

## Original Article

# Microbial inoculants and garbage fermentation liquid reduced root-knot nematode disease and As uptake in *Panax quinquefolium* cultivation by modulating rhizosphere microbiota community

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## ABSTRACT

**Objective:** To find a suitable ecological cultivation measure to solve the problem of root-knot nematode disease of *Panax quinquefolium* (*Panacis Quinquefolii Radix*) and the heavy metals accumulating in its roots.

**Methods:** Three-year-old *P. quinquefolium* was treated with four different combinations of microbial inoculant (MI) and garbage fermentation liquid (GFL) [the joint application of 'TuXiu' MI and Fifty potassium MI (TF), the combination use of 'No. 1' MI and Fifty potassium MI (NF), 'Gulefeng' poly- $\gamma$ -glutamic acid MI (PGA), GFL], and the untreated control (CK). Here, high-throughput sequencing, ICP-MS and UPLC were employed to systematically characterize changes of microbial diversity and structure composition, heavy metals (As, Cd and Pb) content and ginsenoside content among different treatments.

**Results:** The results revealed that different MIs and GFL could increase the root dry weight of *P. quinquefolium*, PGA enhanced it by 83.24%, followed by GFL (49.93%), meanwhile, PGA and GFL were able to lessen root-knot nematode disease incidence by 57.25% and 64.35%. The treatment of PGA and GFL can also effectively reduce heavy metals in roots. The As content in GFL and PGA was decreased by 52.17% and 43.48% respectively, while the Cd and Pb contents of GFL and PGA was decreased somewhat. Additionally, the content of total ginsenosides was increased by 42.14% and 42.07%, in response to TF and NF, respectively. Our metagenomic analysis showed that the relative abundance of particular soil microbial community members related to the biocontrol of root-knot nematode disease and plant pathogen (i.e., *Chaetomium* in NF, *Xylaria* in GFL, and *Microascus* in PGA), heavy metal bioremediation (*Hyphomicrobium* in PGA and *Xylaria* in GFL), and nitrogen fixation (*Nordella* and *Nitrospira* in TF) was significantly increased; notably, potential harmful microflora, such as *Plectosphaerella* and *Rhizobacter*, were more abundant in the control group.

**Conclusion:** MI and GFL could improve the quality of *P. quinquefolium* by modifying its rhizosphere microbial community structure and composition, both of them are beneficial to the development of ecological cultivation of *P. quinquefolium*.

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## 1. Introduction

*Panax quinquefolium* L., a perennial medicinal plant also known as American ginseng, is famous for its pharmacological activities (Li et al., 2019; Szczuka et al., 2019). However, it frequently suffers from severe root diseases. The *P. quinquefolium* nematode disease is one such root diseases, capable of reducing its yield by more

than 60% (Zhang et al., 2009). This disease also aggravates the severity of other co-occurring root diseases and affects the plant quality adversely (Al-Hazmi & Al-Nadary, 2015). To date, the main plant parasitic nematodes are *Meloidogyne*, *Heterodera*, and *Globodera* spp. (East, Zasada, Schreiner, & Moyer, 2019; Vieira & Gleason, 2019; Wilkes & Kirkpatrick, 2020), whose prevention and control depends primarily on organochlorine pesticides (Zhang et al., 2009). Yet this kind of pesticide features stable chemical properties, so it easily accumulates in the roots of *P. quinquefolium*, thus threatening its consumption and therapeutic use. For example, 15 different organochlorine pesticides were detected in

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60 samples of *P. quinquefolium*, namely benzene hexachloride (BHC, including a-BHC, b-BHC, c-BHC, d-BHC), hexachlorobenzene, heptachlor, heptachlor epoxide, aldrin, p,p'-DDE, p,p'-DDD, o,p'-DDT, p,p'-DDT, mirex, endrin, and dieldrin (Wu, Liu, Zhao, & Xu, 2011). Recently, Zhao et al. (2018) reported 16 pesticides in *P. quinquefolium*, of which 15 were organochlorine pesticides. Furthermore, the steady input of pesticides will also worsen soil ecology, as well as diminishing the disease-controlling effect of pesticides. For instance, Liu, Li, Li, and Chen (2008) reported that pentachlorophenol could significantly reduce the diversity of bacterial community and the number of ammonia oxidizing bacteria in soil. Hill and Hausbeck (2008) found that 82% of 210 strains of *Phytophthora cactorum* isolated from *P. quinquefolium* soil in Wisconsin, USA, were resistant to metalaxyl. To sum up, there are sufficient and mounting evidences to show that it affects medicinal plant quality adversely to apply pesticides to treat the nematode diseases of *P. quinquefolium*. Therefore, it is imperative to find a safer way, to inhibit the population of pathogenic nematodes and reduce the production risk in the cultivation process.

