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RESEARCH ARTICLE

Influence of cadmium and copper mixtures to rhizosphere bacterial communities

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HIGHLIGHTS

- The relative abundance of rhizosphere soil bacteria has significantly positive correlation with BCF of Cd and Cu.
- Obvious variations of predominant species of bacterial communities in rhizosphere soil would emerge in the additions with different concentrations of Cd-Cu mixtures.
- In the additions with Cd and Cu, the mean of rhizosphere soil bacterial community diversity index was ranked as: Cu alone > Cd-Cu mixtures > Cd pollution.
- The PCA and PERMANOVA analysis showed that Cu may be the main factor changing the composition of rhizosphere soil bacteria.

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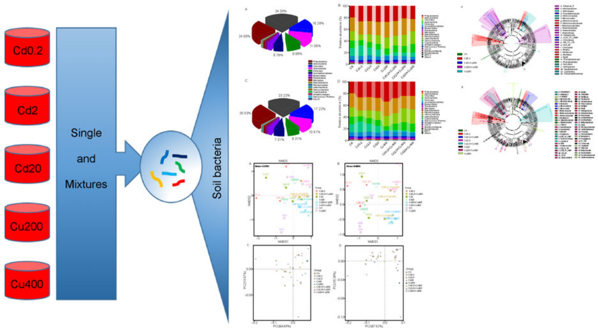
GRAPHICAL ABSTRACT



ABSTRACT

To study the effects of combined Cd and Cu pollution on rhizosphere bacterial community. High-throughput sequencing was used to examine the response of rhizosphere bacterial communities to heavy-metal stress under single and mixed pollution of cadmium (Cd) and copper (Cu). With additions of Cd and Cu, the mean diversity index of rhizosphere bacterial community was in the order Cu alone > Cd-Cu mixtures > Cd alone. In all Cd and Cu treatments, the dominant phyla were Proteobacteria, Actinobacteria, Chloroflexi and Acidobacteria. In the additions with different concentrations of Cd-Cu mixtures, LEfSe indicated that there were differences in the predominant species of rhizosphere bacterial communities. Some genera such as *Streptomyces* and *Microbacterium* belonging to Actinobacteria as biomarkers were significantly enriched in both control and treatments, while some genera such as *Pseudoxanthomonas* and *Rhodopseudomonas* belonging to Proteobacteria as biomarkers were observed to be enriched in the additions with single and mixture of Cd and Cu. According to the Nonmetric multidimensional scaling (NMDS) analysis, the structure of rhizosphere bacterial community was different between treatments and the CK. Principal Component Analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA) showed that there were significant differences among treatments ($p < 0.01$), and that the addition of Cu might be the primary factor affecting the composition of rhizosphere bacterial communities.

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1 Introduction

Because of rapid industrialization and anthropogenic activity, heavy metal pollution of soils has become a world-wide environmental issue (Anjum et al., 2015; Xie et al., 2018). In particular, the application of fertilizers and pesticides leads to the accumulation of metals such as cadmium (Cd) and copper (Cu) in soils (Roy and Mcdonald, 2015). With high accumulation in soil, Cu can be transferred through the food chain and cause serious damage to the human liver, endocrine system and kidneys (Tang et al., 2019; Guo et al., 2020). Cd pollution significantly adversely affects crops, and its accumulation in agricultural products may directly cause serious disease in soil (Shi et al., 2020).

To date, many studies have investigated the effect of combinations of heavy metal on vegetable (An et al., 2004; Feng et al., 2018; Xie et al., 2018; Greco et al., 2019). Hou et al. (2018) found that the synergistic reaction between Cd and Cu might exist, which lead to more harmful effects to vegetables. In addition, the concentration of Cd and Cu had an obvious impact on the occurrence of antagonism and synergism, and the research had found that the antagonistic reaction and synergistic reaction emerged with low concentrations and higher concentrations of Cd and Cu, respectively (Chu et al., 2006). Study had found that Cu and Cd are toxic to plants by competing with other essential elements to bind the active sites of enzymes (Feng et al., 2018). Sunda and Huntsman (1998) also found that metal coordination sites on the cell surface are never entirely specific for a single metal or nutrient, and competition for membrane transport sites and intracellular binding sites can occur for metals with similar ionic radii and coordination geometry. The rhizosphere is one of the most active interfaces in ecosystems because many soil biota are involved in complex biological and ecological processes (Tian et al., 2020). The beneficial interactions between plant roots, and soil microorganisms can improve the tolerance and accumulation ability of plants to metals. Soil microbial community-plant synergism can increase the nutrient supply and enhance the tolerance of plants to excessive toxic trace metals in contaminated soil (Kidd et al., 2009; Wenzel, 2009). Studies have also shown that different heavy metal resistant microorganisms affect plant growth and metal absorption through different mechanisms, including plant hormone production and soil environment improvement (Ju et al., 2019). At present, studies have found that the diversity and structure of rhizosphere microorganisms have significantly changed due to single heavy metal pollution (Gołebiewski et al., 2014; Schneider et al., 2017). But additional research is needed on the effects of combined Cd and Cu pollution on rhizosphere bacterial community.

Soil microorganisms are essential components in most terrestrial ecosystems due to their roles in mineral cycles and their living habitats around plant roots. Moreover, soil microbes can directly increase plant biomass production and tolerance to heavy metals (Hansda et al., 2017). However, heavy metal stress can affect the survival, population and diversity of microorganisms and can reduce growth in plants (Siripornadulsil and Siripornadulsil, 2013; Teste et al., 2017; Li et al., 2018). It is reported that the pollution of Cd and Cu would decrease microbial biomass and soil enzyme activities (Giller et al., 2009; Wyszkowska et al., 2013). Song et al. (2018a) found that soil microbial biomass decreased with increasing soil heavy metal (Cd, Cu) concentrations in both long-term and short-term experiments, and the interaction between soil physicochemical factors (pH, TN, TC) and heavy metals (Cd, Cu) played a major role in change in the bacterial community in long-term polluted soil. Vegetables play important roles in our daily diet as economic crops. Vegetables take up heavy metals through two ways, including absorbing them from the contaminated soils, as well as from deposits on parts of the vegetables exposed to the air from polluted environments (X. Liu et al., 2013). There have been many studies on the adverse effects of heavy metal to vegetable (Liu et al., 2013; Hou et al., 2018; Gong et al., 2019). However, few reports in the rhizosphere bacterial community of vegetable on combined pollution of Cd and Cu. Pakchoi is one of the most important worldwide leaf vegetables, and study has identified some genotypes of pakchoi with significantly different capacities of Cd uptake and accumulation under Cd exposure (Zhou et al., 2016). Therefore, the mechanisms by which heavy metal stress affects the diversity and structure of rhizosphere microbial communities of pakchoi and how those communities respond to the stresses of single and combined heavy metals need to be investigated further.

