hazard. Mater.* **2020**, *400*, 123289. [[CrossRef](#)]
25. Cardelli, V.; De Feudis, M.; Fornasier, F.; Massaccesi, L.; Cocco, S.; Agnelli, A.; Weindorf, D.C.; Corti, G. Changes of topsoil under *Fagus sylvatica* along a small latitudinal-altitudinal gradient. *Geoderma* **2019**, *344*, 164–178. [[CrossRef](#)]
26. Wei, H.; Wang, L.; Hassan, M.; Xie, B. Succession of the functional microbial communities and the metabolic functions in maize straw composting process. *Bioresour. Technol.* **2018**, *256*, 333–341. [[CrossRef](#)]
27. Awasthi, M.K.; Duan, Y.; Awasthi, S.K.; Liu, T.; Chen, H.; Pandey, A.; Zhang, Z.; Taherzadeh, M.J. Emerging applications of biochar: Improving pig manure composting and attenuation of heavy metal mobility in mature compost. *J. Hazard. Mater.* **2020**, *389*, 122116. [[CrossRef](#)]
28. Zhang, R.; Gu, J.; Wang, X.; Yang, L.; Zhang, K.; Yin, Y.; Zhang, X. Contributions of the microbial community and environmental variables to antibiotic resistance genes during co-composting with swine manure and cotton stalks. *J. Hazard. Mater.* **2018**, *358*, 82–91. [[CrossRef](#)]
29. Forsberg, K.J.; Patel, S.; Gibson, M.K.; Lauber, C.L.; Knight, R.; Fierer, N.; Dantas, G. Bacterial phylogeny structures soil resistomes across habitats. *Nature* **2014**, *509*, 612. [[CrossRef](#)]
30. Sui, Q.; Zhang, J.; Chen, M.; Wang, R.; Wang, Y.; Wei, Y. Fate of microbial pollutants and evolution of antibiotic resistance in three types of soil amended with swine slurry. *Environ. Pollut.* **2019**, *245*, 353–362. [[CrossRef](#)]
31. Huang, W.; Li, Y.; Liu, X.; Wang, W.; Zhou, S. Linking the electron transfer capacity with the compositional characteristics of dissolved organic matter during hyperthermophilic composting. *Sci. Total Environ.* **2021**, *755*, 142687. [[CrossRef](#)] [[PubMed](#)]
32. Riaz, L.; Wang, Q.; Yang, Q.; Li, X.; Lixunan, L. Potential of industrial composting and anaerobic digestion for the removal of antibiotics, antibiotic resistance genes and heavy metals from chicken manure. *Sci. Total Environ.* **2020**, *718*, 137414. [[CrossRef](#)] [[PubMed](#)]
33. Ding, S.; Zhou, D.; Wei, H.; Wu, S.; Xie, B. Alleviating soil degradation caused by watermelon continuous cropping obstacle: Application of urban waste compost. *Chemosphere* **2021**, *262*, 128387. [[CrossRef](#)] [[PubMed](#)]
34. Guo, H.; Nasir, M.; Lv, J.; Dai, Y.; Gao, J. Understanding the variation of microbial community in heavy metals contaminated soil using high throughput sequencing. *Ecotoxicol. Environ. Saf.* **2017**, *144*, 300–306. [[CrossRef](#)] [[PubMed](#)]
35. Zhai, W.; Wong, M.T.; Luo, F.; Hashmi, M.Z.; Liu, X.; Edwards, E.A.; Tang, X.; Xu, J. Arsenic Methylation and its Relationship to Abundance and Diversity of *arsM* Genes in Composting Manure. *Sci. Rep.* **2017**, *7*, srep42198. [[CrossRef](#)] [[PubMed](#)]

36. Thomas, J.C., IV; Oladeinde, A.; Kieran, T.J.; Finger, J.W., Jr.; Bayona-Vásquez, N.J.; Cartee, J.C.; Beasley, J.C.; Seaman, J.C.; McArthur, J.V.; Rhodes, O.E., Jr. Co-occurrence of antibiotic, biocide, and heavy metal resistance genes in bacteria from metal and radionuclide contaminated soils at the Savannah River Site. *Microb. Biotechnol.* **2020**, *13*, 1179–1200. [[CrossRef](#)] [[PubMed](#)]
37. Ma, Y.; Rajkumar, M.; Vicente, J.; Freitas, H. Inoculation of Ni-resistant plant growth promoting bacterium *Psychrobacter* sp. strain, S.R.; S8 for the improvement of nickel phytoextraction by energy crops. *Int. J. Phytoremed.* **2010**, *13*, 126–139. [[CrossRef](#)]
38. Fang, Y.; Jia, X.; Chen, L.; Lin, C.; Zhang, H.; Chen, J. Effect of thermotolerant bacterial inoculation on the microbial community during sludge composting. *Can. J. Microbiol.* **2019**, *65*, 750–761. [[CrossRef](#)]
39. Mei, J.; Wu, Y.; Qian, F.; Chen, C.; Shen, Y.; Zhao, Y. Methane-oxidizing microorganism properties in landfills. *Pol. J. Environ. Stud.* **2019**, *28*, 3809–3818.
