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Manipulation of the rhizosphere bacterial community by biofertilizers is associated with mitigation of cadmium phytotoxicity

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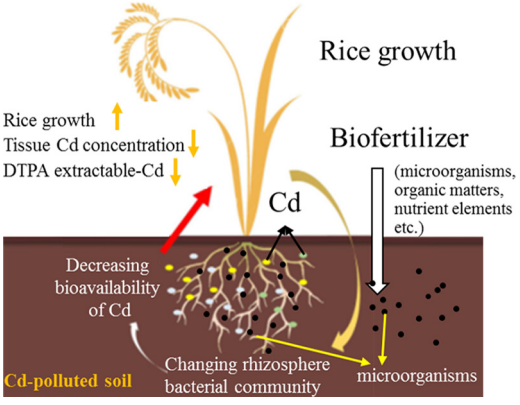
HIGHLIGHTS

- Biofertilizers were effective in mitigation of cadmium phytotoxicity.
- The rhizosphere bacterial community played critical roles in Cd stabilization.
- Effectiveness in mitigating Cd phytotoxicity was dependent on the type of biofertilizer applied.
- Soil physicochemical properties drove the structure of rhizosphere bacterial community.

GRAPHICAL ABSTRACT



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ABSTRACT

The objective of this study was to understand the effect of biofertilizers on cadmium (Cd)-induced phytotoxicity and the rhizosphere bacterial community. The crop specie rice (*Oryza sativa* L.) was planted in Cd-contaminated soils, and Illumina high-throughput sequencing was performed to investigate how the composition of the rhizosphere bacterial community responded to the addition of biofertilizers. Biofertilizers were effective in alleviating Cd phytotoxicity as indicated by the significant increase in plant biomass (up to 85.2% and 48.4% for roots and shoots, respectively) and decrease in tissue Cd concentration (up to 72.2% in roots) of rice receiving fertilizer treatments compared with the CK (no treatment). These positive effects were likely due to the increase in soil pH, which can be attributed primarily to Cd immobilization, and the promotion of beneficial taxa such as Proteobacteria, Bacteroidetes, Gemmatimonadetes, and Firmicutes. In addition, autoclaved biofertilizers tended to have similar beneficial effects and similar bacterial community alpha diversities as the original biofertilizer treatments. This suggests that the change in soil physicochemical properties by biofertilizer addition might drive the structure of rhizosphere bacterial community, and not the biofertilizer microbes themselves. In both the original and sterilized biofertilizer treatments, the effectiveness in mitigating of Cd phytotoxicity was found to be dependent on the type of biofertilizer applied. Comparatively, the biofertilizer denoted as DY was more effective in mitigating Cd phytotoxicity than others. These results demonstrate that biofertilizer addition

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could be a promising approach to immobilize soil Cd by manipulating the rhizosphere bacterial community, thus to facilitate plant growth.

1. Introduction

The rhizosphere is a narrow region of soil that adheres to plant roots, which plays a critical role in maintaining the balance of soil ecosystem, because complex biological and ecological processes, such as degradation of hydrocarbon compounds, the production of antibiotics, nutrients cycling, plant colonization or plant protection occur in this place (Marschner et al., 2003; Shen et al., 2015). Compared to bulk soil bacteria, rhizosphere bacteria live in more close association with plants, and display the advantages of promoting plant nutrient uptake, suppressing plant pathogens, or maintaining plant health (Muehe et al., 2015). Besides, rhizosphere bacteria contribute to the immobilization of metal ions and decrease their bioavailability through different mechanisms, such as extracellular complexation, precipitation, oxidation-reduction reactions or intracellular accumulation (Ahmad et al., 2008; Dennis et al., 2010; Kabeer et al., 2014; Deng et al., 2015).

Soil cadmium (Cd) contamination usually caused by mining, industrial or agricultural activities is considered one of the most severe environmental issues, because Cd is non-degradable and highly toxic, and has negative impacts on the human food chain and health (Bolan et al., 2015; Rizwan et al., 2016; Khan et al., 2017a). In recent years, the development of reliable, safe, environmentally friendly and cost-effective methods for controlling or reducing Cd contamination on agricultural land in China has aroused great interest (Wei et al., 2011; Tang et al., 2016). One potential method for counteracting Cd stress and increasing plant growth is the exogenous application of microbes. For example, the addition of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Cupriavidus taiwanensis* and *Beauveria bassiana* to the soil was found to decrease Cd accumulation in rice (*Oryza sativa*) and increase plant growth and biomass under Cd stress; these beneficial effects were due to the formation of nontoxic insoluble cadmium sulfide (CdS) and adsorption by Cd-binding proteins (Siripornadulsil & Siripornadulsil, 2013; Suksabye et al., 2016). In another study, Moreira et al. (2014) reported that inoculation with plant-growth-promoting rhizobacteria (PGPR) increased maize growth and decreased Cd accumulation in shoots compared to the untreated control. Similar results were obtained by Sangthong et al. (2016) who found that application of Cd-resistant *Micrococcus* sp. TISTR2221 improved maize growth and reduced Cd accumulation in grains. Although the application of soil microorganisms has been widely reported to effectively improve plant health and stabilize soil Cd, the direct application of microorganisms onto fields without a suitable organic substrate is not expected to be stable, especially over the long term (Shen et al., 2015).

Biofertilizers, are usually formed by the solid-state fermentation of agro-industrial waste, they contain both microorganisms and primary nutrients or plant growth regulating substances (Chen et al., 2011). The application of biofertilizers into soil has been shown to improve the production of antibiotics and the biodegradation of soil organic matter, increase nutrient supply, enhance plant tolerance to environmental stress, therefore, biofertilizer has been adopted as a clean and efficient soil conditioner or amendment to improve the quality of soil by agriculturists and plant biologists (Gajdos et al., 2012; Bhardwaj et al., 2014; Shen et al., 2013). The combined benefits of fertilizers and bioagents may be expected to alleviate the effects of Cd toxicity on plant growth. Because the current hypothesis is that manipulation of the rhizosphere bacterial community could suppress Cd phytotoxicity, it is necessary to know how rhizosphere bacterial community composition and plant growth respond to biofertilizer application.