In the present study, a pot experiment with pakchoi and different concentrations of Cd and Cu alone and in mixtures was conducted. The specific aims of this study were the following: (1) to determine the effects of Cd-Cu mixtures on community diversity of rhizosphere bacteria and identify biomarkers taxa; and (2) to evaluate how Cd and Cu pollution alter the structure of rhizosphere bacterial communities using Nonmetric multidimensional scaling, Principal Component Analysis and permutational multivariate analysis of variance.

2 Materials and methods

2.1 Study area and test treatment

The pot experiment was conducted in a greenhouse situated in North-east Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, China (44°0′11″N, 125°23′58″E). The temperature in the greenhouse during the study ranged from 18 to 30°C, and the air humidity was between 55% and 80%. The clay loam soil used to cultivate pakchoi (provided by the seed breeding station in Dehui City, Jilin Province) was from farmland in Changchun, China. Soil physicochemical properties were the following: pH 7.45, organic matter 2.43%, total nitrogen 1.05 g kg^{-1}, total phosphorus 0.58 g kg^{-1}, and the contents of Cd and Cu were 0.09 and 19.64 mg kg^{-1}. After the soil was air-dried and passed through a 2 mm sieve, Cd was added as Cd $(NO_3)_2 \cdot 4(H_2O)$, and Cu was added as Cu $(NO_3)_2 \cdot 3(H_2O)$. To ensure normal growth of pakchoi, fertilizer was added as 0.75 g NH_4NO_3 and 0.44 g K_2SO_4 per kg soil. The specific experimental design details are shown in our previous study (Hou et al., 2018).

In this study, the Cu treatments included low (200 mg kg^{-1}) and high (400 mg kg^{-1}) additions, and the Cd treatments included low (0.2 mg kg^{-1}), medium (2.0 mg kg^{-1}), and high (20 mg kg^{-1}) additions. The treatments with mixtures of Cu and Cd were the following (all concentrations in mg kg^{-1}): (Cd 0.2 + Cu 200; Cd 2.0 + Cu 200; Cd 20 + Cu 200; Cd 0.2 + Cu 400; Cd 2.0 + Cu 400; Cd 20 + Cu 400 mg kg^{-1}). The control (CK) did not receive heavy metal additions, each treatment was prepared in hexaplicate. The pakchoi was harvested after 60 days, and using a toothbrush to collect rhizosphere soil within approximately 2 mm of the root surfaces. All collected soil samples were homogenized by sieving them through a 2-mm mesh sieve to remove stones and plant roots (Yang et al., 2019). One part was air-dried and stored at 4°C for physicochemical analyses, and the other part was stored at −80°C for high-throughput sequencing of the soil microbes. The main properties of the experimental soil are shown in Table 1.

2.2 Determination of the physicochemical properties and heavy metals of soil

Potentiometric (pHS-3B, Leici, Shanghai, China) was used to determine the pH value of the soil (soil-water ratio, 1:2.5), and its organic matter content was determined using the Walkley-Black method (Sun et al., 2013; Gan et al., 2017). Soil total nitrogen was measured by the micro-Kjeldahl method (Mani et al., 2012). Soil total phosphorus was measured using the ammonium-molybdate-spectrophotometric method (Guan et al., 2014).

To determine total Cd and Cu contents, soil samples were air-dried at ambient temperature, crushed and passed through a 0.149-mm nylon sieve and then digested in triplicate with a mixture of HF, $HClO_4$ and HNO_3 (Liu et al., 2013). The total concentration of soil Cd was determined by graphite furnace atomic absorption spectroscopy (GFAAS; AA-6300C, Shimadzu, Japan). The total concentration of soil Cu was determined by flame (air acetylene) atomic absorption spectroscopy (FAAS; AA-6300C, Shimadzu, Japan) (Chaudhuri et al., 2003; Xie et al., 2009; Zhao et al., 2019).

2.3 DNA extraction, Illumina MiSeq, and analysis of sequencing data

Total DNA was extracted from 0.5 g of rhizosphere soil using a DNA isolation kit (Omega, Norcross, GA, USA). The DNA was quantified using a NanoDrop NC 2000 (Thermo Scientific, Waltham, MA, USA), and DNA quality was assayed on 0.8% agarose gel via electrophoresis. Take the bacterial rRNA target sequence as the target, and the corresponding primers were designed according to the conserved regions in the sequence. Sample-specific bar coded sequences were added. Polymerase chain reaction was used amplify of the variable V3-V4 region of the bacterial 16S rRNA gene using the forward primer 338F (ACTCCTACGGGAGGCAGCA) and reverse primer 806R (GGACTACHVGGGTWTCTAAT). The PCR amplification program was the following: 95°C for 3 min; 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and 5 min of final elongation at 72°C. The PCR amplification used NEB's (USA) Q5 high-fidelity DNA polymerase, and the amplified products were detected by 2% agarose gel electrophoresis. The recovery uses AXYGEN's (USA) gel recovery kit and refers to the preliminary electrophoresis. The

Table 1 Main characteristics of the experimental soil (mean±SD).

Treatments	pH	Organic matter (%)	Cd (mg kg^{-1})	Cu (mg kg^{-1})
CK	7.45±0.06 ^{a}	2.43±0.12 ^{ab}	0.16±0.02 ^{c}	19.64±0.10 ^{c}
Cd0.2	7.42±0.29 ^{a}	2.39±0.09 ^{ab}	0.23±0.01 ^{c}	20.70±0.27 ^{c}
Cd2.0	7.40±0.09 ^{a}	2.08±0.14 ^{b}	2.32±0.54 ^{c}	19.70±0.91 ^{c}
Cd20	7.38±0.20 ^{a}	2.04±0.05 ^{b}	14.37±6.72 ^{b}	20.53±1.16 ^{c}
Cu200	7.36±0.06 ^{a}	2.47±0.23 ^{a}	0.05±0.01 ^{c}	289.48±130.13 ^{b}
Cu400	7.41±0.06 ^{a}	2.23±0.28 ^{ab}	0.08±0.02 ^{c}	236.08±45.94 ^{b}
Cd0.2 + Cu200	7.34±0.25 ^{a}	2.38±0.12 ^{ab}	0.25±0.05 ^{c}	193.82±29.85 ^{b}
Cd2.0 + Cu200	7.27±0.11 ^{a}	2.26±0.19 ^{ab}	0.91±0.10 ^{c}	123.52±20.34 ^{c}
Cd20 + Cu200	7.35±0.19 ^{a}	2.08±0.04 ^{b}	22.21±8.25 ^{a}	282.32±199.29 ^{b}
Cd0.2 + Cu400	7.54±0.04 ^{a}	2.12±0.11 ^{b}	0.25±0.02 ^{c}	379.31±79.94 ^{a}
Cd2.0 + Cu400	7.46±0.26 ^{a}	2.36±0.12 ^{ab}	1.10±0.19 ^{c}	194.65±146.40 ^{b}
Cd20 + Cu400	7.39±0.04 ^{a}	2.20±0.06 ^{b}	20.24±0.60 ^{a}	488.98±134.62 ^{a}

Note: Different letters in the upper right corner denote significant differences among treatments ($p < 0.05$).