Biological control is an environment-friendly and low-cost plant disease control way that is increasingly gaining attention. Using a microbial inoculant (MI) which contains plant growth-promoting microorganisms (PGPM), is becoming extremely popular in agriculture. When the PGPM is applied to plants or soils, it can assist plants' growth by enhancing their nutrient absorption or alleviating diseases, such as root rot and nematode diseases (Jiang et al., 2018; Shen et al., 2018). *Bacillus* is the most commonly used PGPM. When *B. subtilis* EA-CB0575 was incubated to tomato rhizosphere, it can produce indole and iron-carrier compounds, dissolve phosphate, and fix nitrogen, was shown to improve the dry weight of tomato by 34.60% (Franco-Sierra et al., 2020). The *B. amyloliquefaciens* AK-0 can release antibiotic compounds to slow down the root rot of *P. ginseng* caused by *Cylindrocarpon destructans* (Kim, Balaraju, & Jeon, 2017). Another PGPM, *B. cereus* BCM2, is able to adhere itself to the surface of nematodes, and its metabolites can quickly identify and kill the second-stage juveniles of *Meloidogyne incognita* (Hu et al., 2020).

Garbage fermentation liquid (GFL) is an organic fermentation product, which is fermented by mixing plant tissues or other biological garbage with water and brown sugar (Arun & Sivashanmugam, 2017). Some PGPM, namely *Lactobacillus* and *Candida*, are the dominant microorganisms produced in this fermentation process (Du et al., 2017). *Lactobacillus* spp. are generally regarded as beneficial microorganisms, which can be used to improve the stress resistance and the control of plant parasitic nematodes (Hussein & Joo, 2018; Seo et al., 2019). *Candida* members can promote the growth of *Vigna radiata* under drought stress, and biodegrade 4-nitroaniline in contaminated land (Silambarasan & Vangnai, 2017). In recent years, more researches are showing that GFL can play a pivotal role in removing impurities and pathogenic bacteria from dairy waste-activated sludge, and improving the effective components of medicinal plants (Arun & Sivashanmugam, 2015; Ouyang & Gu, 2017; Wei, Cao, Wang, & Han, 2020). Under conditions of Cd stress, GFL was capable of reducing the Cd content of *Salvia miltiorrhiza* by 25.31% and increasing its total tanshinone content by 40.08% (Wei, Cao, et al., 2020). Therefore, GFL has a broad application prospect in the cultivation of medicinal plant.

Soil microbial community composition and structure is closely associated with plants' growth and health. The diversity of *Panax notoginseng* soil fungi is decreased significantly after planted three successive years in the same field (Dong, Xu, Feng, Li, & Chen, 2016). Additionally, significant microbial structural differences could be found between the healthy and rusty *P. ginseng* rhizosphere soil. The genera *Fusarium*, *Cylindrocarpn*, *Acrophialophora*, *Alternaria* and *Doratomyces* were mainly concentrated in the

rhizosphere of diseased plants, while *Xenopolyscytalum*, *Arthrobritys*, *Chalara*, *Cryptococcus*, and *Scutellinia* were mainly enriched in healthy ones (Wei, Wang et al., 2020). In the present study, we carried out a metagenomics analysis to study the quality-improvement effect upon *P. quinquefolium* as driven by MIs and GFL. Our aim was (i) to compare the differences of rhizosphere soil microflora of *P. quinquefolium* treated by different MIs and GFL, and (ii) to explore the potential mechanism(s) of biological control of root-knot nematode, biological removal of heavy metals, and promotion of ginsenoside content by various MIs and GFL from the perspective of microorganisms. It is anticipated this study will prove helpful for better understanding the effects of MI and GFL on the quality improvement of medicinal plants.

## 2. Materials and methods

### 2.1. Description of microbial inoculants and garbage fermentation liquid

Four kinds of commercially available MIs and GFL were used. The 'Tuxiu' MI (Beijing Chenao Runze Technology Co., Ltd., China) is a microbial mixture containing *B. licheniformis*, *B. amylolyticus*, *B. subtilis*, actinomycetes and yeast over 500 million propagules per milliliter. The 'Fifty potassium' MI (Beijing Chenao Runze Technology Co., Ltd.) is mainly composed of nitrogen, phosphorus, and potassium (16:3:12) nutrients, whose viable bacteria number more than 500 million propagules per gram. The 'No. 1' MI (Beijing Chenao Runze Technology Co., Ltd.) contains a liquid suspension, comprising of *B. subtilis*, *B. licheniformis*, and actinomycetes, at a concentration exceeding 2 billion propagules per milliliter. The 'Gulefeng' poly- $\gamma$ -glutamic acid MI (Nanjing Xuankai Biotechnology Co., Ltd., China) is composed of *B. subtilis*, *B. amylolytica*, and *B. gelatinosus* over 5 billion propagules per milliliter, and rich in a great quantity of poly- $\gamma$ -glutamic acid. Finally, garbage fermentation liquid (GFL), is a hand-made brown liquid fermented by *Citrus aurantium* fruits, together with brown sugar and water, as described by in Ouyang and Gu (2017), from January to April. After completing the fermentation period, the liquid was separated from the solids for further investigation, which pH is 3.67, and the dissolved oxygen is 1.69 mg/L. The microbial community composition of GFL was tested prior to implementing the experiment via high-throughput sequencing technology: the main microbes in it were *Lactobacillus*, *Pichia*, and *Candida* spp., among others.

