COMPARATIVE EFFECT OF CHINESE MILK VETCH AND WHEAT ON BACTERIAL COMMUNITY STRUCTURE AND DIVERSITY OF RHIZOSPHERE SOIL IN TONGLING COPPER TAILINGS

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Abstract. Mine pollution causes a serious impact on the ecological environment surrounding the mine site. It provides great theoretical value to explore the effects of planting different plants on soil characteristics and bacterial community structure around the mine site for vegetation restoration. In this study, we mainly assessed the rhizosphere bacterial community structure and diversity of wheat and Chinese milk vetch seedlings in copper tailings by 16S rRNA high-throughput sequencing and also soil properties. The results showed that planting wheat and Chinese milk vetch seedlings led to a decrease in soil pH, but both could increase the content of total nitrogen, organic matter, and available nitrogen in the rhizosphere soil, as well as enhance the activities of urease and alkaline protease. In addition, a total of 1,133,919 active sequences and 13,199 operational taxonomic units were obtained. The microorganisms in the substrate samples belong to 34 phyla and 81 classes, *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the top three dominant group. In general, Chinese milk vetch or wheat enriched the diversity of bacterial community in rhizosphere soil, the results showed that the number of rhizosphere bacteria in Chinese milk vetch was higher than that in wheat. The structure of the rhizosphere bacterial community of wheat and Chinese milk vetch seedling was similar, but the bacterial abundance was different. Mantel tests showed that Chao index significantly correlated with ammonium nitrogen. These results not only expand our understanding of rhizosphere bacteriomes but also provide theoretical basis for improving the soil quality of industrial and crop yields.

Keywords: *soil properties, high-throughput sequencing, rhizosphere bacteria, community structure and diversity, soil quality*

Introduction

Copper (Cu) has been applied in electronics, construction, transportation, and information technology industries. With the rapid development of social economy and industrialization, the demand for Cu resources has continued to increase. Heavy metal pollution of soil is a significant environmental problem, and the mining of Cu resources has become increasingly intensive. In addition, various types of waste rocks and tailings also caused serious pollution with heavy metals (HMs) to the original ecosystem around the site. Soil pollution with HMs is generally a common problem with a negative potential impact on agriculture and human health (Adrees et al., 2015; Chen et al., 2017; Janczak-Pieniazek et al., 2023; Mourad et al., 2021; Shao et al., 2010). So much more low-cost, environmentally friendly remediation strategies are urgently required. Phytoremediation has an advantage in cost and environmental suitability, which allows plant species to extract pollutants from the environment, to immobilize or to turn them into harmless species (Helmisaari et al., 2007). To fulfill the demand for protecting the ecological environment, heavy metal tolerant plants have been investigated on a large scale. Liu et al. found that kentucky bluegrass (*Poa pratensis* Linn.), jersey cudweed (*Gnaphalium affine* D. Don) and Chinese brake fern (*Pteris vittata* L.) were Pb-tolerant plants; and that

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horseweed (*Conyza canadensis* (L.) Cronq.), kentucky bluegrass (*Poa pratensis* Linn.) and jersey cudweed (*Gnaphalium affine* D. Don) were Cd-tolerant plants, and that radix phytolaccae (*Phytolacca acinosa* Roxb) had a great tolerance to Mn (Liu et al., 2006), Crofton weed (*Ageratina adenophora*) is a known hyperaccumulator of chromium (Cr) and lead (Pb) (Dai et al., 2020). Presently, oldham groundsel (*Senccio oldhamianus*), curlytop knotweed (*Polygonum lapathifolium*), sticky snakeroot (*Ageratina Adenophora*), sourgrass (*Paspalum conjugatum*) were introduced to restore mines, but alien species may eventually pose a threat to local biodiversity, these studies tended to focus on vigorous weeds, invasive alien species, and were not applicable to farm land remediation because of some limitations including low efficiency, high cost in China, in addition, the treatment of heavy metals after absorption by plants has become a problem. Therefore, it is of great significance to screen the ideal different crop species for more economical and efficient phytoremediation in mining areas.

Fast-growing local plant species are preferable because their adaptation to the soil and climate conditions favors their self-propagation. As one of the most broadly cultivated and consumed food crops worldwide, wheat (*Triticum aestivum* L.) has a certain tolerance to copper tailings stress (Babeanu et al., 2010; Karaman et al., 2013; Liu et al., 2001). For instance, Kutuluka variety was identified as metal-resistant wheat variety and recommended for cultivation on soils polluted with Cu and Pb (Alybayeva et al., 2019). Yang et al. screened 229 rice (*Oryza sativa* L.) varieties for Cu tolerance or sensitivity, and gave possible Pb tolerance mechanism in the tolerant rice varieties (Yang et al., 2000), in addition, Chinese milk vetch (*Astragalus sinicus* L.) plays an important role in maintaining nitrogen cycle in farmland ecosystem because of the innate ability to fix nitrogen. It has been widely used as green fertilizer among rice to increase soil nutrients and rice productivity in southern China. Additionally, Wang et al. found that Chinese milk vetch could maintain a stable physiological state and strong nitrogen fixation ability in Cu-contaminated soil (Wang et al., 2023). So it may be feasible to restore heavy metal contaminated soil with wheat or Chinese milk vetch.

However, Karatas et al. reported that the increased the HMs (Cu, Cr, Mn, Ni, Pb and Zn) content in the root, body and grain of wheat was due to wastewater irrigation (Karatas et al., 2006). Liu et al. observed the lowest value of Pb for the winter wheat grain (0.99-1.01 $mg{\cdot}kg^{-1}$) that was close to concentrations of Pb in the wheat grain approach the Chinese national food guideline limit (1.0 $mg{\cdot}kg^{-1}$). These toxic metals could accumulate in the body through the food chain and become health risks to living organisms when they exceed safety limit. The role of Pb, cadmium (Cd), Cr, arsenic (As), and Hg in carcinogenesis has been investigated (Parida and Patel, 2023). For instance, Zhang et al. reported that Cd can induce liver damage (Zhang et al., 2022a), some researchers found the presence of Cr, iron (Fe) and Cu in renal cell carcinoma samples, and thought HMs was related to oncogenic pathway of renal cell carcinoma (Sa et al., 2016). So it is essential for us to select or breed crop genotypes with high heavy metal resistance, high accumulation of HMS in straw but low-HMS concentration in edible part.

Generally, rhizosphere microbiota are regarded as the second genome of plants (Berendsen et al., 2012), controlling the activation, uptake and utilization of nutrients in plants, rhizosphere bacterial diversity and composition in different plant grown in mine tailing (Jia et al., 2020; Lin et al., 2022). Soil bacteria are particularly important in ecological function and biological stability, so soil bacterial communities are the indicator of soil quality (Hermans et al., 2020). Microbial biomass was negatively affected by the elevated metal Cu/Zn levels (Wang et al., 2007). Microbial parameters were negatively correlated with the metals, with inhibition increasing with the bioavailability of the metals in century old landfill soil (Bhattacharyya et al., 2008). Zampieri et al. found that HMs influenced the population size, diversity, and metabolic activity of bacteria (Zampieri et al., 2016), Cu has a harmful effect on rhizobiales (Alengebawy et al., 2021), numbers of soil bacteria were significantly inhibited (Wang et al., 2018). However, Santo et al. isolated copper-resistant bacteria (Santo et al., 2010), Silva et al. screened bacterial tolerance to Cr, silver (Ag) and mercury (Hg) (Silva et al., 2012), further, heavy metal–resistant bacteria was isolated and performed test resistance against Cr, nickel (Ni), Cu, cobalt (Co), Cd and Hg (Jing et al., 2012). With multiple plant growth-promoting traits, as resistant bacteria alleviated as toxicity in rice (Etesami et al., 2023), and it suggested they could promote rice growth. More and more plant growth-promoting bacteria (PGPB) were isolated and cultured (Das et al., 2023; Fan et al., 2018), Han et al. isolated eighteen functional strains with - heavy metal resistance and growth-promoting characteristics, and verified that functional bacterial strains also reduced heavy metal accumulation in wheat (Han et al., 2019). However, the effect of phytoremediation through crop species on rhizosphere soil microorganisms in copper tailings reclamation area is still unclear.