PCR amplification and recovery products were subjected to fluorescence quantification, the fluorescence reagent was Quant-iT PicoGreen dsDNA Assay Kit, and the quantification instrument was Microplate reader (BioTek, FLx800) (Zhao et al., 2020).

The amplified products were sent to Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) for sequencing on the Illumina MiSeq high-throughput sequencing platform. Quantitative Insights into Microbial Ecology (QIIME) (v1.8.0, http://qiime.org/) was used to identify the sequences. USEARCH (v5.2.236, http://www.drive5.com/usearch/) was called by QIIME to check and eliminate the chimeric sequences (Huang et al., 2020). The results obtained by sequencing were filtered using QIIME (Lauber et al., 2008). To cluster operational taxonomic unit (OTUs), the Uclust method in QIIME was used to cluster high-quality sequences by 97% similarity and select the longest sequence of each class as the representative sequence (Edgar, 2010). The default parameters in QIIME were used, and the representative sequence of an OTU class was compared with the template sequences of a corresponding database (in this study, the Greengenes database: http://greengenes.secondgenome.com/) to obtain the taxonomic information. For different types of sequences, the template sequences of specific databases were used to OTUs (Zhao et al., 2019). To obtain the taxonomic information on each OUT, the BLAST method in QIIME was used to compare sequences in the database.

2.4 Statistical analysis

To assess species richness and diversity, the diversity indices Chao1, ACE, and Shannon were calculated using QIIME (Jiang et al., 2019). Following one-way ANOVA, a least significant difference test was used to test means for significant differences at $p < 0.05$. The analysis was performed in SPSS (V26.0). The LEfSe method was used to determine the features as biomarkers which can explain significant differences between different treatments. An LDA threshold score > 3.0 and a significance value of 0.05 were set (Wang et al., 2020). The PCA, NMDS, and other graphs were analyzed using R software (V3.6.3) and the Genescloud platform (https://www.genescloud.cn/). QIIME was used to perform PERMANOVA based on Bray-Curtis similarity indices, and 999 permutation tests to determine whether the differences between groups were statistically significant. The 16S rRNA data have been submitted to the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession number PRJNA721125 (Registration date: 11-Apr-2021).

3 Results

3.1 Abundance and structural diversity of rhizosphere bacterial communities

A total of 76104 OTUs were identified following Illumina MiSeq sequencing. Venn diagram in Fig. 1 show the numbers of shared and unique OTUs in different treatments. As shown in Fig. 1A, 5595 OTUs were shared among the five groups. The number of unique OTUs in the treatment of Cd 0.2 was similar to that in CK, whereas only two unique OTUs were identified in the treatment of Cd 20. Analogously, there were more unique OTUs numbers in the mixed treatment of Cd 0.2 + Cu 200 than in the Cd 20 + Cu 200. As shown in Fig. 1B, the number of

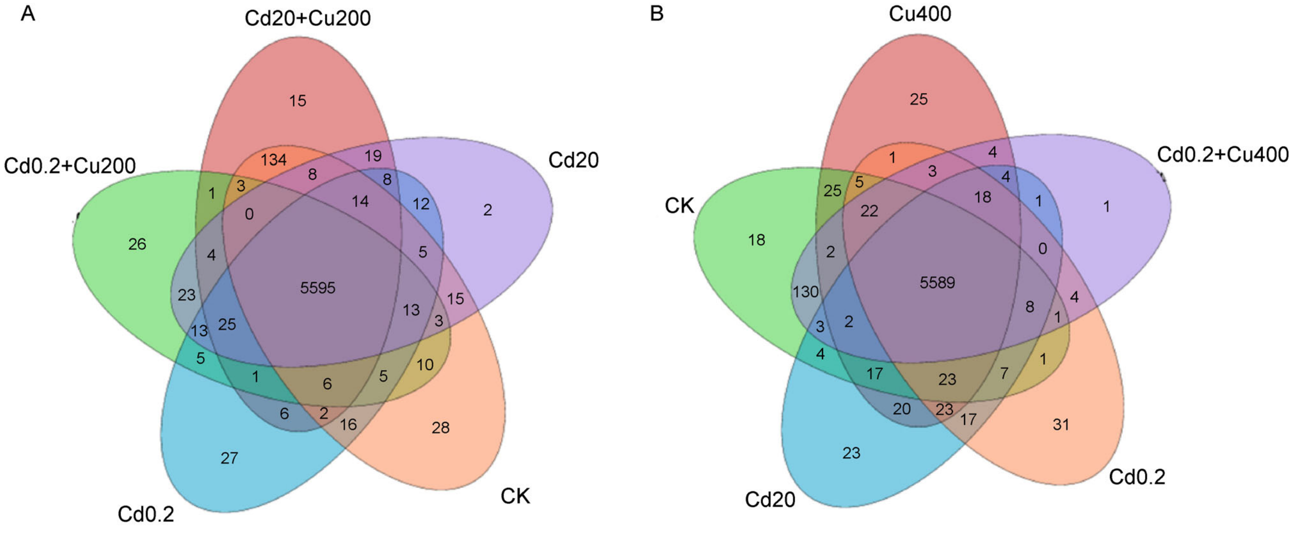

Fig. 1 Venn diagram of OTU numbers in rhizosphere soil samples (A: CK, treatment of Cd (0.2, 20 mg kg^{-1}) + Cu (0, 200 mg kg^{-1}); B: CK, treatment of Cd (0.2, 20 mg kg^{-1}), Cu 400 mg kg^{-1} and Cd 0.2 mg kg^{-1} + Cu 400 mg kg^{-1}. CK in A and B were the same control group. Different colors represent different treatments, the numbers of overlapping sections represent the number of OTUs common in multiple samples, and the numbers of non-overlapping sections represent the number of OTUs unique to the corresponding sample).

unique OTUs in the treatments with only Cd or Cu was similar to that in CK. The treatment of Cd 0.2 + Cu 400 had the smallest number unique OTUs, with only one. Notably, in the treatments with different Cd-Cu mixtures (Fig. 1A and 1B), the number of shared OTUs among all treatments was much greater than the number of unique OTUs in each treatment.