### 2.2. Field experiment and soil collection

*P. quinquefolium* had been cultivated for 3 years in Rongcheng City, Shandong Province (37.2027 N, 122.4298E, mean elevation: 52 m), in eastern China, the region which is well known as the country's main cultivation area of this medicinal plant. The As, Cd, and Pb soil concentrations at this site were 2.73  $\mu\text{g/g}$ , 0.03  $\mu\text{g/g}$  and 11.42  $\mu\text{g/g}$ , respectively. Five treatments were applied in a field divided into 15 plots randomly (each 8 m  $\times$  1.5 m): Tuxiu MI + Fifty potassium MI (Tuxiu irrigation + Fifty potassium fertilizer application), No. 1 MI + Fifty potassium (No. 1 irrigation + Fifty potassium fertilizer application), Gulefeng poly- $\gamma$ -glutamic acid MI (Gulefeng poly- $\gamma$ -glutamic acid irrigation), GFL (GFL irrigation), and the untreated control (water irrigation), hereon TF, NF, PGA, GFL, and CK respectively, with three plot replicates per treatment. A 1.5  $\times$  1.5 isolation zone was set between adjacent replicate. These soil treatments were applied according to the manufacturer's instructions and the principles described previously (Ouyang & Gu, 2017). At harvest time, 15 rhizosphere soil (0–20 cm depth layer) of *P. quinquefolium* roots were collected by the five-spot-sampling method and labeled accordingly (i.e., TF,

NF, PGA, GFL, and CK respectively) (Wang, Li, Li, Pan, & Zhang, 2018). All soil samples were homogenized by 2-mm sieve and then stored at  $-80^{\circ}\text{C}$  for further study.

### 2.3. DNA extraction, PCR amplification and sequencing

Total soil DNA was extracted by using the soil genomic DNA kit (Tiangen Biotech Beijing Co., China) and following the manufacturer's instructions. The V4-V5 region of the bacterial 16S rRNA gene was amplified with the primer pair 515F/907R (5'-GTGCCAGCMGCCGCGG-3', 5'-CCGTCGAATTCMTTTRAGTTT-3') (Fu et al., 2017), while the internal transcribed spacer (ITS) region of the rRNA gene was amplified by the fungal-specific primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and ITS2R (5'-GCTGCGTTCTTCATCGATGC) (Li, Ma, Mark Ibekwe, Wang, & Yang, 2018). Both PCR amplification and purification were carried out following previously (Rodrigues et al., 2013), with the ensuing amplicons pooled in equimolar ratios for sequencing. The purified PCR products were sequenced on an Illumina HiSeq platform (Shanghai Biozeron Co., Ltd., China).

### 2.4. Herb yield and health status

In October, the  $4.5 \times 1.5$  area of *P. quinquefolium* roots in the middle of each replicate was harvested. The roots were removed from soil to assess its incidence of disease (i.e., of the root-knot nematode disease). For a given individual replicate, the number of infected plants and total plants of harvested was recorded to calculate the incidence of disease. The roots were rinsed and oven-dried at  $50^{\circ}\text{C}$  to a constant weight for counting the average dry weight of each root in different duplicons.

$$\text{Incidence of disease} = \frac{\text{Amount of infected plants}}{\text{Total amount of plants}} \times 100\%$$

$$\text{Average dry weight} = \frac{\text{Total constant weight}}{\text{Total amount of plants}} \times 100\%$$