The objectives of this study were (1) to evaluate the effectiveness of biofertilizer addition on the mitigation of Cd phytotoxicity and (2) to determine the response of the rhizosphere bacterial community to biofertilizer amendment. Different biofertilizers, including autoclaved controls, were applied to Cd-polluted soil, and the biomass and Cd uptake of rice plants grown under each treatment were used to evaluate the efficiency of remediation. Illumina high-throughput sequencing of 16S rRNA was also applied to analyze the differences in the composition of the rhizosphere bacterial community after applying different biofertilizers. Based on the results of this study, a promising approach as applying biofertilizers into soil to immobilize soil Cd and optimize the composition of the rhizosphere bacterial community could be explored.

2. Materials and methods

2.1. Collection of samples

The naturally polluted soil samples (0–20 cm soil layer) used for laboratory experiments were collected from Hengyang (HY), Hunan province, China. The soil at this site was traditionally tilled for rice, and the climate and soil characteristics are shown in Table 1. Soil material was homogenized, air-dried and crushed. The soil was sieved (2-mm mesh size), and soil properties were determined according to standard methods. Soil pH was measured using a soil/water ratio of 1:5. Soil organic matter content was determined by combustion analysis (Marriott & Wander, 2016). Cation exchanging capacity (CEC) was measured using 1 mol/L ammonium chloride, pH 7.0 after pretreatment to remove the soluble salts (Oorts et al., 2007). Soil texture was analyzed as described by Tan (2005). Soil Cd concentration was determined using a PerkinElmer 1100B atomic absorption spectrometer. The biofertilizers used as amendments for remediation of Cd polluted soils were kindly supplied by the Center for Quality Supervision and Test for Microbial Fertilizers and Mushroom Spawn of the Ministry of Agriculture, Beijing, China. Biofertilizers were prepared using a solid fermentation method. Specifically, the first biofertilizer (DY) was prepared under aerobic conditions, using cattle manure supplemented with micronutrients and additives to stimulate fermentation. The second biofertilizer (AM) was produced under aerobic fermentation, and the organic substrates included oil rapeseed cakes and pig manure compost (1:1, w/w). The third fertilizer (HM) was prepared by fermenting bagasse and chaff at a ratio of 3:1 (w/w) using peat as the carrier in an aerobic environment. The biofertilizers were stored at 4 °C prior to use in pot experiments. The nutrient and bacterial compositions (determined as described in Section 2.3) of each biofertilizer are shown in Table 2.

2.2. Pot experiment

A 500 g soil sample was ground to pass through a 2 mm mesh sieve and placed into a plastic pot. Biofertilizers were blended into HY at a rate of 3%. To control for the effect of bacteria in biofertilizers on the remediation of Cd-polluted soils, biofertilizers were autoclaved in a steam pressurized vessel at 120 °C for 1 h and applied at the same ratio. Thus, the treatments in this study included (1) soils without any biofertilizer (CK); (2) soils with biofertilizer DY (DY); (3) soils with autoclaved DY (ADY); (4) soils with biofertilizer AM (AM); (5) soils with autoclaved AM (AAM); (6) soils with biofertilizer HM (HM); and (7) soils with autoclaved HM (AHM). NPK basal fertilizer containing 0.25 g Urea/kg soil, 0.15 g KH_2PO_4/kg soil and 0.04 g KCl/kg was first dissolved in deionized water and evenly mixed with the soil in each pot, and then all pots were incubated for 5 weeks with the moisture maintained at 75% of the

Table 1
Chemical and physical properties of the test soil.

Treatment	pH (water/soil = 2.5:1)	CEC (cmol+/kg)	Soil organic carbon (%)	Total N (g/kg)	Total P (g/kg)	Total K (g/kg)	Cd background value (mg/kg)
Before planting							
Paddy soil	6.55	16.65	1.86	1.69	0.59	18.6	1.32
After planting							
CK	6.46	16.42	1.90	1.71	0.61	17.9	1.31
DY	7.71	19.43	2.96	3.68	3.30	21.4	1.30
ADY	7.80	18.62	2.72	3.59	3.19	20.9	1.30
AM	6.94	18.38	2.74	4.86	2.46	19.6	1.31
AAM	6.97	18.28	2.61	5.13	2.59	19.1	1.28
HM	7.11	17.74	2.46	8.96	3.49	22.5	1.30
AHM	7.06	17.52	2.39	9.21	3.32	23.1	1.29

water holding capacity. The reason for incubation is that the nutrients contained in biofertilizers requires some time to release into soil, and similarly, when the microbes in biofertilizers transfer to soil, the incubation is beneficial for microbes to accommodate soil environment and act effectively against Cd stress. The paddy rice seeds (XS09) were sterilized by soaking in 5% hydrogen peroxide for 5 min, rinsed with distilled water, and placed in a culture dish containing two pieces of filter paper. After germination, eight rice seedlings were transplanted into each plastic pot containing HY soil based on different treatments. The pots used in the experiment were arranged in a randomized block design with three replicates for each treatment. During rice growth, each pot was irrigated every three days with distilled water to maintain soil moisture at approximately 60–70% of water holding capacity. After 5 weeks of growth under a normal diel light cycle in a semi-closed greenhouse, the plant samples were washed with tap water, rinsed 3–4 times with deionized water, and then the shoots were collected. Rhizosphere soil samples were collected following plant harvest. Specifically, for each pot, soil cores were collected from three random sites under the root base at a depth of 0–5 cm using a small shovel, and these samples were pooled to obtain a composite root sample. Root pieces were collected from the cores by hand using a disposable glove and were pooled together for each plot. All root pieces were gently shaken by hand to remove soil and then added to a 250 ml Erlenmeyer flask. The rhizosphere soil was collected by centrifuging the remaining soil suspension in the flask at 4000 ×*g* for 10 min. The collected soil samples were immediately divided into two parts: one part was stored at −80 °C for molecular analysis and the other one was air-dried immediately for chemical analysis. The roots and shoots were then oven dried (60 °C) until a constant weight was reached. The plant samples were ground using a stainless steel mill and passed through a 0.25 mm sieve.

Table 2
The main components and bacterial composition (Phyla) of each biofertilizer.