Table 2 lists the richnesss and diversity indices of rhizosphere bacterial communities in treatments with varying amounts of Cd and Cu. The richness and diversity indices (Chao1, ACE and Shannon indices) were not significantly different in the different treatments ($p > 0.05$). Thus, only the average value was compared in each treatment. In the heavy metal treatments, the Shannon index changed slightly compared with that in CK. In the treatments with only Cd or Cu, the Chao1 and ACE richness indice decreased compared with those in the control. However, in the treatments with mixtures of Cd and Cu, the Chao1 and ACE indices were similar to those in the control. In addition, the bacterial community diversity indices decreased (after the application of only Cd or Cu alone (except in Cu 200, in which the decrease was not significant; Table 2). In the comparisons between single Cd treatments and those with Cd at the same level mixed with the low level of Cu, the mean bacterial community richness indices were higher in the mixtures than with Cd alone: Cd 0.2 + Cu 200 > Cd 0.2; Cd 2.0 + Cu 200 > Cd 2.0; Cd 20 + Cu 200 > Cd 20. However, in the same type of comparisons with Cu, the mean bacterial community richness index in the combined treatments was slightly smaller.

3.2 Effects of heavy metals on dominant phyla of bacterial communities

Figure 2A and 2C show the relative abundances of phyla of bacteria in all treatments. In the treatments with only Cd or mixtures of Cd (0.2, 2.0, 20 mg kg^{-1}) and a low level of Cu (200 mg kg^{-1}) (Fig. 2A), there were six dominant phyla: Proteobacteria (24.9%), Actinobacteria (24.1%), Chloroflexi (16.3%), Acidobacteria (11.1%), Firmicutes (9.89%) and Gemmatimonadetes (6.79%). In treatments with only Cd or mixtures of Cd (0.2, 2.0, 20 mg kg^{-1}) and a high level of Cu (400 mg kg^{-1}) (Fig. 2C), the dominant bacteria phyla were also Proteobacteria (26.5%), Actinobacteria (23.2%), Chloroflexi (17.2%), Acidobacteria (10.6%), Firmicutes (8.31%) and Gemmatimonadetes (7.31%).

Figure 2B and 2D show the abundance of phyla in individual treatments. The lowest abundance of Proteobacteria (19.9%) was in the control, whereas the highest abundance (33.9%) was in the Cu 400 mg kg^{-1} treatment. Notably, although Firmicutes was a dominant phylum in the control (14.0%) and Cd only treatments (11.5% to 18.2%), it only accounted for 2.91% in Cu 200 mg kg^{-1} treatment (Fig. 2B) and 1.23% in Cu 400 mg kg^{-1} treatment (Fig. 2D). In addition, in the mixed treatments with Cd (0.2, 2.0, 20 mg kg^{-1}) and Cu (400 mg kg^{-1}), the relative abundance of Firmcutes accounted for only 1.23% to 3.13% (Fig. 2D).

3.3 Biomarkers of soil bacterial community under heavy metal mixtures pollution

In this study, the specific bacteria from phylum to genus within different treatments were evaluated by conducting biomarker analysis using the LEfSe method (Wang et al., 2020). As shown in Fig. 3A, 30-two bacterial clades showed significant differences with a LDA threshold of 3.0. Similarly, as shown in Fig. 3B, 60-two bacterial clades showed significantly differences with a LDA threshold of 3.0.

Table 2 Bacterial community diversity index (sequence similarity 97%) level (mean±SD).

Treatments	No. OTUs	Chao1	ACE	Shannon
CK	3386±237 ^{a}	4096±241 ^{ab}	4491±292 ^{a}	10.31±0.25 ^{ab}
Cd0.2	3003±420 ^{a}	3782±373 ^{ab}	4012±546 ^{a}	9.92±0.59 ^{b}
Cd2.0	3142±390 ^{a}	3756±453 ^{ab}	4101±529 ^{a}	10.15±0.46 ^{ab}
Cd20	3048±543 ^{a}	3523±934 ^{b}	3789±578 ^{a}	10.28±0.23 ^{ab}
Cu200	3363±159 ^{a}	4245±135 ^{ab}	4556±160 ^{a}	10.54±0.05 ^{a}
Cu400	2958±327 ^{a}	3595±554 ^{ab}	3928±657 ^{a}	10.08±0.24 ^{ab}
Cd0.2 + Cu200	3252±96 ^{a}	4237±56 ^{ab}	4532±77 ^{a}	10.36±0.12 ^{ab}
Cd2.0 + Cu200	3340±137 ^{a}	4307±288 ^{a}	4683±308 ^{a}	10.31±0.25 ^{ab}
Cd20 + Cu200	3149±415 ^{a}	3756±795 ^{ab}	4095±901 ^{a}	10.36±0.22 ^{ab}
Cd0.2 + Cu400	3201±169 ^{a}	4095±324 ^{ab}	4461±265 ^{a}	10.24±0.27 ^{ab}
Cd2.0 + Cu400	3407±42 ^{a}	4093±82 ^{ab}	4493±100 ^{a}	10.54±0.51 ^{ab}
Cd20 + Cu400	3224±34 ^{a}	3972±185 ^{ab}	4354±116 ^{a}	10.34±0.10 ^{ab}

Note: Each number in the table represents the average value of three measurements; Different letters in the upper right corner denote significant differences among treatments ($p < 0.05$).