40. Chen, X.; Zhao, Y.; Zhang, C.; Zhang, D.; Yao, C.; Meng, Q.; Zhao, R.; Wei, Z. Speciation, toxicity mechanism and remediation ways of heavy metals during composting: A novel theoretical microbial remediation method is proposed. *J. Environ. Manag.* **2020**, *272*, 111109. [[CrossRef](#)]
41. Zhu, L.; Zhao, Y.; Zhang, W.; Zhou, H.; Chen, X.; Li, Y.; Wei, D.; Wei, Z. Roles of bacterial community in the transformation of organic nitrogen toward enhanced bioavailability during composting with different wastes. *Bioresour. Technol.* **2019**, *285*, 121326. [[CrossRef](#)] [[PubMed](#)]
42. Du, J.; Zhang, Y.; Qu, M.; Yin, Y.; Fan, K.; Hu, B.; Zhang, H.; Wei, M.; Ma, C. Effects of biochar on the microbial activity and community structure during sewage sludge composting. *Bioresour. Technol.* **2019**, *272*, 171–179. [[CrossRef](#)] [[PubMed](#)]
43. Liu, H.; Zhou, Y.; Qin, S.; Awasth, S.K.; Liu, T.; Liu, H.; Zhang, Z.; Awasthi, M.K. Distribution of heavy metal resistant bacterial community succession in cow manure biochar amended sheep manure compost. *Bioresour. Technol.* **2021**, *335*, 125282. [[CrossRef](#)]
44. Harindintwali, J.D.; Zhou, J.; Yu, X. Lignocellulosic crop residue composting by cellulolytic nitrogen-fixing bacteria: A novel tool for environmental sustainability. *Sci. Total Environ.* **2020**, *715*, 136912. [[CrossRef](#)]
45. Rajapaksha, A.U.; Chen, S.S.; Tsang, D.C.; Zhang, M.; Vithanage, M.; Mandal, S.; Gao, B.; Bolan, N.S.; Ok, Y.S. Engineered/designer biochar for contaminant removal/immobilization from soil and water: Potential and implication of biochar modification. *Chemosphere* **2016**, *148*, 276–291. [[CrossRef](#)] [[PubMed](#)]
46. Peng, H.; Gu, J.; Wang, X.; Wang, Q.; Sun, W.; Hu, T.; Guo, H.; Ma, J.; Bao, J. Insight into the fate of antibiotic resistance genes and bacterial community in co-composting green tea residues with swine manure. *J. Environ. Manag.* **2020**, *266*, 110581. [[CrossRef](#)] [[PubMed](#)]
47. Cui, H.; Ou, Y.; Wang, L.; Yan, B.; Li, Y.; Bao, M. Critical passivation mechanisms on heavy metals during aerobic composting with different grain-size zeolite. *J. Hazard. Mater.* **2021**, *406*, 124313. [[CrossRef](#)]
48. Chen, Y.; Chen, Y.; Li, Y.; Wu, Y.; Zeng, Z.; Xu, R.; Wang, S.; Li, H.; Zhang, J. Changes of heavy metal fractions during co-composting of agricultural waste and river sediment with inoculation of *Phanerochaete chrysosporium*. *J. Hazard. Mater.* **2019**, *378*, 120757. [[CrossRef](#)]
49. Wu, D.; Wei, Z.; Qu, F.; Mohamed, T.A.; Zhu, L.; Zhao, Y.; Jia, L.; Zhao, R.; Liu, L.; Li, P. Effect of Fenton pretreatment combined with bacteria inoculation on humic substances formation during lignocellulosic biomass composting derived from rice straw. *Bioresour. Technol.* **2020**, *303*, 122849. [[CrossRef](#)]
50. Aponte, H.; Meli, P.; Butler, B.; Paolini, J.; Matus, F.; Merino, C.; Cornejo, P.; Kuzyakov, Y. Meta-analysis of heavy metal effects on soil enzyme activities. *Sci. Total Environ.* **2020**, *737*, 139744. [[CrossRef](#)]
51. Chen, Y.; Liu, Y.; Li, Y.; Wu, Y.; Chen, Y.; Zeng, G.; Zhang, J.; Li, H. Influence of biochar on heavy metals and microbial community during composting of river sediment with agricultural wastes. *Bioresour. Technol.* **2017**, *243*, 347–355. [[CrossRef](#)] [[PubMed](#)]