At present, few studies highlights on the differences in bacterial community structure and diversity within rhizosphere soil between leguminous and non-leguminous plants. In this study, we conducted a pot experiment with two types of plants, and collected rhizosphere soil samples from wheat and Chinese milk vetch at seedling stage in a Cu tailing dam area. Our objectives were to: (1) investigate the difference and similarity of physicochemical properties and enzymatic activities between rhizosphere and bulk soils; (2) investigate the difference and similarity of rhizosphere bacterial communities between Leguminous Chinese milk vetch and Gramineous wheat; (3) investigate and identify the key environmental factors correlated with diversity indexes. This study can provide scientific knowledge for the development of agroforestry ecosystems to improve the soil quality of industrial and mining reclamation.

Materials and methods

Experimental design

The pot experiment was conducted in outdoor, Anhui normal University, Wuhu, China, during 2022 using a wheat cultivars, namely Yangmai-2 ((*Triticum aestivum* L. cv. Yangmai 2), and Chinese milk vetch (*Astragalus sinicus* L.) gifted from Wuhu Qingyijiang seed industry Co., wheat seeds gifted from the Crop Institute of Anhui Academy of Agricultural Sciences, both was popular crop cultivated in the area, our experiment was conducted from October 2022 to December 2022.

The potting soil was collected from the top 20 cm layer of soil in the farmland adjacent to the Laoyaling tailing pond in Anhui province (30°54′N, 117°53′E). The pot experiment was divided into three groups: non-rhizosphere soil was used as the control group (CK), wheat rhizosphere soil group (W) and Chinese milk vetch rhizosphere soil group (CMV), with three replicates for each treatment. Each pot was 100× 30 × 23 cm in size, and contained 25 kg of soil. Three plots in each group were chosen for determination of surface soil properties.

Seeds were firstly surface sterilized with 0.2% ammonium molybdate for 10 min, rinsed thoroughly with sterilized distilled water, then soaked overnight in sterile water at room temperature, and germinated in sterilized moist quartz sand. At two leaf stage, plant seedlings were selected for uniformity and transplanted into pots.

In December 2022, rhizosphere soil was sampled as follows: 6 plants were randomly selected by the “S” sampling method for the determination of soil physicochemical indexes, soil microorganism diversity.

Rhizosphere soil sample collection

The soil samples were collected at 11 weeks after planting, respectively. After shaking off the loosely adhering soil, the tightly adhering soil (rhizosphere soil) was collected using a brush. After removal of the roots, the remaining soil (excluding the top 2 cm) was mixed and considered to be rhizosphere soil. All soil samples were sieved to < 2 mm to remove visible roots and sub-samples were stored at -20°C for DNA extraction, and another part of rhizosphere soil was naturally air-dried and screened for soil physicochemical properties determination, and fresh soil was used for enzyme activity determination.

Physicochemical properties and enzymatic activities

The physicochemical properties including pH, total nitrogen(TN), available nitrogen(AN), nitrate nitrogen(NO_3^--N), ammonium nitrogen($NH4^+$-N), organic matter(OM), the enzymatic activities of soil urease and alkaline protease (ALP) were detected, respectively. Soil pH was determined in leaching solution (soil: water = 1:2.5) by a PHS-3C pH meter (Shanghai Youke Instrument Co., Ltd., China); soil OM was determined by oxidation with potassium dichromate; the total nitrogen content was determined by Kjeldahl method; AN was determined by the alkaline diffusion method; NH_4^+-Nwas determined by Nessler’s reagent colorimetry; NO_3^-–N was determined by Griess-Ilosvay method.

The fresh soil samples from the surface layer (0-20 cm) were air-dried and sifted through a 50 mesh. The activity of urease and alkaline protease studied. Urease activity was measured by the phenol sodium hypochlorite colorimetric method, and the soil alkaline protease (ALP) activities were determined with soil alkaline protease (S-ALP) activity detection kits (BC0885, Solarbio, Beijing, China). Each sample was taken in triplicate.

DNA extraction and PCR amplification

DNA was extracted using the FastDNA R Spin Kit (Tiangen, China), following the manufacturer’s instructions, after each extraction, the concentration and purity of the extracted DNA was measured by a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, United States). Thereafter, DNA samples were sent to Kaitai Biotechnology Co. (Hangzhou, China) for V3-V4 regions amplification. V3-V4 regions of the 16S rRNA gene were amplified using two universal primers B341F (CCTACGGGNGGCWGCAG) and B785R (GACTACHVGGGTATCTAATCC) Using T100™ Thermal Cycler (Bio-RAD Inc. Shanghai, China); The first round PCR reaction was performed in a total volume of 25 μL containing 0.25 μL of both primers (25 μM), 12.5 μL of 2-fold Phanta Max Master Mix (Vazyme Biotech Co., Ltd. Nanjing, China) and 10 ng template DNA. The first round PCR procedure included an initial denaturation at 95°C for 3 min, 25 cycles of denaturing (30 s at 95°C), annealing (30 s at 55°C), and extension (45 s at 72°C), and a final extension at 72°C for 5 min. PCR products were purified with AGENCOURT®AMPURE® XP Kit (Beckman Coulter, Inc.) according to the manufacturer's instructions. The purified PCR product 2.5 μL was used as the second round template, other components and procedures of the reaction system were the same as those of the first round PCR. The second round PCR procedure included an initial denaturation at 95°C for 3 min, 8 cycles of denaturing (30 s at 95°C), annealing (30 s at 55°C), and extension (30 s at 72°C), and a final extension at 72°C for 5 min.

Illumina MiSeq sequencing

The amplified PCR products were examined by using 2% agarose gel electrophoresis. Subsequently, DNA libraries of 350 bp fragments were prepared, after quality inspection, the libraries were mixed and denatured, finally, DNA libraries were sequenced on an Illumina Novaseq 6000 sequencing platform by paired-end sequencing (Hangzhou Kaitai Biotechnology Co., Ltd., Hangzhou, China).

Processing of sequencing data

To obtain high-quality sequencing data, the raw reads were quality-filtered and processed by using cutadapt (Whelan and Surette, 2017) and VSEARCH (Rognes et al., 2016). Subsequently, Operational Taxonomic Units (OTU) were used for clustering of effective sequences with similarity greater than 97.00%, Using the mothur software's "classify.seqs" command, identify the species information that has the highest similarity to the OTU sequence and a confidence level exceeding 80%, to obtain OTU taxonomic classification, the mothur software was applied to identify the species information that had the highest similarity to the OTU sequence and a confidence level exceeding 80%(Schloss et al., 2009). Metabolism function of bacterial community was analyzed by PICRUSt (Douglas et al., 2020).

Data statistics and analysis

The characteristics of the soil were analyzed by an ANOVA, with the treatment as the main factors, each of the three blocks was considered a replication. Once a significant treatment effect was indicated ($p < 0.05$), the abundance accounting Shannon and Simpson diversity indices were computed using OTUs by software R. To investigate significant species difference between different groups, a non-parametric analysis by Metastats and the LEFse function were carried out (Segata et al., 2011; White et al., 2009). We employed R software (2.15.3) to create the dilution curve. We produced a heatmap, and utilized the non-weighted paired average hierarchical clustering method to draw the phylogenetic tree with the R language tools (Yu et al., 2017). We also conducted Mantel tests (Dixon, 2003) using the Spearman method with 1000 permutations to determine the correlation of different diversity indexes diversity indexes with the physicochemical properties and enzyme activities.

Results

Soil properties

It has been reported that soil physicochemical properties can be influenced by different vegetation restoration types. In this study, the pH, total nitrogen, organic matter, total nitrogen, organic matter, available nitrogen, urease activity and alkaline protease activity were measured, as shown in *Table 1*, compared with the control group, the cultivation of wheat and Chinese milk vetch resulted in changes in soil property parameters, although the trends of these changes were not entirely consistent. Specifically, compared with the control group, planting wheat significantly decreased pH and levels of NO_3^--N and NH_4^+-N, while significantly increased AN content and ALP activity; it also promoted the increase of TN, OM content and urease activity, but the effect was not significant. On the other hand, planting Chinese milk vetch also led to a significant decrease in pH and NH_4^+-N levels, but a significant increase in the content of OM, AN and NO_3^--N; similar to W group, TN level and Soil urease activity had no significant change, but they showed an increasing trend. Furthermore, the CMV group exhibited significantly higher levels of OM as well as NO_3^--N and NH_4^+-N when compared with W group, it was impossible that Chinese milk vetch increased the content of OM as well as NO_3^--N and NH_4^+-N to maintain growth and development by nitrogen fixation without fertilization. However, there were no significant differences in TN and urease activity among the three groups, we supposed that it was produced mainly due to the low room temperature and the low seedling age of the plants. The result suggested that both wheat and Chinese milk vetch seedlings partially affected the physical and chemical properties and soil enzymes of the rhizosphere soil.