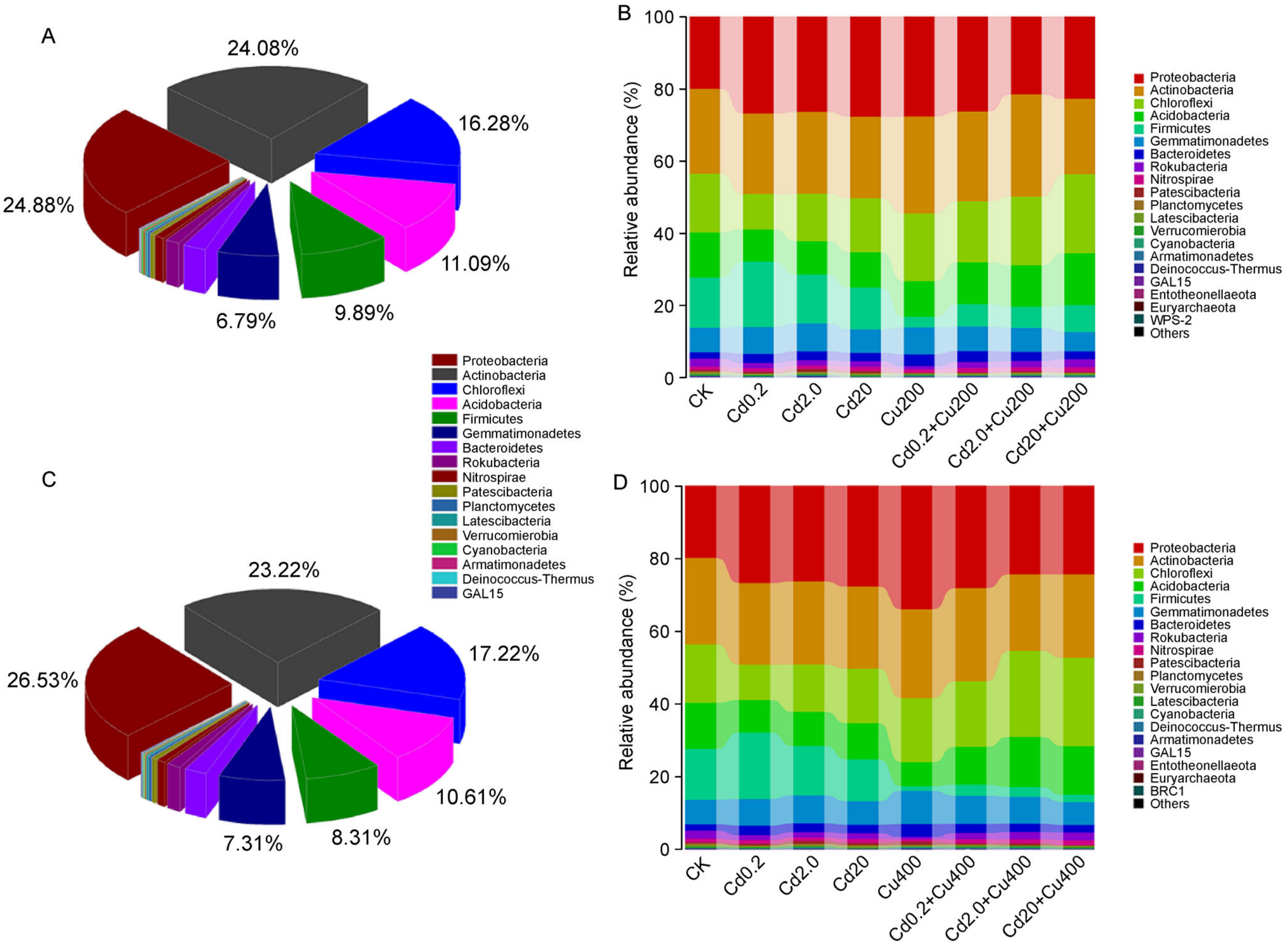

Fig. 2 Relative abundance of bacteria at phylum level. (A, C: Percentage of bacterial relative abundance of all treatments; B, D: Relative abundance of bacteria under different treatment. A, B: Treatment of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 200 mg kg^{-1}); C, D: Treatment of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 400 mg kg^{-1})).

At the genus level, of these, *Streptomyces* belonging to Actinobacteria as biomarkers was significantly enriched in the control ($p < 0.05$). In the additions with only Cd or Cu (Fig. 3A and 3B), because of significant enrichment ($p < 0.05$), the following were identified as biomarkers: *Aquabacterium* (belonging to Proteobacteria), *Bacillus* (belonging to Firmicutes) and *Micromonospora* (belonging to Actinobacteria) in Cd 0.2 treatment; *Fictibacillus* (belonging to Firmicutes) in the Cd 2 treatment ($p < 0.05$); *Paenibacillus* (belonging to Firmicutes), *Roseomonas* and *Variovorax* (belonging to Proteobacteria) in the Cd 20 treatment; *Arthrobacter* (belonging to Actinobacteria), *Ferrovibrio, Sphingopyxis, Cupriavidus* and *Pseudoxanthomonas* (belonging to Proteobacteria) in the Cu 200 treatment; *Microbacterium* (belonging to Actinobacteria), *Sphingopyxis, Cupriavidus, Rhodanobacter* and *Pseudoxanthomonas* (belonging to Proteobacteria) in the Cu 400 treatment. In the additions with Cd and Cu mixtures (Fig. 3A and 3B), because of significant enrichment ($p < 0.05$), the following were identified as biomarkers: *Arthrobacter* (belonging to Actinobacteria) in the treatment of Cd 0.2 + Cu 400; *Pseudarthrobacter* (belonging to Actinobacteria) in the treatment of Cd 2 + Cu 200 ($p < 0.05$); *Vicinamibacter* (belonging to Acidobacteria), *Afipia* (belonging to Proteobacteria), in the treatment of Cd 2 + Cu 400; *Microbacterium* (belonging to Actinobacteria), in the treatment of Cd 20 + Cu 200; *Hydrogenispora* and *Methlobacillus* (belonging to Firmicutes), *Rhodopseudomonas, Ferrovibrio* and *Altererythrobacter* (belonging to Proteobacteria) in the treatment of Cd 20 + Cu 400. These results indicated the predominant taxa of rhizosphere bacterial communities were affected by additions with different concentrations of Cd-Cu mixtures.

3.4 Beta diversity of the bacterial communities

The unweighted UniFrac NMDS based on OTUs and relative abundance showed that the single and mixed Cd and Cu treatments affected rhizosphere bacterial communities (Fig. 4A and 4B). In the treatments with Cd (0, 0.2, 2.0, 20 mg kg^{-1}) + Cu (200 mg kg^{-1}), the distance among the mixed treatments was smaller than that among other