Biofertilizer		DY	AM	HM
Total viable count (CFU/g)		7.8×10^8	5.2×10^8	6.3×10^8
Bacterial composition (Phyla) (%)	Acidobacteria	0	1.04	0.11
	Actinobacteria	0.60	49.65	57.50
	Bacteroidetes	0.13	7.08	1.48
	Chloroflexi	0	11.96	2.76
	Firmicutes	43.31	8.03	22.99
	Gemmatimonadetes	0	3.99	1.44
	Proteobacteria	55.49	15.67	13.09
	Others	0.47	3.62	0.74
pH		8.41	7.26	7.48
Organic matter (g/kg)		412	346	265
N (g/kg)		62.1	112	243
P (g/kg)		97.2	62.2	105
K (g/kg)		124.3	74.6	156
Ca (g/kg)		6.6	9.75	13.2
Mg (g/kg)		7	6.4	9.6
S (g/kg)		1.8	1.0	1.2
Fe (g/kg)		4.9	3.9	3.7
Mn (g/kg)		0.29	0.52	0.24
Zn (g/kg)		0.35	0.43	0.14
Cd (g/kg)		ND	ND	ND

ND not detectable.

2.3. Sampling and analyses

2.3.1. Soil and plant tissue Cd

The "total" (strong acid-extractable) Cd concentration in the soil before and after planting was measured by digesting approximately 1 g of air-dried soil with 4.5 ml HCl (37%), 1.5 ml HNO_3 (65%) and 1 ml H_2O_2 (30%) in a teflon bomb placed in a microwave digestion apparatus (Milestone MLS 1200 Mega). A similar procedure was used to digest plant materials but without HCl addition. The amount of diethylenetriamene pentaacetate (DTPA)-extractable Cd in soils was determined using 0.005 mol/L DTPA +0.01 mol/L $CaCl_2$ + 0.1 mol/L triethanolamine, pH 7.30 at a soil-to-solution ratio (w/v) of 1:2. The Cd concentrations in both the digests and extracts of plant and soil samples were determined using a PerkinElmer 1100B atomic absorption spectrometer.

2.3.2. DNA extraction and PCR amplification

Total soil genomic DNA was extracted from 0.5 g of frozen soil sample (three replicates per treatment) using the FastDNA SPIN Kit for Soil (MP Biomedicals; Solon, OH, USA) and the FastPrep-24 instrument (MP Biomedicals) according to the manufacturer's instructions. DNA quality and quantity was determined using a NanoDrop 2000 Spectrophotometer (Bio-Rad Laboratories Inc., USA.). The V4-V5 region of the bacterial 16S ribosomal DNA (rDNA) was amplified using a LightCycler® 480 (Roche Applied Science) real-time quantitative PCR (qPCR) system. The assay was performed in a 20 μl volume containing 10 μl of SYBR® Premix Ex Taq (Tli RNaseH Plus, 2×, Takara Bio, Japan), 1 μl of 10 mM forward primer 341f (5′-CCTAYGGGRBGCASCAG-3′) and 1 μl of 10 mM reverse primer 806r (5′-GGACTACNNGGGTWTCTAAT-3′) (Kaiya et al., 2012), 7 μl Milli-Q water, and 1 μl of 10-fold diluted DNA. The qPCR reaction was performed in triplicate. The PCR conditions were an initial denaturation at 95 °C for 30 s (ramp rate of 4.4 °C/s), followed by 30 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, 95 °C for 5 s and then elongation at 60 °C for 1 min, with a final extension at 50 °C for 30 s (Liu et al., 2016). The PCR products were pooled and purified using an AxyPrepDNA purification kit (AXYGEN, Inc.). The purified amplicons (465-bp fragments) from all samples were submitted to Lingen Biotechnology Co., Ltd. (Shanghai, China) for paired-end sequencing on the Illumina HiSeq platform.

2.3.3. Analysis of Illumina HiSeq sequencing data

The QIIME software package (version 1.8.0) was used to analyze the raw Illumina HiSeq sequencing data (Caporaso et al., 2010). The reads were quality trimmed by discarding quality scores below 20 and sequence lengths below 400 bp. In total, 2,301,922 high quality and chimera-free reads with an average length of 415 bp were obtained.

After preprocessing the reads, the number of sequences among the different samples ranged from 37,859 to 74,131. The unique sequences among these remaining reads were used to define operational taxonomic units (OTUs) using Usearch (version 7.1 http://drive5.com/uparse/) with a threshold of 97% similarity. The taxonomic identities of the phylotypes were determined using the Ribosomal Database Project (RDP) Classifier (version 2.2 http://sourceforge.net/projects/rdpclassifier/) at a confidence threshold of 70%.

2.4. Statistical analyses

Coverage, richness (Chao and ACE indexes), and diversity (Shannon and Simpson indexes) were used to estimate the alpha diversity of each sample. Mothur (version 1.34.0) was used to generate hierarchical cluster dendrograms (with Bray-Curtis distance dissimilarities) and perform principal component analysis (PCA) to compare the bacterial community structures across all soil samples (Schloss et al., 2009). The correlations between the abundant bacterial phyla and soil characteristics were determined by the Mantel test, and redundancy analysis (RDA) was carried out using the R vegan package (Oksanen et al., n. d.). SPSS v20.0 (SPSS Inc., USA) was used to perform one-way analysis of variance (ANOVA) with posthoc Tukey's honest significant difference (HSD) tests and to calculate Spearman's rank correlations.

3. Results and discussion

3.1. The effect of biofertilizers on the mitigation of Cd toxicity to rice

3.1.1. Plant growth responses and Cd accumulation

Plant growth is a direct indicator of Cd phytotoxicity in contaminated soils. We found that rice dry biomass was significantly higher under all treatments with biofertilizer addition compared to the CK (Fig. 1A). However sterile biofertilizers did not significantly increase rice biomass, except for one condition (AHM treatment with increased root biomass), indicating that the bacterial communities in biofertilizers may play a critical role in improving plant resistance to soil Cd stress. This may be because microbes can stabilize Cd in the soil and reduce its toxicity to roots, thereby allow plants to take up more nutrients (Derakhshan et al., 2017). The different fertilizers also had significantly different effects on plant biomass. For example, rice root and shoot biomass under the DY treatment increased up to 85.2% and 48.4%, respectively, compared to the CK; this increase was much higher than that observed for the AM and HM treatments. This suggests that the effectiveness of Cd remediation was biofertilizer specific.