Table 1. *Comparison of soil physicochemical properties and enzymatic activities*

Treatment	pH	TN	OM	AN	NO_3^--N	$NH4^+$-N	Urease	ALP
CK	8.20±0.02A	1.14±0.08A	20.51±0.38B	77.36±3.71B	10.09±1.29B	3.73±0.32A	0.07±0.003A	0.31±0.013B
W	8.04±0.01B	1.23±0.02A	21.16±0.18B	91.06±4.54A	5.88±0.31C	1.81±0.25C	0.08±0.002A	0.35±0.016A
CMV	8.05±0.01B	1.18±0.01A	22.69±0.52A	92.04±2.22A	13.00±0.95A	3.01±0.25B	0.08±0.003A	0.33±0.004AB

Different capital letters represent significant differences

Richness of soil bacteria

The 16S rRNA sequencing was performed to investigate the changes of microbial communities. A total of 1,133,919 effective sequences were obtained through high-throughput sequencing of soil samples from three groups, the sample sequencing coverage was above 98.5%. The Shannon index of bacteria in the samples tended to be stable (*Fig. 1*), indicating that the diversity of bacteria in the samples did not change with the increase of the amount of soil bacteria sequenced. The sparse curve (*Fig. 2*) tends to reach the saturation plateau, indicating that the microbiome of the sample is large enough.

Through sequencing, total 13,199 OTUs were obtained through clustering. As shown in *Figure 3*, the number of OUTs in the CK, W and CMV group, was 84,204, 92,114 and 98,869, respectively. The number of shared OUTs in three groups was 5952, accounting for 70.69%, 64.60% and 60.03% of the total rhizosphere OTUs in CK, W and CMV group, respectively; CK and W groups shared 6699 OTUs, CK and CMV groups shared 6616 OTUs, and W and CMV groups shared 367,940 OTUs, there were unique 1057 OTUs in CK group, 1526 OTUs in W group, and 2265 OUTs in CMV group, indicating that wheat and Chinese milk vetch enriched the bacterial communities, and the effect of Chinese milk vetch was more obvious.

The Chao 1 and Shannon index values indicate the richness and diversity of the microbial community, respectively. As shown in *Figure 4*, compared with the CK group, CMV and W groups exhibited an increase in indexes containing Chao, Shannon, Ace, and Simpson, but none of these reached statistical significance, it demonstrated that the cultivation of Chinese milk vetch and wheat enhanced soil bacterial richness. Furthermore, the Chao and Ace indices were higher in the CMV group compared with the W group, indicating a greater community richness in CMV group than in the W group.

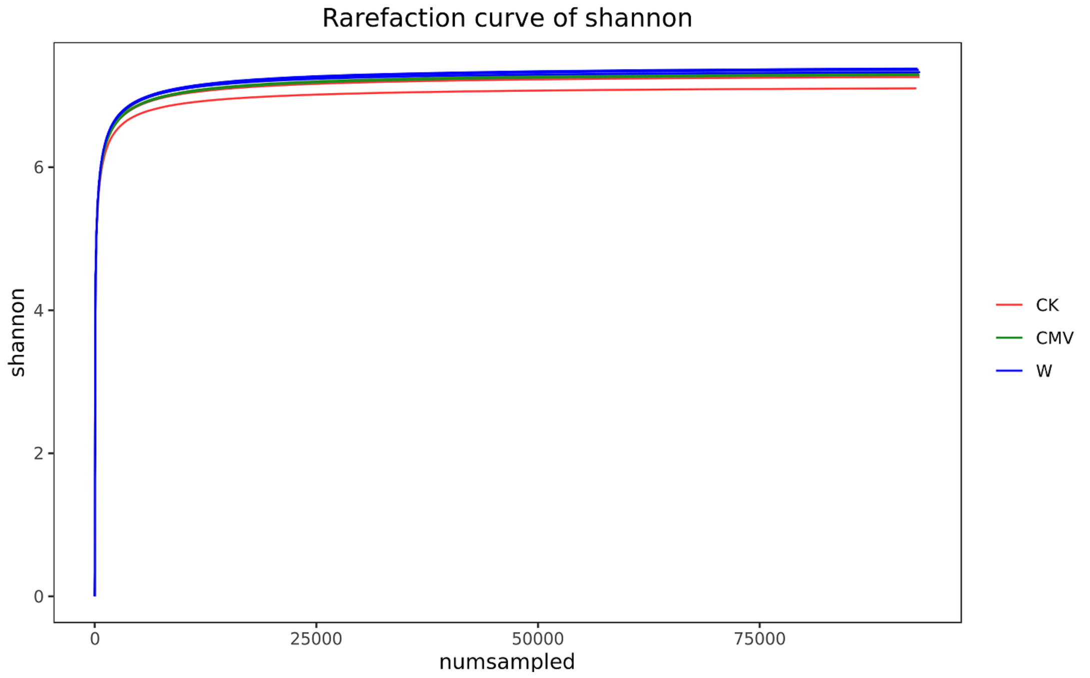

Figure 1. *Shannon index of bacterial community composition in the rhizosphere soils*

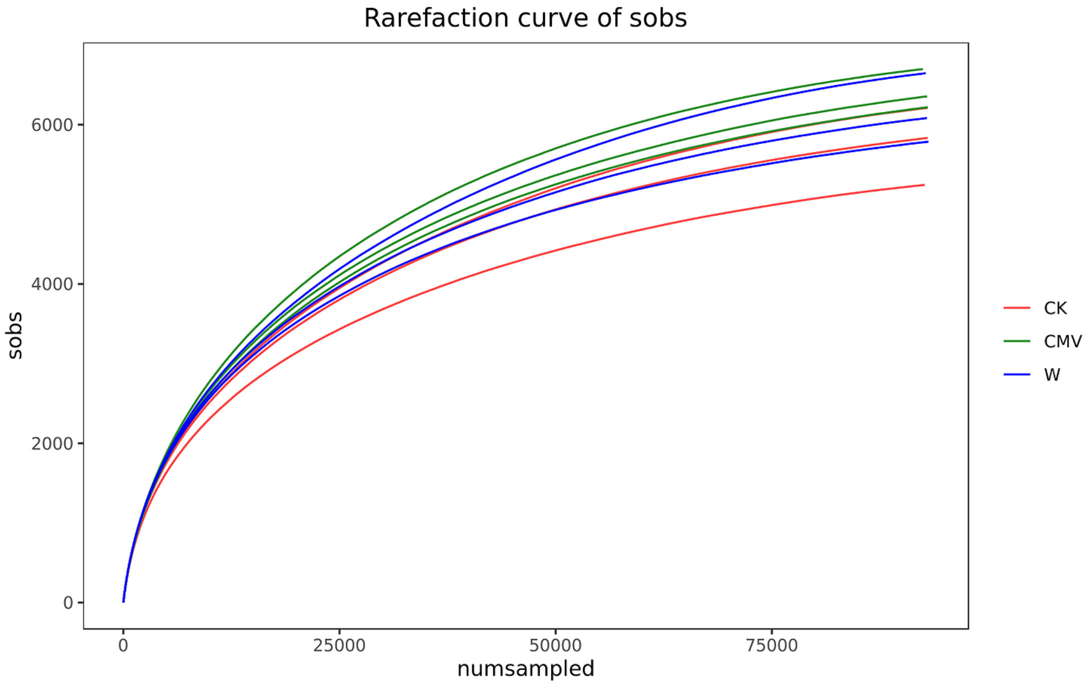

Figure 2. *Rarefaction curves of bacterial community composition in the rhizosphere soils*