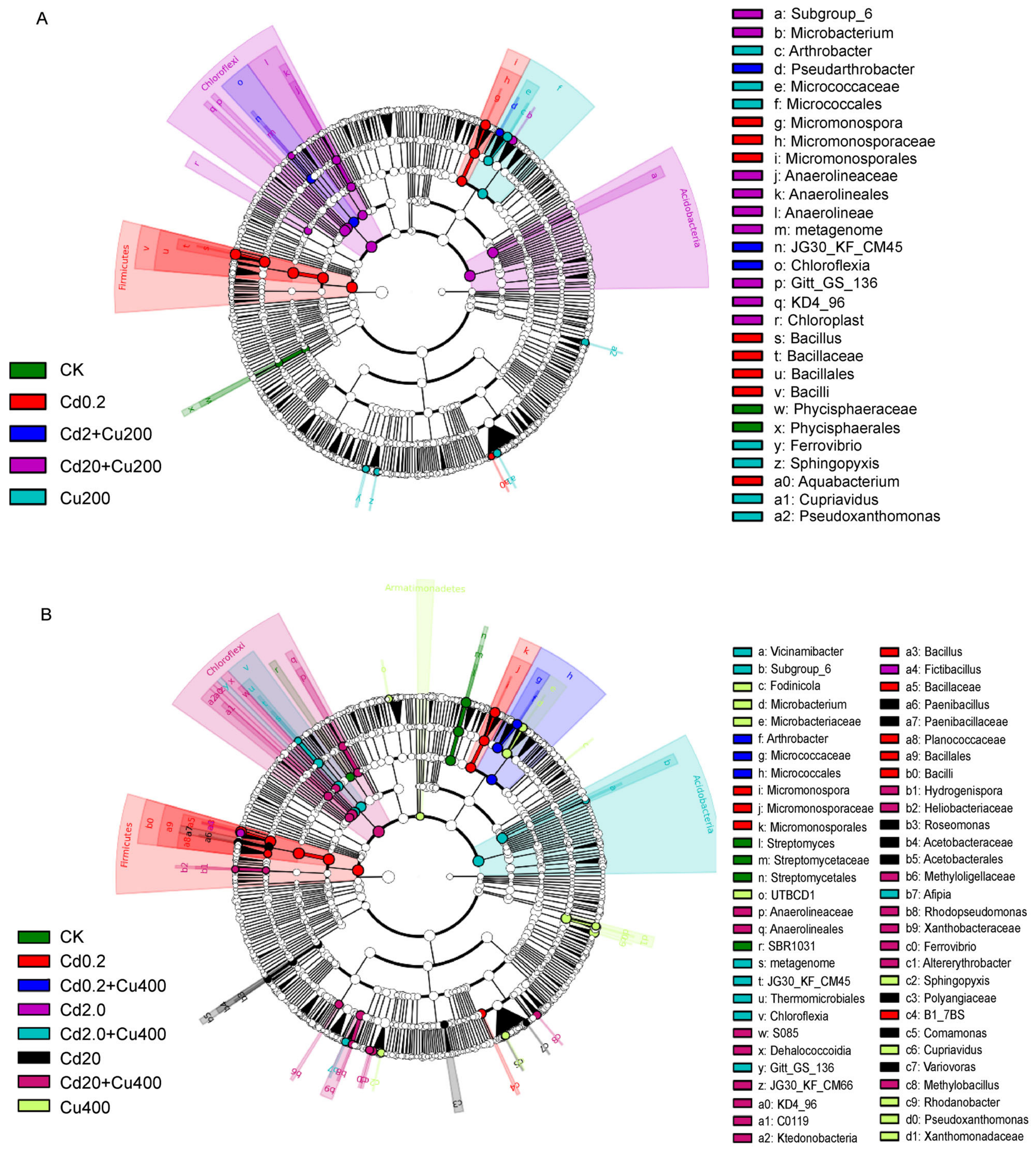

Fig. 3 Taxonomic dendrogram of biomarker bacteria (A: Treatments of CK, Cd0.2 and Cd (0, 2, 20 mg kg^{-1}) + Cu (200 mg kg^{-1}); B: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu 0, 400 mg kg^{-1})). The circle diameter is proportional to the taxon abundance, and each ring represents a taxonomic level in order from the center to the periphery: phylum, class, order, family, and genus.

treatments. The distance between CK and heavy metal treatments was relatively large, and therefore, they could not be considered a cluster (Fig. 4A). Thus, compared with the CK, the additions of Cd and Cu changed the abundance of soil bacteria and shaped the structure of rhizosphere bacterial communities. The results in Cd (0, 0.2, 2.0, 20 mg kg^{-1}) + Cu (400 mg kg^{-1}) treatment (Fig. 4B) were similar to those with Cu at 200 mg kg^{-1} (Fig. 4A). However, the difference among mixed treatments was greater.

PCA was used to assess the degree to which Cd and Cu

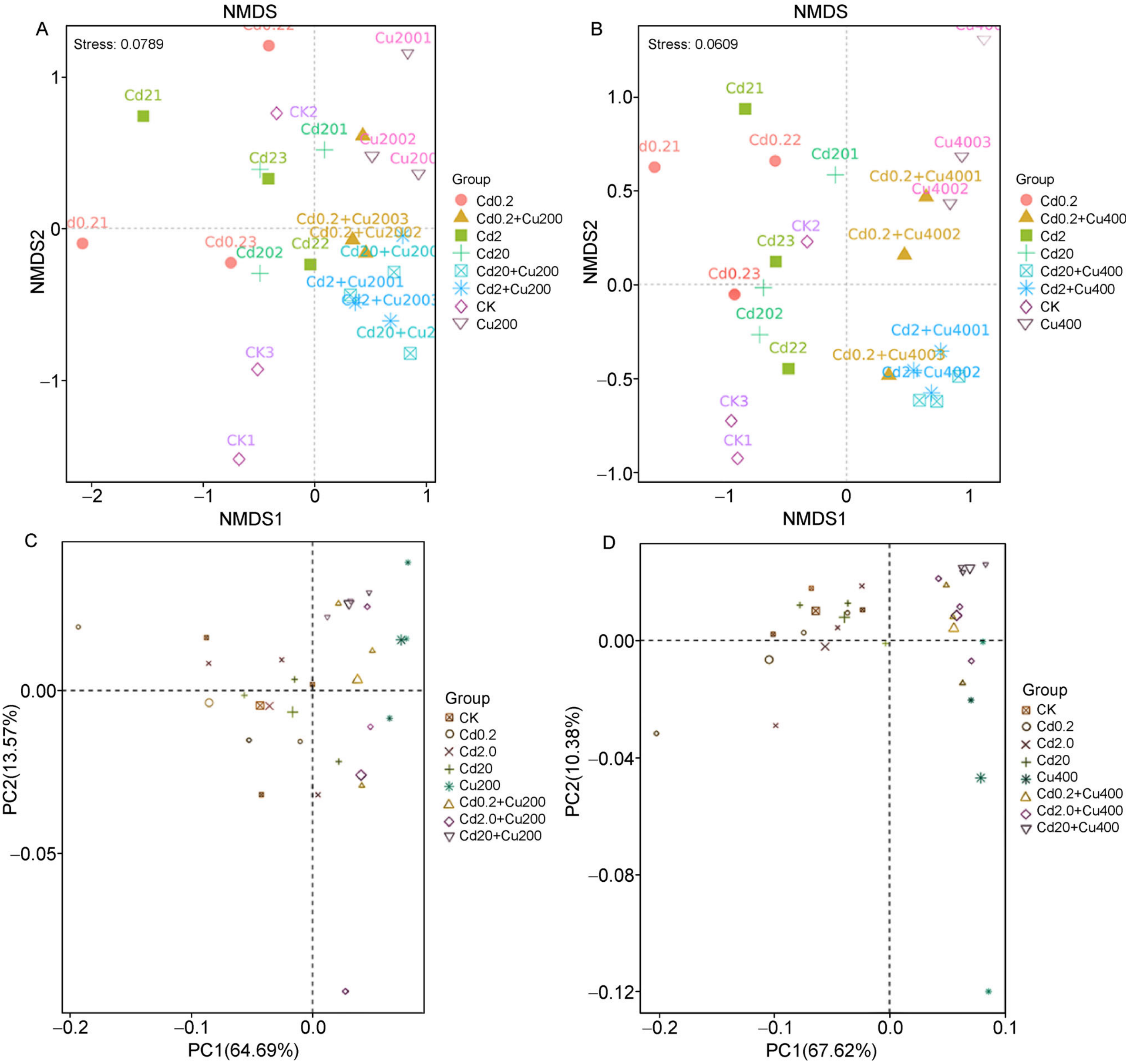

Fig. 4 Beta diversity of rhizosphere soil bacterial community. (A, B: Ordination plot of treatments according to unweighted UniFrac NMDS based on relative abundance bacteria taxa at OTU level in rhizosphere soil; C, D: PCA based on the composition structure of rhizosphere soil microbial community at the genus level. A, C: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 200 mg kg^{-1}); B, D: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 400 mg kg^{-1})).