Fig. 1B shows the effect of biofertilizer on tissue Cd concentration. As expected, biofertilizer addition indeed significantly decreased rice root and shoot Cd concentrations, indicating that biofertilizer may effectively stabilize Cd in soils and inhibit its translocation into rice tissues. Significantly lower tissue Cd concentrations compared with the CK were also observed for all autoclaved fertilizers except for AHM. This reduction in the transfer of Cd into plants in the presence of autoclaved biofertilizers could be due to improved physical health of the soil and nutrient availability. Because biofertilizers are produced by the solid-state fermentation of agro-wastes, they are soil conditioners and contain high organic matter content; this organic matter could convert the soluble/exchangeable Cd into organic bond fraction (Khan et al., 2017a). The nutrients in the autoclaved biofertilizer may also enhance native bacterial activity in the organic-amended soils, resulting in increased nutrient cycling, hormone production and establishment of plant symbioses, which may improve plant stress tolerance (Farrell et al., 2010; Oldare et al., 2011). However, it should be noted that although the addition of autoclaved biofertilizers significantly decreased tissue Cd concentration, their additions did not significantly increase tissue biomass, while unautoclaved biofertilizer did (Fig. 1).

Compared with all other treaments, the DY treatment resulted in the lowest tissue Cd concentration; DY roots had 72.2% lower levels of Cd than CK roots. This provides further evidence that the DY treatment was more effective in restricting Cd uptake and accumulation in rice. This might be due to the higher pH and organic matter content of DY compared with the other fertilizers (Table 2), both of which play an important role in retaining Cd in soils by promoting the formation of stable metalo-organo complexes (Khan et al., 2017). Cd is likely to form Cd $(OH)^{+}$ at high soil pH (>7), which results in the enhancement of Cd adsorption to soils. Organic matter is similarly helpful in retaining Cd because it adsorbs or forms stable complexes with Cd (Kashem & Singh, 2011). It also should be noted that rice has been identified as a Cd-sensitive species and an accumulator of Cd, frequently containing >0.10 mg Cd kg^{-1} dry matter (Grant et al., 2008). Thus, the presence of biofertilizers that delay Cd uptake by the roots and accumulation in the shoots has the potential to dramatically decrease tissue Cd concentrations and improve the growth of rice in Cd-contaminated soil.

3.1.2. Soil pH and DTPA-extractable Cd

The DY and AM treatments noticeably increased soil pH compared to the CK, but the HM treatment significantly decreased soil pH (Fig. 2A). This could be explained by the original pH of the biofertilizers (Table 2). The rankings of treatments by soil pH (Fig. 2), organic matter content (Table 2) and decrease in tissue Cd concentration (Fig. 1B) were the same (DY>AM>HM). This is consistent with the fact that soil pH and organic matter are two of the most important parameters that control Cd availability. Although the HM application had no or a reduced effect on the pH of the test soil, this treatment resulted in significantly lower Cd concentrations in rice tissues than the CK (no treatment) (Fig. 1B). This indicates that factors other than soil pH, such as the nutrients

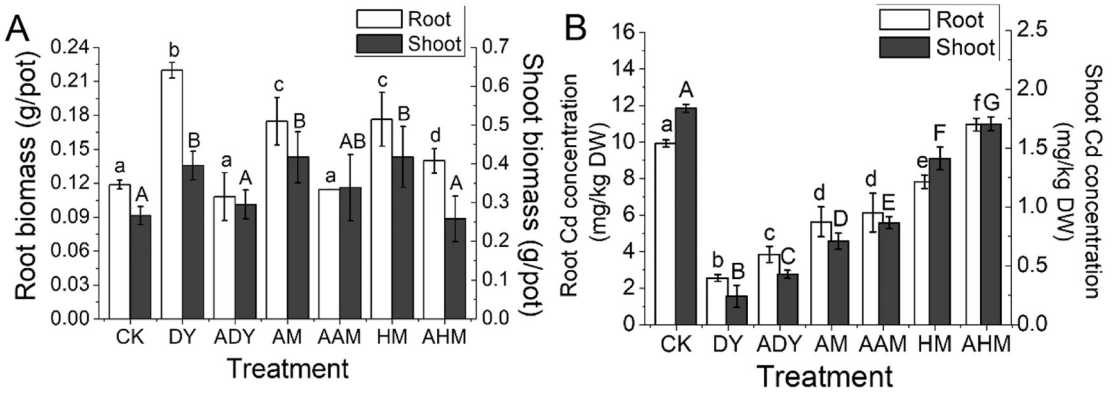

Fig. 1. Dry biomass (A) and Cd content (B) in rice after applying various biofertilizers. Error bars represent standard deviations, and bars with different letters are significantly different at $P < 0.05$. Comparisons were done separately for roots (lowercase letters) and shoots (uppercase letters). Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

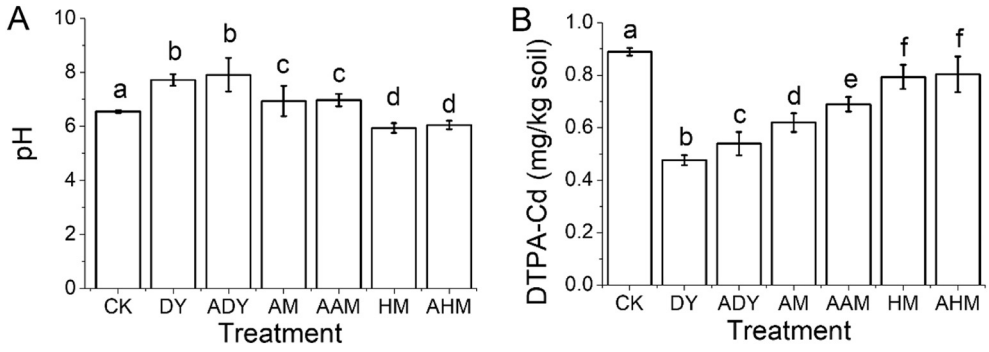

Fig. 2. Soil pH (A) and DTPA-Cd concentration (B) of CK soil and soil treated with different biofertilizers. Error bars represent standard deviations, different letters indicate statistically significant differences between treatments. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

(e.g. nitrogen [N], phosphorus [P], potassium [K], calcium [Ca], Zinc [Zn]) and microbes in the biofertilizers need to be considered (Catherine et al., 2006; Sun et al., 2007). It is also interesting that the addition of autoclaved biofertilizers had the same effect on soil pH as unautoclaved soils. This seems to explain why biofertilizers remained effective in deceasing Cd accumulation in plant tissues even after sterilization (Fig. 1B).