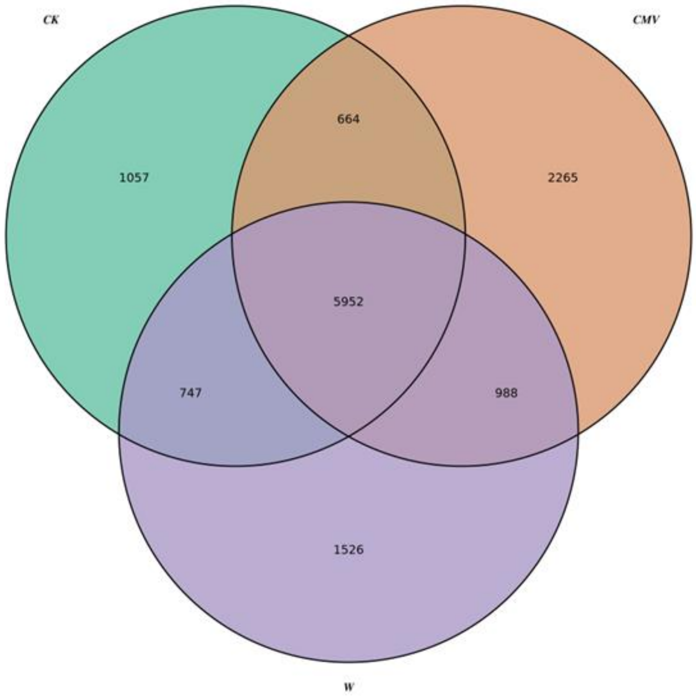

Figure 3. *Venn diagram of soil sample characteristics*

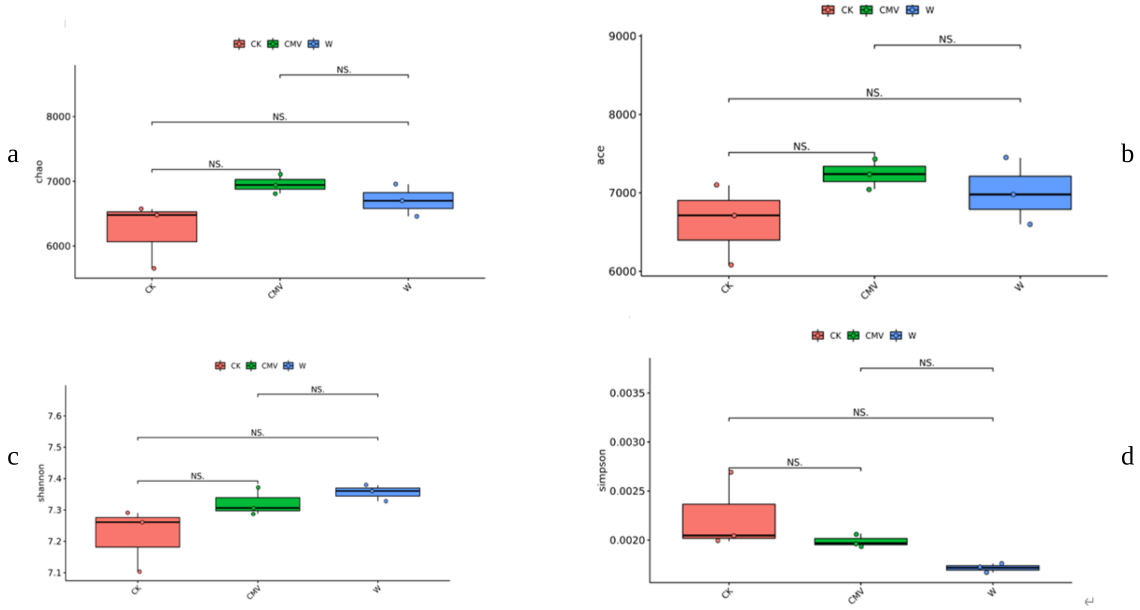

Figure 4. *Boxplot of Alpha diversity index differences between groups. (a) Chao1, (b) ACE, (c) Shannon, (d) Simpson*

Comparison of bacterial community structures

To address whether phylogenetic bacterial community structure in the mine area was associated with rhizosphere from three treatment groups, an assessment analysis was carried out. A total of 32 phyla, 110 classes, 255 orders, 454 families, and 896 genera were isolated from the nine samples. It can be seen from the Venn diagram at phyla level (*Fig. 5*) that the rhizosphere soil of CK, W and CMV group contain 30, 28 and 31 gates, respectively, among which the common number of phylas among the three groups is 90.00%, 96.42% and 87.10% of rhizosphere phyla s in control group (CK), wheat group (W group) and CMV group, respectively. The results showed that Chinese milk vetch enriched the composition of rhizosphere bacteria.

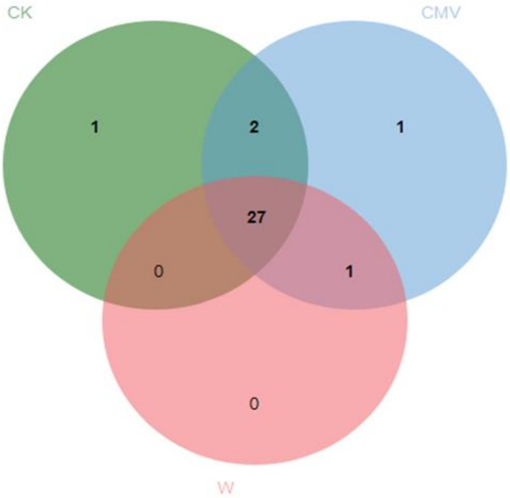

Figure 5. *Venn diagram of soil sample characteristics at the phyla level*

For bacteria, the phyla with the more than 1% relative abundance in the soil under all treatments were *Proteobacteria* (29.30%-31.63%), *Acidobacteria* (28.45%-31.11%), Actinobacteria (9.52%-11.96%), *Gemmatimonadetes* (7.50%-9.21%), *Chloroflexi* (4.75%-5.10%), *Bacteroidetes* (4.07%-6.45%), *Planctomycetes* (2.51%-2.87%), *Verrucomicrobia* (2.00%-2.52%), and others (the abundance of less than 1%). The composition of the dominant phyla was nearly same, the abundance of each phylum in the three groups was different but similar (*Fig. 6a*). It suggested that there were no significant differences in the dominant bacterial phyla among the groups. But *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Gemmatimonadetes* were relatively abundant, the abundances of *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia* were relatively low. Compared with the control group, the abundance of *Gemmatimonadetes* decreased in W group and CMV group.

At the class level (*Fig. 6b*), *Subgroup_*6 (17.43%-14.14%), *Gammaproteobacteria* (15.63%-12.74%), *alphaproteobacteria* (13.15%-10.77%) and Blastocatellia_(Subgroup_4) (9.46%-8.01%) were the dominant bacteria in the 3 groups with relative high abundance. The other bacteriae with relative abundance from high to low were *Gemmatimonadetes* (7.96%-6.67%), Bacteroidia (6.33%-3.96%), *Deltaproteobacteria* (5.38%-4.56%), *Actinobacteria* (5.36%-4.43%), *Thermoleophilia* (3.23% - 20.5%), *Acidimicrobiia* (3.09%-1.84%) *Verrucomicrobiae* (2.52%-2.00%), *NC10* (1.68%-1.03%), *Planctomycetacia (*1.62%-1.34%) *Holophagae* (1.59%-1.21%), *Acidobacteriia* (1.50%-1.32%), *Chloroflexia* (1.19%-1.02%) , in addition, *Anaerolineae* (1.26% - 2.12%) was present in the rhizosphere soil but not bulk soil (supplement material). It suggested that there were no significant differences in the major bacterial class among the groups under the same condition.