affected the richness and diversity of rhizosphere bacteria (Fig. 4C and 4D). Figure 4C shows the PCA of single or mixtures of Cd (0.2, 2.0, 20 mg kg^{-1}) and Cu (200 mg kg^{-1}) treatments. Two principal components with eigenvalues greater than 1 were extracted, which explained 78.26% of the changes in the composition of the rhizosphere bacterial community. On the basis of the communalities obtained in the analysis, the use of only two components to represent the variables was adequate. The distance between all single Cd additions and CK was smaller than that among the other treatments (Fig. 4C), which indicated that the composition of rhizosphere bacteria was similar between Cd only treatment and CK. According to the PCA, with the addition of 200 mg kg^{-1} Cu, the treatments of 200 mg kg^{-1} Cu and the three mixed groups clustered together. Simultaneously, the distance increased between those treatments and CK (Fig. 4C). The PERMANOVA results (Table 3) confirmed those obtained in the PCA, with significant differences detected among treatments (p = 0.002). Figure 4D shows the PCA of single or mixtures of Cd and 400 mg kg^{-1} Cu. The results were similar to that of Fig. 4C. PERMANOVA results (Table 3) also detected significant differences among

Table 3 *p*-values and F-values generated from one-way PERMANOVA of Bray-Crutis similarity indices among treatments.

Treatments	Degree of freedom	*p*-value	F-value
A	3	0.861	0.6853
B	3	0.001**	3.6329
C	7	0.002**	2.6894
D	7	0.002**	2.6660
E	7	0.001**	3.3950
F	7	0.002**	1.9063
G	7	0.001**	3.0055

Note: A: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}); B: Treatments of Cu (0, 200, 400 mg kg^{-1}); C: Treatments of Cd (0, 0.2 mg kg^{-1}) + Cu (0, 200, 400 mg kg^{-1}); D: Treatments of Cd (0, 2 mg kg^{-1}) + Cu (0, 200,400 mg kg^{-1}); E: Treatments of Cd (0, 20 mg kg^{-1}) + Cu (0, 200, 400 mg kg^{-1}); F: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 200 mg kg^{-1}); G: Treatments of Cd (0, 0.2, 2, 20 mg kg^{-1}) + Cu (0, 400 mg kg^{-1}). **Correlation is significant at 0.01 levels.

treatments (p = 0.001). Remarkably, according to the PERMANOVA, there was no significant difference among treatments with the addition of only Cd (p = 0.861; Table 3).

4 Discussion

4.1 Effects of Cd and Cu additions on the diversity and structure of rhizosphere bacterial community

In this study, with all possible shared and unique relationships among the five treatments of samples, more than 30 partitions observed in the Venn diagram. The addition of other treatments would overcrowd the diagram and sharply increase the complexity and greatly reduce the readability. To avoid the problem of too many treatments, most studies select specific treatments for comparison in Venn diagram (He et al., 2017; Yu et al., 2020). Therefore, in this study, the treatments of Cd (0.2, 20 mg kg^{-1}) + Cu (200 mg kg^{-1}) were selected to examine the effects of high and low concentrations of Cd combined with Cu (Fig. 1A); To study the effects of Cd or Cu alone, Cd low (0.2 mg kg^{-1}) and high (20 mg kg^{-1}) concentrations and the Cu (400 mg kg^{-1}) high concentration were selected (Fig. 1B). According to He et al. (2017), shared OTUs can potentially delineate the core rhizosphere microbiome of a soil sample set. Therefore, in this study, the results in the Venn diagram might be explained by bacterial adaptation to soils with different concentrations of Cd-Cu mixtures contamination.

Richness and diversity indices, such as Chao1, ACE and *Shannon* indices, are used to evaluate the abundance, and evenness of biological communities (Sánchez-Moreno and Navas, 2007; Yuebing et al., 2020). In this study, the Shannon index in different treatments was similar, indicating that the evenness in bacterial communities was similar among treatments (Table 2). The results are consistent with those of previous studies, and Will et al. (2010) and Nacke et al. (2011) found that with the additions of lead and zinc, the species diversity of bacterial communities in polluted soils was similar to that in unpolluted soils. In the additions with single and mixtures of Cd and Cu, the richness indice, Chao1 and ACE, showed different degrees of change (Table 2). Exposure to Cd or Cu decreased the richness of rhizosphere bacterial communities. In the treatments with Cd-Cu mixtures, an antagonistic relation between Cd and Cu might have occurred, which reduced the toxic effects of heavy metals on soil bacteria. Sun et al. (2020) also found that exposure to heavy metal pollution affects the abundance, structure, diversity and uniformity of bacterial communities. In addition, Chodak et al. (2013) found that the diversity of soil bacteria (Chao1) was negatively affected by heavy metal pollution. With combinations of heavy metals, there can be antagonistic or synergistic effects that are related to their concentrations (Lanier et al., 2019).

According to the analysis of bacterial community beta diversity, compared with the CK, the addition of Cu might be the primary factor that altered the composition of rhizosphere bacterial communities (Fig. 4, Table 3). High concentrations of Cu can affect the numbers, biomass, activity and composition of soil microbial communities (Wang et al., 2007; Fernández-Calviño et al., 2012; Song et al., 2018). Xu et al. (2015) studied the effects of TiO_2 and CuO nanoparticles on the community structure of soil microbes in flooded paddy soil, the results showed that CuO nanoparticles was the major factor that reduced the composition and diversity of soil microbial community, and the authors speculated that the bioavailability of CuO nanoparticles increased the toxicity to microbes. The CuO nanoparticles may have also indirectly affected soil microbes by changing nutrient bioavailability. The formation of reactive oxygen species and the subsequent oxidative stress are the likely mechanisms causing changes in microbial community structure at high concentrations of Cu, which also leads to the oxidation of proteins, DNA and lipids and ultimately cell death (Li et al., 2014). However, this does not mean that Cd has no effect on the structural changes of rhizosphere microorganisms in soil, though competition and antagonism of Cd and Cu in soil, Cu and Cd play a synergistic role in the change of bacterial community. Exchangeable Cd has strong mobility in surface layer (0 to 15 cm) and cannot be degraded by microorganisms (Zia et al., 2018). Cd also indirectly affects the richness and structure of rhizosphere microbial communities by affecting soil respiration and metabolism (Vig et al., 2003).