The concentration of DTPA-extractable heavy metals (including Cu, Zn, Fe, Mn, and Cd) in soil can be used as an indicator of the available metal pool in soils. All treatments were effective in decreasing the level of DTPA-extractable Cd (Fig. 2B). The lowest DTPA-extractable Cd concentration was observed for the DY treatment, where a 46% reduction in Cd level compared to the test soil was observed. For the HM treatment, the extractable Cd was approximately 10% lower than that of CK (Fig. 2B). The lower concentration of DTPA-extractable Cd in biofertilizer-treated soil could due to the fact that biofertilizers (especially DY and AM) impart alkaline properties that raise soil pH, thereby promoting the formation of insoluble Cd precipitates, complexes, and secondary minerals (Nejad et al., 2017). Although the addition of all biofertilizers significantly lowered available Cd concentrations compared with the CK, this decrease was lower for the unautoclaved biofertilizers than their corresponding unautoclaved controls, further indicating that the microbes in the biofertilizers (Table 1) play a vital role in stabilizing Cd in soils. However, careful inspection of Fig. 2B reveals that not only did the ADY treatment display a much lower extractable Cd concentration than AAM and AHM, this concentration was even lower than that observed for AM or HM. This seems to suggest that in this case, differences in the soil physical structure or chemical composition/fertility of the biofertilizers drove the stabilization of Cd in soils. However, more investigation is needed to test this hypothesis.

3.2. The effect of biofertilizers on the rhizosphere bacterial community in Cd-contaminated soil

3.2.1. Sequencing results and diversity indices

Diverse bacterial communities and different phylogenetic OTUs (2439 to 3059, each defined based on a sequence similarity level of 97%) in the soil samples were revealed from high-quality sequencing reads (Table 3). The coverage value for each soil sample was approximately 0.99, indicating that the sequencing depth was sufficient to completely reveal the bacterial diversity. Alpha diversity metrics, including species richness (Chao), evenness (ACE), and Shannon and Simpson, which are regularly used to evaluate bacterial diversity, were also calculated using all sequences from each sample and found to be consistent with the number of OTUs (Table 3). Based on OTUs, the lowest richness and diversity was observed in the DY treatment, while the highest was observed for HM. Moreover, there was no significant difference between the CK and AM treatment. This indicates that the application of biofertilizer on Cd-contaminated soils might have a significant effect on rhizosphere bacterial diversity, but this effect is dependent on the type of biofertilizer applied. In addition, it is important to note that similar alpha diversities were observed between corresponding unautoclaved and autoclaved biofertilizer treatments, suggesting that the chemical components of the soil amendments or changes in the soil physical structure might drive the changes in rhizosphere bacterial community rather than the microbes included in the biofertilizer.

Table 3
Summary of bacterial 16S sequencing data and diversity estimates for each treatment. 95% confidence intervals are given in parentheses, and significant differences are indicated by different lowercase letters ($P < 0.05$) (these comparisons were done separately for each alpha diversity metric). Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

Sample	Reads	OTUs	Coverage	ACE	Chao	Shannon	Simpson
CK	60,571 ± 7052	2943 ± 310	0.993	3212 a (3167,3266)	3072 a (3042,3111)	6.29 a (6.28,6.31)	0.0081 a (0.0079,0.0084)
DY	57,740 ± 6128	2698 ± 269	0.993	2986 b (2938,3043)	2832 b (2801,2872)	5.92 b (5.99,5.93)	0.015 b (0.0146,0.0154)
ADY	61,652 ± 5834	2954 ± 258	0.994	3185 a (3144,3234)	3062 a (3035,3097)	6.32 a (6.31,6.34)	0.0071 a (0.0069,0.0073)
AM	65,194 ± 6749	3013 ± 319	0.994	3259 a (3217,3310)	3118 a (3093,3153)	6.22 a (6.21,6.23)	0.0098 a (0.0096,0.0101)
AAM	61,907 ± 5398	2981 ± 364	0.993	3247 a (3202,3301)	3097 a (3069,3133)	6.34 a (6.33,6.36)	0.0075 a (0.0073,0.0077)
HM	63,843 ± 7649	2479 ± 196	0.994	2756 c (2709,2814)	2617 c (2585,2659)	5.45 c (5.44,5.47)	0.0269 c (0.0262,0.0275)
AHM	61,010 ± 8102	2439 ± 354	0.993	2726 c (2677,2785)	2578 c (2546,2620)	5.73 c (5.71,5.74)	0.0166 c (0.0162,0.0171)

3.2.2. Bacterial community composition

3.2.2.1. Composition similarity. Matrix of bacterial community distance based on the Bray-Curtis distance (Table 4) indicated that the community structural patterns differed significantly between biofertilizer treatments. The DY and HM bacterial communities were distinct from CK communities, which displayed high similarity with AM communities. It is also important to note that there was no significant difference in community composition between the corresponding unautoclaved and autoclaved biofertilizer treatments when compared with the differences between biofertilizers. This pattern was confirmed by principal component analysis (PCA; Fig. 3). Bacterial communities in soil samples treated with the same type of biofertilizer clustered closely together, while samples from different treatments were distant from each other. The second principal component (PC2) discriminated the bacterial community associated with the DY treatment from those associated with AM and HM, and the first principal component (PC1) discriminated AM from HM. CK was close to AM, but it is interesting to note that although the AM and CK soils had similar bacterial diversities and communities (Table 4 and Fig. 3), their effect on stabilizing soil Cd and plant growth varied significantly (Figs. 1 and 2). There are at least two possible explanations for this. One reason might be that more low-abundance OTUs were detected in the AM-treated rhizosphere soil than in the CK soil (data not shown), which probably played a critical role in plant resistance to Cd toxicity. Another reason may be that the biofertilizer treatment could affect the number and composition of soil microbes other than bacteria, such as arbuscular mycorrhizal fungi, that can form symbionts with the majority of plant species. These microbes have been shown to improve plant development by increasing water and nutrient absorption and to enhance plant tolerance under various stresses, such as heavy metal, drought, and salinity (Hassan et al., 2013). Therefore, the effect of biofertilizer addition on these microbes could be crucial. However, more investigation is needed to test this hypothesis.