Fig 6a

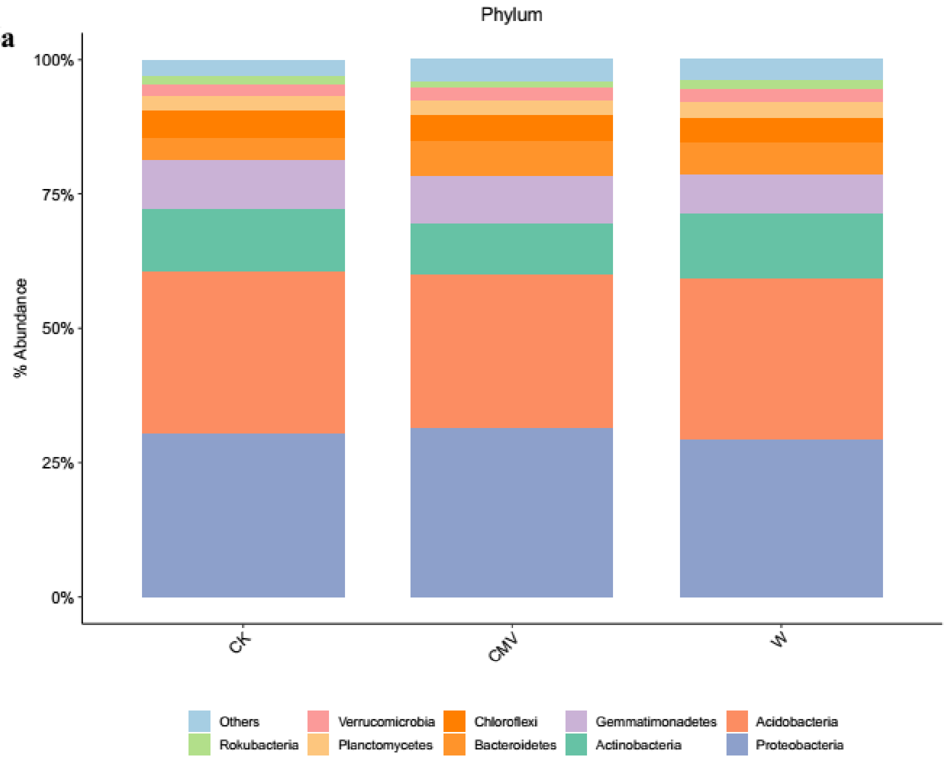

Fig 6b

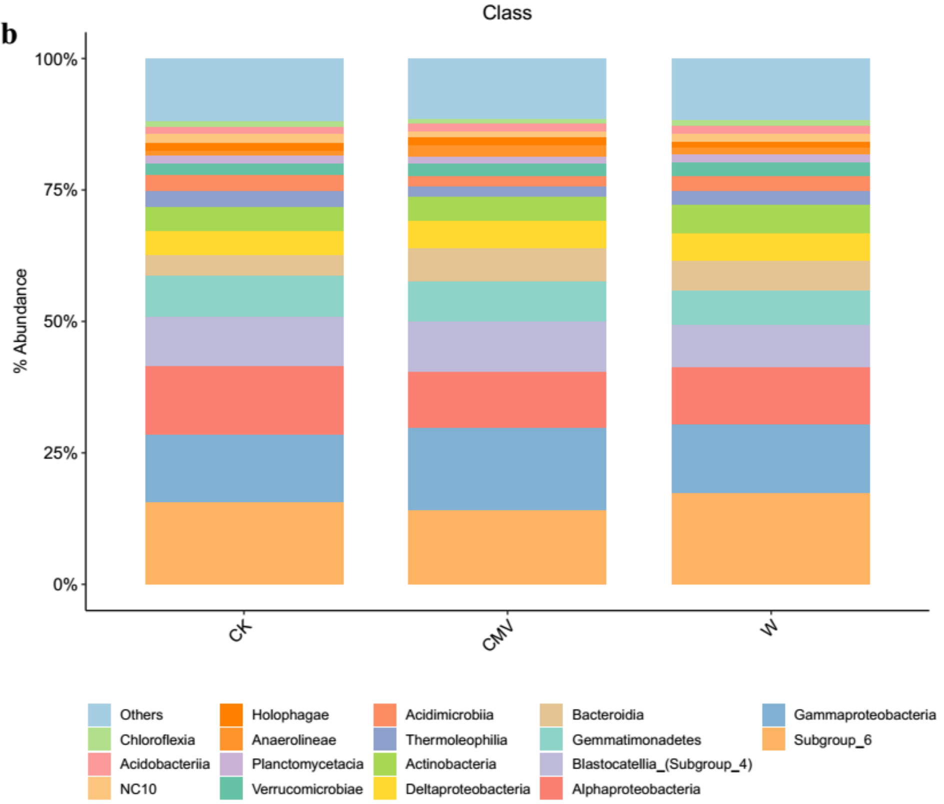

Fig 6c

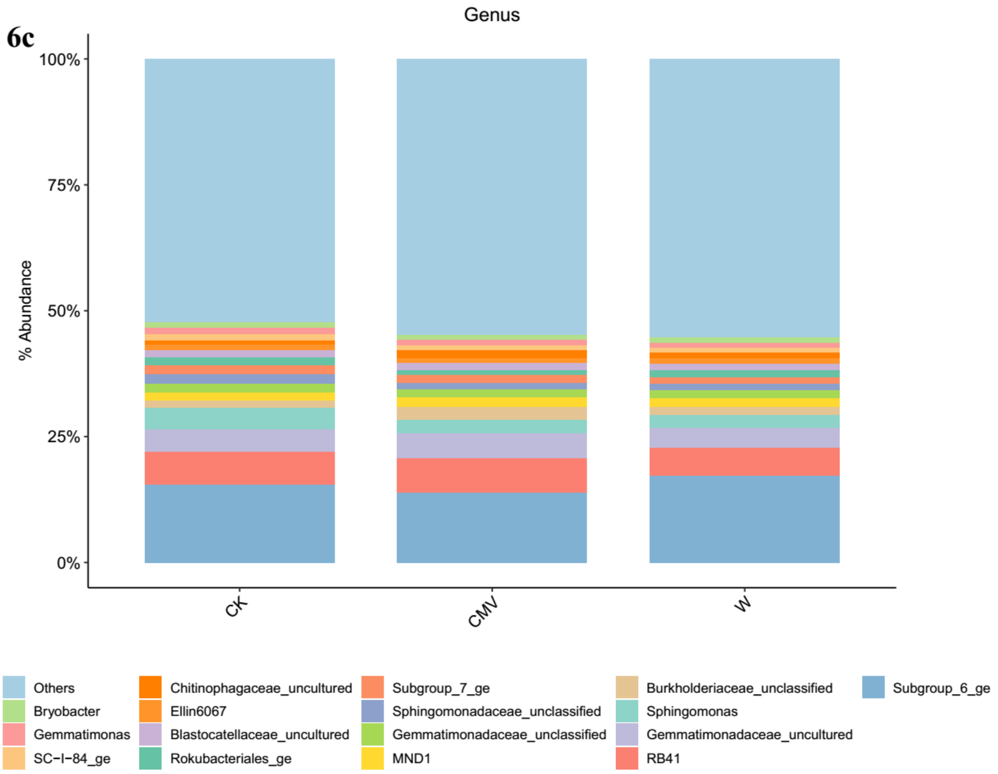

Figure 6. *Species distribution heatmap of soil samples at the different levels. (a) Phyla level, (b) class level, (c) genus level*

At the genus level (*Fig. 6c*), the common dominant genus is *Subgroup_6_ge* (13.92%-17.20%), *RB*41 (5.67%-6.76%), *Gemmatimonadaceae_ uncultured* (4.56%-4.93%), *Sphingomonas* (2.41%-4.27%), *Sphingomonadaceae_unclassified* (1.31%-2.01%), *Gemmatimonadaceae_ unclassified* (1.46%-1.82%), *Rokubacteriales_ge* (1.03%-1.67%) *Subgroup_7_ge* (1.21%-1.59%), *MND*1 (1.50%-1.91%), *Burkholderiaceae_unclassified* (1.46%-2.60%), *Blastocatellaceae_uncultured* (1.17%-1.31%), W and CMV group shared *Chitinophagaceae_uncultured* (1.12%-1.45%) and *Pedosphaeraceae_ge* (1.06%-1.08%), The CMV group is specialized in *Bacteria_unclassified* (1.13%), *Bryobacter* (1.10%), *PLTA13_ge* (1.06%), TRA3-20_ge (1.06%) and *UTCFX*1 (1.13%), it suggested that there were significant differences in the major bacterial genus among the groups.

According to the similarity between samples, the top 50 abundant classes were clustered and heatmapped (*Fig. 7*). To distinguishing high-abundance and low-abundance taxa, a color gradient was utilized to depict the similarity of community composition among samples. As observed from the heat map, *WD2101_soil_group_ge*, *TK10_ge*, *Gaiellales_uncultured_ge*, *Longimicrobiaceae_ge*, *Blastocatellaceae_unclassified*, *Subgroup_10*, *Sphingomonas*, *Massilia*, *S085_ge*, *Ellin6055*, *Gemmatirosa*, *Sphingomonadaceae_unclassified*, *Micromonosporaceae_unclassified*, *Gemmatimonas*, and *JG30− KF−CM66* were the relative high abundant microbial genera in CK group; *Latescibacteria_ge*, *Subgroup_6_ge*, *Roseiflexaceae_uncultured*, *MB−A2−108_ge*, *Luedemannella*, *Actinobacteria_unclassified*, *Lechevalieria*, *Actinophytocola*, *Chthoniobacter*, *Pirellula*, and *Xanthobacteraceae_unclassified* were predominant genera with high abundance in W group; In addition, the microorganisms with high abundance in CMV group mainly included *UTCFX1*, *Betaproteobacteriales_unclassified*, *Rubrobacter*, *Burkholderiaceae_unclassified*, *Chitinophagaceae_unclassified*, *Nitrospira*, *Bryobacter*, *Rhizobacter*, *UTBCD1*, *Saprospiraceae_uncultured*, *Gemmatimonadaceae_uncultured*, *S0134_terrestrial_group_ge*, *Flavisolibacter*, and *Steroidobacter*. Therefore, we inferred that Chinese milk vetch seedlings had a more significant impact on the microorganism genera richness of the rhizosphere soil than wheat seedlings under the same condition.