4.2 Effects of Cd and Cu additions on the abundance of dominant bacteria

The relative abundance of Preteobacteria is positively correlated with Cu and zinc pollution (Feris et al., 2003; Yan et al., 2020). According to Feris et al. (2003), the Proteobacteria have the strongest tolerance to heavy metals, and can even increase in abundance at certain concentrations. The results in this study are consistent with those of previous research. In the treatments with heavy metals, there were changes in rhizosphere soil bacterial communities, and the relative abundance of Proteobacteria increased (Fig. 2). The phylum Proteobacteria is one of the most abundant and widespread phyla of soil bacteria (Narendrula-Kotha and Nkongolo, 2017), It is also the dominant phylum of bacteria in heavy metal contaminated soil (Idris et al., 2004; Song et al., 2018b). The predominant bacterial phyla observed in this study are similar to those in observed in other heavy metal contaminated soils (Liu et al., 2015; Zhang et al., 2016).

In this study, Firmicutes (7.20%–28.5%) was the dominant phylum in the rhizosphere with the addition of Cd only, however, it was not with the addition of Cu (Fig. 2). Similarly, Ferreira et al. (2015) found that Cu inhibits the growth thermophilic bacterial communities in the Firmicutes. Therefore, the results of this study and those of Ferreira et al. (2015) suggest that members of the Firmicutes are sensitive to the addition of Cu, and may be inhibited by high concentration. Cu is a micronutrient required for multiple metal-dependent enzymes but at higher concentrations it presents toxicity (Ferreira et al., 2015). Study has found that excess copper avidly binds to many biomolecules such as proteins, lipids, and nucleic acids, regardless of its valence state (Solioz et al., 2010). But compared to other toxic metals such as Cd and lead, Cu is an essential trace nutrient. So bacteria evolved tight copper homeostatic control mechanisms, involving copper binding and transport and the regulation of gene expression by copper (Solioz et al., 2010).

4.3 Identification of biomarkers in rhizosphere bacterial communities in single and mixture additions with of Cd and Cu

The organisms that passed the pairwise Wilcoxon test were defined as biomarkers (Jiao et al., 2018). LEfSe analysis (linear discriminative analysis (LDA) coupled with effect size measurement for significant differences) is effective method for identifying biomarkers (Zhang et al., 2018; Wang et al., 2020). Heavy metals have important effects on the structure of rhizosphere and endophytic microbial communities (Lin et al., 2021). Study has showed that the richness of Actinobacteria was found corresponding to the variation of soil physicochemical characters (Yu et al., 2020). The results in this study are consistent with those of previous studies (Zhang et al., 2016; Yu et al., 2020). The Actinobacteria genera *Streptomyces, Micromonospora, Arthrobacter* and *Microbacterium* were significantly enriched in contaminated or uncontaminated soil (Fig. 3A and 3B). The results demonstrate the important role of the phylum Actinobacteria in providing biomarker in bacterial communities (Zhou et al., 2011; Yu et al., 2020). In addition, Proteobacteria are the most tolerant to heavy metals, and their relative abundance can even slightly increase at certain metal concentration (Feris et al., 2003; Lin et al., 2021). In this paper, compared with CK, in the treatments with heavy metal additions, some genera of Proteobacteria were enriched to varying degrees, including *Micromonospora, Roseomonas, Pseudoxanthomonas* and *Rhodopseudomonas* (Fig. 3A and 3B). Those genera are heavy metal resistant and ecologically beneficial and therefore, could promote plant growth and facilitate phytoremediation (Liu et al., 2018; Lin et al., 2021). Our results were consistent with those of Lin et al. (2021) who also found that some genera of Proteobacteria are significantly enriched in different degrees under heavy metal stress, including *Kaistobacter, Lysobacter, Cellvibrio* and *Pseudomonas*. Besides, some genera in the Firmicutes, such as *Bacillus* were enriched in the additions with Cd only, while there was no significantly difference in the additions with Cu only (Fig. 3A and 3B). We speculated the reason may be that Firmcutes is sensitive to the addition of Cu, and the high concentration of Cu may inhibit the Firmcutes (Ferreira et al., 2015). Study has found that *Bacillus* could promote plant growth (Lin et al., 2021), and in this study, in the addition with single Cd, *Bacillus* was enriched. This indicated that rhizosphere bacterial community may protect plants from heavy metals by enriching *Bacillus*. In addition to the enrichment of genera in the Actinobacteria and Proteobacteria in the additions with Cd-Cu mixtures, some genera in the Firmicutes were also enriched, including *Hydrogenispora* and *Methlobacillus* (Fig. 3A and 3B). Therefore, at different concentrations, there might be antagonistic interaction between Cd and Cu that alleviated the inhibitory effect of Cu on the Firmicutes. The reason may be that heavy metal coordination sites on the cell surface are not specific for a single metal, and competition for membrane transport sites and intracellular binding sites can occur for metals with similar ionic radii and coordination geometry (Sunda and Huntsman, 1998). The emergence of antagonism and synergism between metals at different concentrations has been observed previously (He et al., 2004; Wang et al., 2015; Cai et al., 2019).

5 Conclusion

The effects of the addition of either Cd or Cu, or mixtures of the two, on the abundance and structure of rhizosphere bacterial communities were examined in this study. The results showed that in the addition of Cd (20 mg kg^{-1}) + Cu (400 mg kg^{-1}) mixture, the dominant phyla of bacteria were Proteobacteria, Actinobacteria, Chloroflexi and Acidobacteria. In the additions with single and mixture of Cd and Cu, compared with CK, the genera *Pseudoxanthomonas* and *Rhodopseudomonas* in the Proteobacteria as biomarkers were enriched to varying degrees. In addition, it is worthy that in the additions with Cd-Cu mixtures, beside the bacteria belonging to Proteobacteria, the genera *Hydrogenispora* and *Methlobacillus* in the Firmicutes were also identified as biomarkers because of significant enrichment. The PCA and PERMANOVA showed there were significant differences among treatments ($p < 0.01$), and that the addition of Cu might be the primary factor affecting the composition of rhizosphere bacterial communities. Cu may affect bacterial communities because it not only binds to enzyme molecules, but also to enzyme substrate complexes, and decrease of enzyme activity, heavy metal pollution will affect the absorption of heavy metals by plants. The result indicated that rhizosphere soil microorganisms participate in the mechanism of promoting plant growth, the number of rhizosphere soil microorganisms is not only related to the types and concentration of heavy metals, but also to their tolerance. At the same time, the rhizosphere soil microbial abundance was also related to the plants planted. The results of this study have increased understanding of the mechanisms of Cd and Cu toxicity to rhizosphere microorganisms, and thus can provide a new theoretical basis for the remediation of metal contaminated soil and plant management.

Authorship contribution

Qirui An: Performed the most of the experiments, writing-original draft. Yuyang Li: Performed the part of the experiments. Na Zheng: Funding acquisition, writing–review and editing, and supervision. Jincai Ma and Chunmei Zhao: Formal analysis, and supervision. Sujing Wang and Siyu Sun: Software. Pengyang Li: Formal analysis. Xiaoqian Li and Shengnan Hou: Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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