3.2.2.2. Phyla abundance. Proteobacteria and Actinobacteria were the most abundant phyla under each treatment (27.5–49.5%) (Fig. 4), followed by Chloroflexi (4.38%–8.67%) and Acidobacteria (5.48%–8.65%). Other major phyla accounting for >1% of the overall bacterial community in rice rhizosphere soils included Saccharibacteria, Firmicutes, Gemmatimonadetes, Bacteroidetes, Thaumarchaeota and Nitrospirae. These phyla have been described as common bacterial groups in different agricultural systems, although their relative abundances vary with the type of soil (Chodak et al., 2013). Based on phyla abundance analysis (Fig. 4), biofertilizer application increased the abundance of Proteobacteria, Actinobacteria, Firmicutes, Gemmatimonadetes and Nitrospirae, but decreased the abundance of Chloroflexi, Acidobacteria, Saccharibacteria and Thaumarchaeota in the rhizosphere.

Though Proteobacteria and Chloroflexi were previously found to the most abundant bacterial phyla in rice soils (Liu et al., 2014), the two most abundant phyla in this study were Proteobacteria and Actinobacteria (Fig. 4). This is consistent with a previous study where these two phyla were found to serve as the active bacterial fraction in heavy metal-polluted soils (Margesin et al., 2011), and also with the finding that Proteobacteria are the most metal-tolerant microorganisms in heavily contaminated soils (Burkhardt et al., 2011).

Another important phylum detected in rice rhizosphere soil was Actinobacteria. Species in this phylum are widely distributed in soil, water, and compost and play important roles in suppressing pathogenic microorganisms and degrading recalcitrant compounds (Franke-Whittle et al., 2009; Alvarez et al., 2012). This bacterial phylum is also dominant in heavy metal contaminated soils and has been usually used to indicate heavy metal contamination (Margesin et al., 2011). In this study, we found that the relative abundance of Actinobacteria in Cd-contaminated soil treated with biofertilizers was significantly increased by up to 16.1%, compared to the CK (Fig. 4). This increased abundance might be due to the metabolic versatility of these microorganisms, which are able to obtain energy from various organic and inorganic compounds (Trujillo, 2008). Thus, increased nutrient availability in rhizosphere soil containing biofertilizers likely did influence the

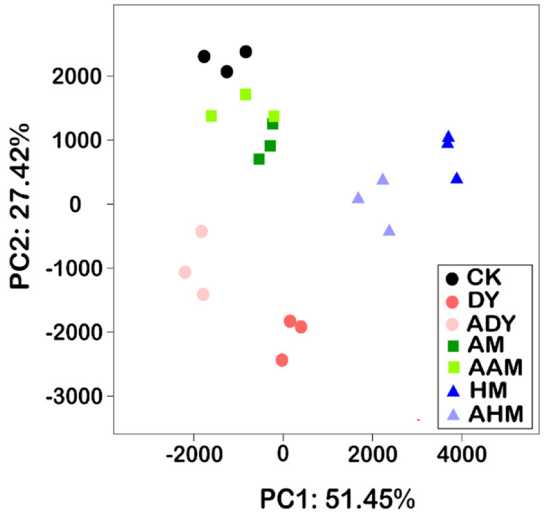

Fig. 3. Principal component analysis (PCA) of the rhizosphere bacterial communities of Cd-contaminated soils amended with different biofertilizers based on OTUs (defined based on 97% sequence similarity). Each value in the figure represents the mean of three replicates. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

Table 4
Matrix of bacterial community distance after different treatments based on Bray-Curtis of dissimilarity. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

	CK	DY	ADY	AM	AAM	HM	AHM
CK	0.000	0.436	0.405	0.252	0.208	0.399	0.387
DY		0.000	0.260	0.371	0.414	0.410	0.393
ADY			0.000	0.394	0.398	0.490	0.475
AM				0.000	0.197	0.316	0.308
AAM					0.000	0.362	0.349
HM						0.000	0.222
AHM							0.000

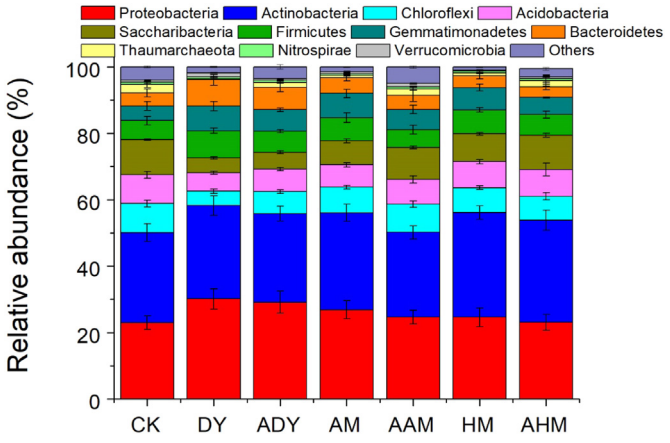

Fig. 4. The relative abundances of bacterial phyla (RA > 0.5%) and protecobacterial classes in rice rhizosphere soils amended with different biofertilizers. Each value represents the mean of three replicates. Error bars represent standard deviations. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

activity and distribution of Actinobacteria in soils, in addition to ecological factors such as pH, salinity, and temperature. However, it should be noted that the inclusion of a group of Actinobacteria that are dominant in ecosystems with high metal concentrations could result in more Cd accumulation in the soil (Baker & Banfield, 2003; Rawlings & Johnson, 2007). This may partially explain why the AM and HM treatments, which contained high proportions of Actinobacteria (49.65% and 57.5% respectively) compared with DY (0.6%), were less effective in reducing DTPA-Cd and inhibiting Cd uptake by plants (Figs. 1, 2 and Table 3).