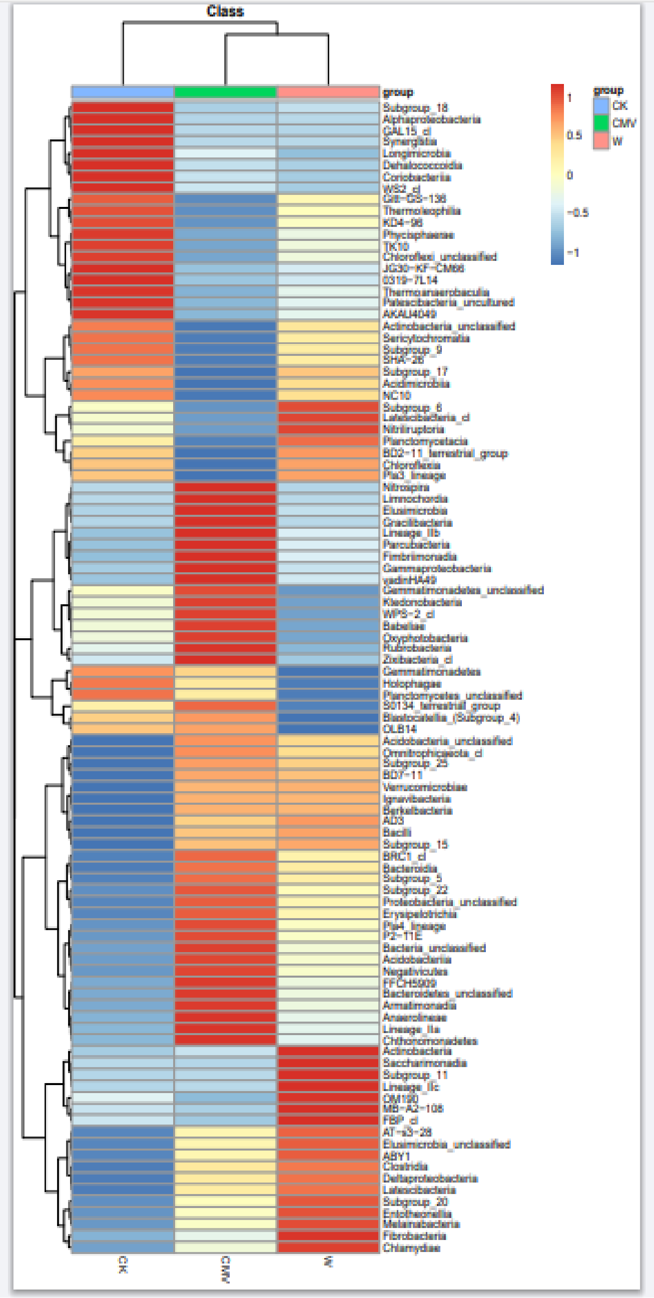

Figure 7. *Hierarchical cluster heatmap of bacterial taxa from different groups at class level*

Principal component analysis

Bray_curtis distance was calculated according to the bacterial species composition data (OTUs) of each sample, and principal component–analysis (PCA)–was performed based on the bray_curtis distance matrix (*Fig. 8a*). The results showed that the contribution rates of principal component 1(PC1) and principal component 2(PC2) were 39.72% and 22.55%, which could explain 66.37% of all samples. PCOA (Principal Co-ordinate~~s~~ Analysis) is a visual method of studying data similarities or differences. In the PCOA scatter plot (*Fig. 8b*), the points of the three groups of samples showed aggregation within the group except one pot in W group, indicating that both wheat seedlings and Chinese milk vetch seedlings cloud had certain effects on the root bacterial community.

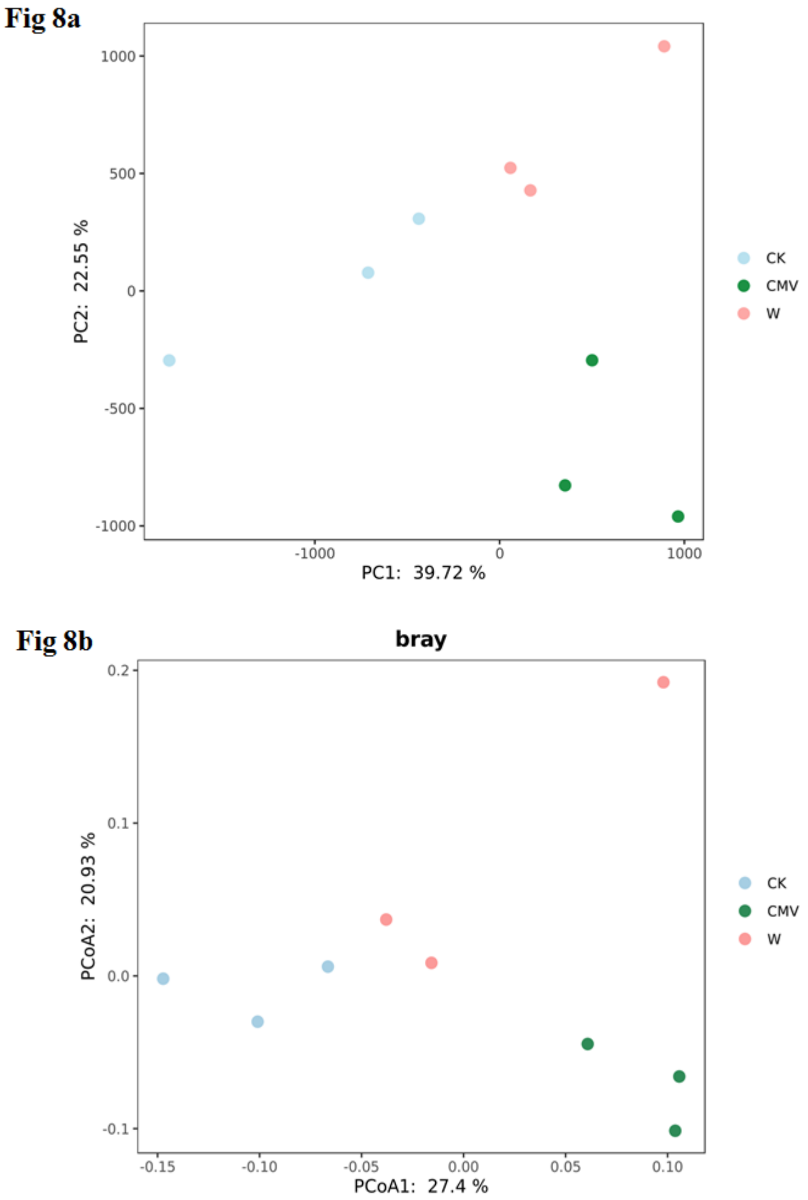

Figure 8*. PCA and PCOA of soil microorganisms in different groups. (a) PCA, (b)PCOA*

In addition, it can also be found that the lattice distance between the 4 samples in the control group and the wheat group is relatively close, indicating that the bacterial community composition of the two groups is similar, and the influence of wheat on the root bacterial community is not significant. The lattice distance between the 3 samples in CMV and CK group was relatively far, and the corresponding scattered points in the

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group showed mutual aggregation. In short, the phylum and class levels revealed a striking similarity in the composition of soil bacterial communities between the two species inhabiting the same area, while exhibiting distinct differences in both composition and abundance. These results indicated that the effect of Chinese milk vetch seedlings on the community composition and diversity formation of rhizosphere bacteria was higher than that of wheat.