The relative abundance of Acidobacteria in rice rhizosphere soils containing various biofertilizers was significantly lower compared to CK soil, and HM soil had a higher abundance of Acidobacteria than AM and DY soil (Fig. 4). This may be due to the pH values of these treatments (Fig. 2); the lower the soil pH, the greater the expected abundance of Acidobacteria. Another reason might be the lower organic matter content in HM (Table 2); the abundance of Acidobacteria, which possess genes involved in the degradation of complex organic matter, is negatively correlated with organic C availability (Jones et al., 2009).The relative abundances of the Bacteroidetes and Firmicutes phyla increased with biofertilizer addition (Fig. 4). Although these phyla have not been previously been shown to be involved in heavy metal tolerance, Bacteroidetes is used to indicate soil health, and the presence of Firmicutes can effectively suppress soil-borne disease (Sanguin et al., 2009). Conversely, the relative abundances of Chloroflexi, Nitrospira phyla were significantly decreased in rhizosphere soil treated with various biofertilizers. While the ecological functions of Chloroflexi are still not well known. The effect of biofertilizers on Nitrospira abundance was probably due to the high levels of ammonium and heavy metals in the amended soils (Tian et al., 2015).

When carefully comparing the effect of different biofertilizers on bacterial phyla abundance (Fig. 4), it was clear that the relative abundances of phyla beneficial for reducing Cd phytotoxicity, mainly including Proteobacteria, Firmicutes and Bacteroidetes, were highest in the DY treatment, followed by AM and HM. Abundance of these phyla was also positively correlated with tissue biomass and negatively correlated with tissue Cd concentration. One reason of this benign effect in DY may be that DY contains a large number of bacteria from beneficial phyla (55.49% of the bacteria in DY are Proteobacteria and 43.31% are Firmicutes; Table 2). The addition of DY to soils could be helpful for building a benign microenvironment that promotes the growth advantageous microorganisms. Another reason might be the change in soil physical and chemical characteristics, including increased soil pH and organic matter, caused by the addition of biofertilizer. In addition, it is important to note that although autoclaved fertilizers did not contain any active bacteria when they were applied to the Cd-contaminated soil, after planting, the bacterial communities were surprisingly similar to their corresponding unautoclaved treatments, which indicates that the change in physicochemical properties caused by the addition of biofertilizer played a dominant role in shaping the bacterial community.

In this study, high-throughput sequencing provided the opportunity to perform an in depth study on the bacterial community composition in rice rhizosphere as shown in Fig. 5. The microbial community analysis identified the dominant genera with an average abundance of >1%. These organisms could be related to dynamically biogeochemical cycling in rice rhizosphere. For example, the genera like *Bacillus*, *Clostridium*, *Rhodobacter*, *Sphingomonas*, and *Acidibacter* play important roles in iron or sulfate cycling in rice rhizosphere (Yu et al., 2017; Liu et al., 2018). The members of Proteobacteria, *Sphingomonas* and *Pseudomonas* were one of the two most abundant genera (Fig. 5). These genera are widespread and are usually involved in various soil processes. For example, *Sphingomonas* species encode multiple heavy metal oxidase genes that were found to be involved in heavy metal resistance (Altimira et al., 2012). Nilgiriwala et al. (2008) reported that *Sphingomonas* sp. BSAR-1, could potentially bioprecipitate Cd present in soils, because it expresses high amounts of alkaline phosphatase. Therefore, the presence of an OTU belonging to this genus provides further evidence of the capacity of these bacteria to survive in Cd-contaminated soils, making them potential candidates for bioremediation applications. Similarly, *Pseudomonas* strains are usually involved in nitrogen cycling, degradation of pollutants and can promote plant growth or increase plant health (Haas & Défago, 2005; Lalucat et al., 2006). Gomez-Balderas et al. (2014) demonstrated that Zn and Cd contamination could significantly decrease the relative abundance of *Pseudomonas* in rhizosphere soils, though they are highly resistant as Zn concentration elevated. Similarly, among the dominant genera, *Rhodobacter* and *Stenotrophomonas* are metal resistant and could show passive or active uptake of metals; *Steroidobacter* was found to closely relate to Cd availability (Hong et al., 2015). *Acidobacteria* possesses genes involved in the degradation of complex organic matter and the reduction of nitrate to nitrite (Rodrigues et al., 2014). *Cyanobacteria* plays a role in producing extracellular polymeric substances, mainly polysaccharide, which could adsorb heavy metals dispersed in the environment (Hong et al., 2015). In this study, we observed significant increases in the number of these genera with biofertilizer addition (Fig. 4), indicating that biofertilizer played a critical role in changing rhizosphere bacterial composition, and can be an effective amendment for Cd-contaminated soils.

3.3. Correlation between environmental parameters (pH and DTPA-Cd) and bacterial community structure

The Mantel test was performed to analyze the correlation between environmental variables and bacterial community structure in Cd-polluted soils. Both soil pH ($P < 0.01$), DTPA-Cd ($P < 0.05$), total P ($P < 0.05$) and organic carbon ($P < 0.05$) were identified as the most influential environmental factors driving the changes in community composition. The first bacterial community ordination axis was positively correlated with soil pH, organic carbon and negatively correlated with DTPA-Cd and total P (Fig. 5). Bacterial communities in the DY and ADY treatments in particular were positively correlated with pH; soil pH was elevated by >1 unit with the addition of DY and ADY (Fig. 2). It should be noted that the intracellular pH of most microorganisms is usually neutral within 1 pH unit, this increase in pH is sufficient to impose a stress and likely influences the bacterial diversity. Similarly, soil organic carbon as the carbon source of bacteria is also considered important. Moreover, the abundances of Bacteroidetes, Gemmatimonadetes, and Proteobacteria were positively correlated with pH and organic carbon (Fig. 5), and these phyla were also dominant in DY and ADY treatments (Fig. 4). Therefore, it is fair to say that these parameters played active roles in shaping the indigenous microbialbacterial communities. The microbialbacterial communities in the CK, AM and AAM soils were positively correlated with DTPA-Cd, and some Cd-coexistencee bacteria, such as Chloroflexi, Acidobacteria, Saccharibacteria and Thaumarchaeota, might have become dominant due to the presence of high Cd concentrations in rhizosphere soils. In addition, the solubility and availability of soil P was determined by the specific bacterial activities, while soil P concentration plays an important role in determining Cd phytotoxicity (Fig. 6).