Under the condition of setting the LDA Score value of 2, the species with significant differences in abundance between different groups are shown, that is, the abundance within each group is significantly higher than the Biomarker of other groups. As can be seen in *Figure 9,* the nine biomarker in W group included *Micromonosporaceae_uncultured*, *BIrii41_ge*, *Rubritaleaceae*, *Gracilibacillus*, *BIrii41*,*Caulobacterale*s, *Luteolibacter*, *Saccharothrix*, *Actinobacteria_unclassifie*, etc. The thirty-five biomarkers in CMV group included *Steroidobacteraceae*, *Chitinophagaceae_uncultured*, *Methylophilaceae*, *Steroidobacter*, *Polyangiaceae*, *Variovorax*, etc; then 26 biomarkers in CK group included *Gaiella*, *Massilia*, *Ilumatobacter*, etc.

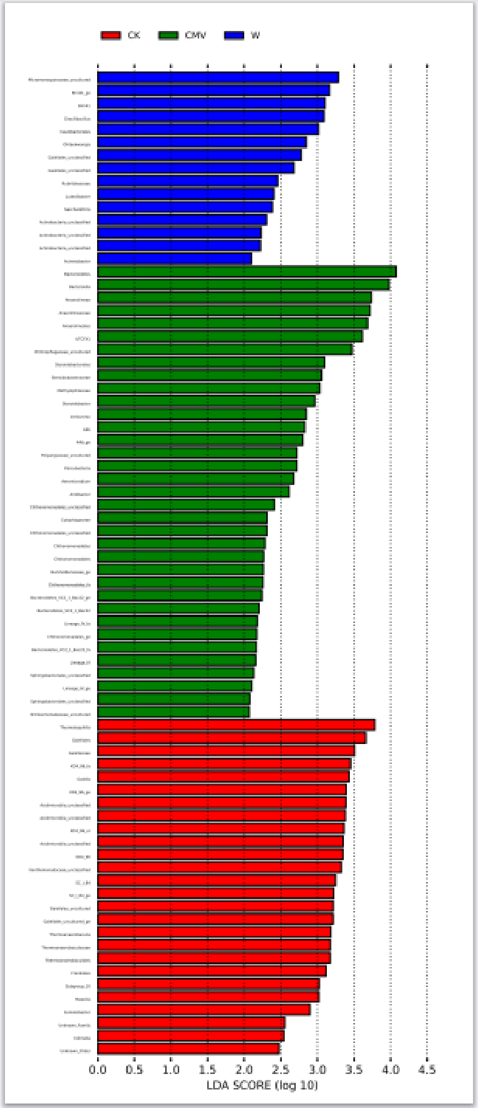

Figure 9. *Principal biomarkersin different groups*

Comparison of soil bacterial community function

To obtain metabolites for key changes in the rhizosphere soil and to analyze the effects of soil metabolites on microbial function and soil nutrient cycling, PICRUSt2 was used to predict the metabolic function of the microbial community based on the results of 16S rRNA amplicon sequencing. The predicted results were compared with the KEGG database, it was found that the types of metabolic pathways were the same, but the types and abundance of bacteria involved were different, there were 6 types of metabolic functions involved in all samples at first level (*Table 2*). In the primary functional metabolic pathway of KEGG, the metabolic function system constituted over 80% of the total, so genes related to “Metabolism” exhibited the highest abundance, then followed by “Genetic information processing”, “Cellular processes”, “Environmental information processing”, “Human diseases”, and “Organic systems”, moreover, the abundance value of CMV and W groups participating in “Metabolism” is higher than that of CK group. There are 33 types of metabolic functions with relative abundance greater than 1% at the second level (*Fig. 10*), and the abundances of metabolic functions of different samples are close to each other. The top 10 KEGG pathways included “Amino acid metabolism”, “Carbohydrate metabolism”, “Metabolism of cofactors and vitamins”, “Metabolism of terpenoids and polyketides”, “Metabolism of other amino acids”, “Lipid metabolism”, “Xenobiotics biodegradation and metabolism”, “Energy metabolism”, “Replication and repair” and “Glycan biosynthesis and metabolism”. But no significant differences existed in the bacterial community function in the rhizosphere soils of the two crops.

Table 2. *KEGG pathways level1*

level1	**CK1**	**CK2**	**CK3**	**CMV1**	**CMV2**	**CMV3**	**W1**	**W2**	**W3**
Cellular processes	198178.1	201140.4	207789.4	208150.1	210476.7	203729.4	194308.7	199006.8	196606.7
Environmental information processing	94896.87	96407.73	97854.31	101542	101248.2	99539.48	94558.77	98641.06	96324.19
Genetic information processing	604101.2	605516.3	607041.1	613443.9	610779	608600.2	605066.2	610796.8	606628.9
Human diseases	15154.13	20517.47	16073.94	16350.86	21441.54	16185.44	14960.54	15697.32	15212.47
Metabolism	4116027	4122938	4135017	4220302	4169123	4153085	4124966	4211119	4162186
Organismal systems	12935.15	14154.37	12917.76	13633.54	15128.19	14941.29	14358.43	14739.18	14601.41

Figure 10. *Prediction of metabolism functions according KEGG level 2 of archaea by PIC RUSt2*

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Correlation analysis of diversity indexes with soil physicochemical parameters, soil enzyme activities

Mantel test was conducted to reveal the effects of the key environmental factors on bacterial diversity indexes. As shown in *Figure 11*, the result showed that that Chao index showed a highly significant positive correlation with ammonium nitrogen, which indicated that the soil bacterial community may had a higher ammonium nitrogen demands in Cu tailings. But not with other factors, the Chao1 method is commonly employed in ecological studies to estimate the overall species richness, a higher value indicates a larger total number of species. In addition, soil physicochemical parameters and soil enzyme activities could possibly promote the diversity of microbial community in the soil, but the other diversity indexes (Shannon, Ace, Simpson and Invsimpson) did not significantly correlated with these parameters, we supposed it may result from limitation of our experimental design.

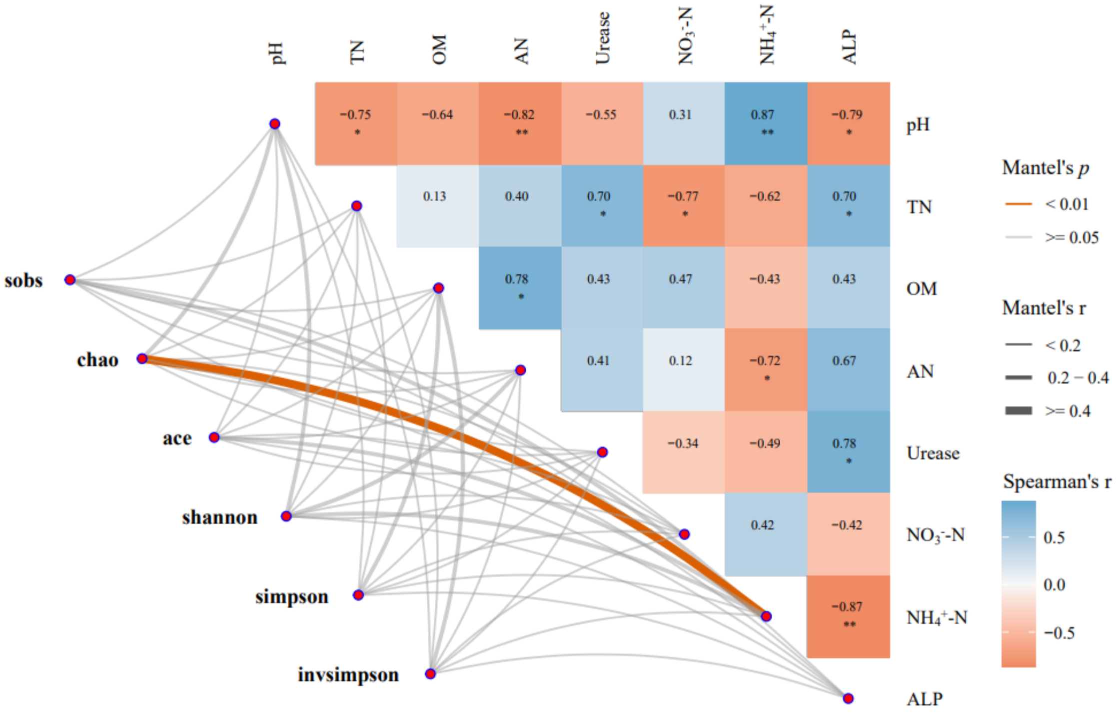

***Figure 11.** Correlation analysis*

Discussion

Effects of wheat and Chinese milk vetch on physical and chemical properties of rhizosphere soil