4. Conclusions

The application of biofertilizer was effective in alleviating the phytotoxicity of Cd in soils by changing the composition of the rhizosphere bacterial community. The addition of biofertilizer to Cd-contaminated soils increased rice root and shoot biomass by up to 85.2% and 48.4%, respectively, and decreased the Cd concentration in roots by up to 72.2% compared to the CK. The positive effect of biofertilizer application could be due to an increase in soil pH and increased abundance of beneficial taxa such as Bacteroidetes, Gemmatimonadetes, and Proteobacteria in the rhizosphere, which stabilize soil Cd and decrease its bioavailability. Similar bacterial community alpha diversities were observed for the biofertilizer treatments and their autoclaved controls,

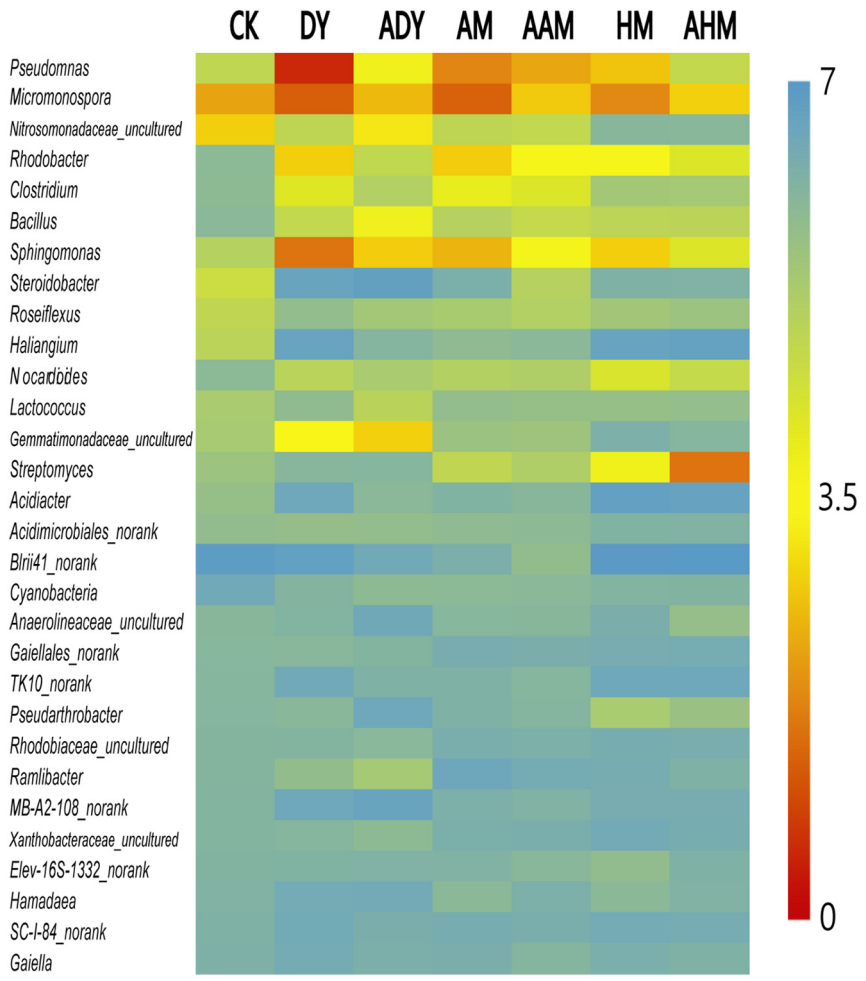

Fig. 5. Bacterial distribution of the top 30 abundant genera among the treated soils. The heatmap plot depicts the relative abundance (%) of each bacterial genera (variables clustering on the vertical axis) within each sample. The relative values for bacterial genera are indicated by color intensity with the legend indicated on the right side. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

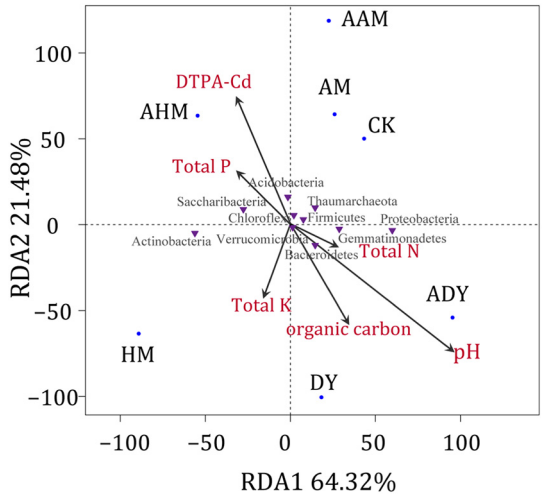

Fig. 6. Redundancy analysis (RDA) of the abundant bacterial phyla and soil properties in individual samples after applying various biofertilizers. Each value represents the mean of three replicates. $^{**}P < 0.01$, $^{*}P < 0.1$. Biofertilizer treatments: CK, soils without any biofertilizer; DY, soils with biofertilizer DY; ADY, soils with autoclaved DY; AM, soils with biofertilizer AM; AAM, soils with autoclaved AM; HM, soils with biofertilizer HM; AHM, soils with autoclaved HM.

suggesting that the change in soil physicochemical properties by biofertilizer addition might be the main factor affecting the rhizosphere bacterial community. In both autoclaved and unautoclaved biofertilizer treatments, the effectiveness in mitigating Cd phytotoxicity was dependent on the type of biofertilizer applied. This study introduced a new idea for development of effective strategies in remediation of Cd-contaminated soils and improvement of plant growth in agricultural land.

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