Mine tailings are detrimental to natural plant growth due to their physicochemical characteristics, such as high pH, high salinity, low water retention capacity, high heavy metal concentrations, and deficiencies in soil organic matter and fertility (Wang et al., 2017). The physicochemical properties and enzyme activities of soils are typically considered to be important indicators of soil quality (Wang et al., 2022b). In this study, significant differences were observed in the physical and chemical properties of rhizosphere and non-rhizosphere soil of wheat and Chinese milk vetch. The pH, organic matter (OM), available nitrogen (AN), nitrate nitrogen (NO_3^--N), and ammonium nitrogen (NH_4^+-N) contents in the rhizosphere soil reached significant levels compared with the non-rhizosphere soil ($p < 0.05$). These findings suggest that different vegetation of wheat and Chinese milk vetch can significantly influence the physical and chemical properties of soil. More and more studies have shown that there are great differences in physicochemical properties between rhizosphere and non-rhizosphere soils of different species in tailings. It is consistent with the results of previous studies, colonization of reed (*Phragmites communis*) significantly changed the bacterial community and the chemical properties of Cu mine tailings (Chen et al., 2008), Sun et al. found that dysophylla (T*ypha angustifolia*), dwarf elephant grass (*Pennisetum purpereum*), and Sudan grass (*Sorghum sudanense*) improved soil properties in rhizosphere when compared with bulk soil of copper mine tailings (Sun et al., 2018).

In this study, both wheat seedling and Chinese milk vetch seedling significantly caused the decrease of pH, this might result from soil organic acids, some scholars agreed that the decrease of rhizosphere soil pH was caused by organic acids secreted by plant roots and microorganisms (Wang et al., 2017), in addition, we found that the contents of TN, OM and AN in the rhizosphere soil were higher than those in the bulk soil, which is consistent with the previous findings.Rhizosphere soil had higher content of TN when compared with loose soil (Moreno-Espíndola et al., 2018), Chen et al. reported that the contents of organic matter, total nitrogen, and ammonium acetate-extractable potassium in vegetated reed (*Phragmites communis*) mine tailings were significantly higher than those in the bare Cu mine tailings (Chen et al., 2008). The NO_3^--N content in the CMV group was significantly higher than that in the W group, which may be due to their high level of *Nitrospirae*. Although there was no significant change observed in some indicators, and even a certain degree of fluctuation exists, these findings still suggest that the two crop seedlings have a discernible impact on the amelioration of copper tailings contaminated soil.

Effects of wheat and Chinese milk vetch on soil enzyme activities in rhizosphere

Soil health has been defined as the “continued capacity of a specific kind of soil to function as a vital living system, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, to maintain or enhance the quality of air and water environments, and to support human health and habitation (Alkorta et al., 2003). Soil enzyme activities have been considered as indicators of soil function and Soil health (Alkorta et al., 2003; Wang et al., 2022a). Urease, acid phosphatase, invertase, dehydrogenase and catalase are important for the soil ecosystem and are widely involved in the material cycle and energy metabolism of soil (Li et al., 2022). In this study, wheat and Chinese milk vetch both increased urease activity and alkaline protease activity in rhizosphere soil, this was consistent with the previous research, 79–89% of urease activity in soils is derived from extracellular enzymes adsorbed to soil colloids. In soil, most of the urease comes from syntheses realized by microorganism and plant materials. Ureases are widely distributed in bacteria (Carlini and Ligabue-Braun, 2016). It was observed that while wheat had a significant impact on ALP, there were no notable differences in urease activity and alkaline protease activity of rhizosphere soil between Chinese milk vetch and wheat. We suppose that this difference may be due to the effects of low ambient temperatures and crop age.

Effects of wheat and Chinese milk vetch on bacteria community and diversity

In this paper, CMV and W groups exhibited an increase in indexes containing Chao, Shannon, Ace, and Simpson when compared with the CK group, it demonstrated that the cultivation of Chinese milk vetch and wheat enhanced soil bacterial richness. Furthermore, the Chao and Ace indices were higher in the CMV group when compared with the W group, indicating a greater community richness in CMV group than in the W group. Consistently, the composition of the bacterial community from abandoned copper mining tailings was more impacted by plant establishment than by differences in the physicochemical properties of the substrates (Gazitúa et al., 2021).

We found that the most dominant taxa in three groups belonged to *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*. This is similar with the previous researches, Chen et al. (Chen et al., 2019), they also found that the most abundant phyla in paddy soil and ditch sediment under the combined effect of plant development and nitrogen fertilization were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Chloroflexi, Bacteroidetes, Planctomycetes*, *Verrucomicrobia*, but the abundance varied between our data and their data. Jia et al. reported that the five prevailing bacterial phyla found in *Bothriochloa ischaemum* rhizosphere were *Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, and Planctomycetes* (Jia et al., 2020). Zhang et al. reported that most predominant phylum included *Proteobacteria, Acidobacteria, Chloroflexi, Bacteroidetes,* and *Actinobacteri* were the domain of the phytostabilizer Athyrium wardii (Hook.) grown in a Pb/Zn mine tailing (Zhang et al., 2022b), except *Proteobacteria* and *Gemmatimonadetes,* they are mainly involved in carbon and nitrogen cycles (Kalam et al., 2020; Larsbrink and McKee, 2020; Sichert et al., 2020; Wiegand et al., 2018). *Acidobacteria* can reduce the pH of alkaline soil through metabolic acid production (Zhalnina et al., 2018), and in this study, pH also showed a very significant decrease.

The spatial distributions of bacterial communities may be driven by multiple environmental factors (Ding et al., 2015). Compared with the control group, planting wheat significantly decreased pH and levels of NO_3^--N and NH_4^+-N, this study is similar to previous studies, Ding et al. reported that the NH_4^+ concentration decreased with increasing depth, which was positively correlated with the relative abundance of *Alphaproteobacteria* in the Poyang Lake beach wetland (Ding et al., 2015). Some scholars generally agree that soil microbial diversity and community structure depend on factors including soil pH, temperature, moisture content, nature and amount of root exudates, nutrient status and agricultural practice, genotypes of plants, pathogens, the pressure of heavy metals and plant protection chemicals (Agler et al., 2016). consistentlly, the Mantel test showed that Chao index was significantly associated with ammonium nitrogen ($p < 0.05$) for rhizosphere soil in Tongling copper tailings.

Comparison of soil bacterial community function of the rhizosphere between wheat and Chinese milk vetch

To clarify if any alteration of bacterial community structure or composition has functional consequences, furthermore, enrichment in metabolic functions was conducted. At the KEGG firstly functional level, the functional metabolic pathway of KEGG, genes related to metabolism exhibited the highest abundance, which indicating that metabolism played an extremely important role in the growth of soil bacteria.

Meanwhile, at the KEGG secondary functional level, the top ten metabolic pathways in bacterial abundance mainly involved in “Amino acid metabolism”, “Carbohydrate metabolism”, “Metabolism of cofactors and vitamins”, “Metabolism of terpenoids and polyketides”, “Lipid metabolism”, etc. “Amino acid metabolism” can help bacteria absorb amino acids, Keshri et al. reported that the level of Amino acid metabolism reflected the capacity of the bacterial populations in the silage to synthesize amino acid de novo (Keshri et al., 2019). Ryu et al. reported that rhizosphere-associated microorganisms acquired essential nutrients from plants through host root exudates, then crucial nutrients can be transformed by microorganisms into utilizable forms such as sugars, organic acids, amino acids, and peptides before being assimilated by the plant (Ryu et al., 2004). Consistent with this study, the abundance value of CMV and W groups participating in metabolism is higher than that of CK group, which was coincide with the higher bacterial abundance and diversity in the treatment groups than in the control group. But our findings showed bacterial metabolic function did not significantly vary among three groups, one possible explanation for these results was that similar bacterial communities might result from similar soil properties, which led to the almost identical metabolic functions.

Conclusion

In this study, by profiling 16S rRNA genes sequencing, we investigated and compared effect of Chinese milk vetch and wheat on bacterial community structure and diversity of rhizosphere soil in Tongling copper tailings. The results showed that both Chinese milk vetch and wheat seedlings had significant impacts on soil pH, nitrate nitrogen, and ammonium nitrogen. Chinese milk vetch and wheat enriched the diversity of bacterial community in rhizosphere soil, furthermore, the effects on bacterial diversity were stronger for nitrogen-fixing plant Chinese milk vetch than they were for wheat, Chao index showed a highly significant positive correlation with ammonium nitrogen.

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