### 2.5. Detection of ginsenosides (Rb1, Re, Rg1) and heavy metals

Next, the concentrations of three ginsenosides (Rb1, Re and Rg1) were determined by ultra-performance liquid chromatography (UPLC). The samples and standards were prepared, according to the guidelines established by the Pharmacopoeia of the People's Republic of China (Pharmacopoeia Committee of P. R. China, 2015). The standards of ginsenoside Rb1, Re, and Rg1 were purchased from the National Institute for Food and Drug Control (China). Acetonitrile and methanol were obtained from Fisher Scientific, with an ACQUITY UPLC BEH  $\text{C}_{18}$  chromatographic column (2.1 mm  $\times$  50 mm, 1.7  $\mu\text{m}$ ) used in the UPLC (WATERS, USA). The mobile phase consisted of 100% acetonitrile (solvent A) and 100% water (solvent B) (gradient elution: 0–2 min, 18% acetonitrile; 2–5 min, 20% acetonitrile; 5–11 min, 40% acetonitrile; 11–11.1 min, 18% acetonitrile; 11.1–12 min, 18% acetonitrile; the flow rate was set to 0.45 mL/min; the column temperature was  $30^{\circ}\text{C}$  with an injection volume of 2  $\mu\text{L}$ . All samples were detected at 203 nm (Yang, Zheng, Jiang, Zhou, & Xiao, 2008).

Arsenic (As) cadmium (Cd), and lead (Pb) in *P. quinquefolium* roots were then detected by inductively coupled plasma-mass spectrometry (ICP-MS). The fine powder of dried roots of *P. quinquefolium* (20–30 mg) was digested in 5 mL of concentrated  $\text{HNO}_3$  for 24 h. After adding 3 mL  $\text{H}_2\text{O}_2$  to each tube, all samples were heated at  $180^{\circ}\text{C}$  for 4–5 h until the tissues were completely digested. Then the digested samples were diluted with ultrapure

water and analyzed by ICP-MS (Thermo X7, Waltham, MA, USA) (Xu, Xin, Lin, & Rui, 2010).

### 2.6. Statistical analyses

After the FASTQ data is converted into the fasta format, the original metagenome file is demultiplexed and quality filtered by QIIME according to standard pipeline (Caporaso et al., 2010). Sequences sharing 97% identity were assigned to the same operational taxonomic unit (OTU), by using the UPARSE tool (Edgar, 2013). The online resources Silva (<http://www.arb-silva.de>) and Unite (<http://unite.ut.ee/index.php>) were used to determining the taxonomic identities of the bacteria and fungi, respectively (Koljal et al., 2013; Quast et al., 2013). Alpha diversity of the bacterial and fungal communities was analyzed using the Shannon ( $H'$ ) and Chao 1 indices (Schloss et al., 2009), both calculated in Mothur software ([http://www.mothur.org/wiki/Schloss\\_SOP#Alpha\\_diversity](http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity)). A principal coordinate analysis (PCoA) was used to illustrate the  $\beta$ -diversity based on Bray-Curtis distances metric (Oh et al., 2020). The linear discriminant analysis (LDA) effect size (LEfSe) was used to further confirm whether differences in the abundances of individual taxa existed among different groups, which were considered significant if they had an LDA score greater than 2 and  $P$ -value  $< 0.05$  (Segata et al., 2011). The metagenome functional of all bacterial communities was predicted by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software package using 16S rRNA gene sequencing based on the kyoto encyclopedia of genes and genomes (KEGG) database (Liu et al., 2020).

The five treatments' data were expressed here as means  $\pm$  standard errors ( $n = 3$  replicates). Statistical significance was determined using a one-way analysis of variance (ANOVA) when  $P < 0.05$  between the treatment groups and the control, implemented in SPSS v22 (SPSS Inc., Chicago, IL, USA).

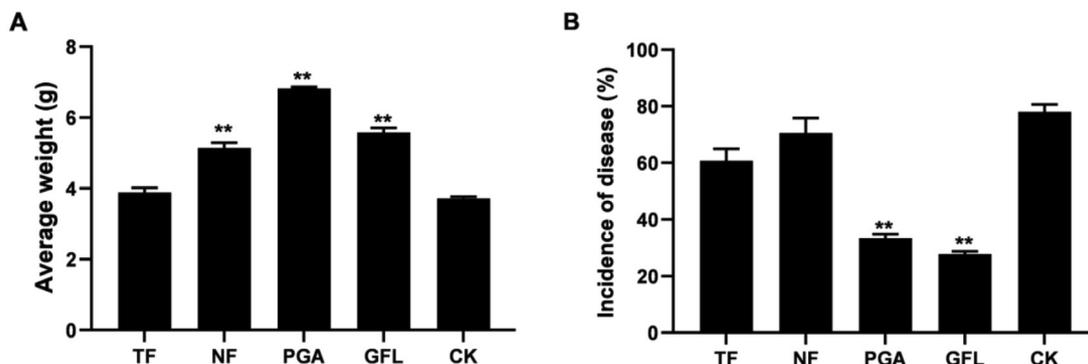
## 3. Results

### 3.1. Effects of MIs and GFL on dry weight and nematode disease

At harvest time of *P. quinquefolium*, its average dry weight (i.e., root weight) and the incidence of disease (i.e., root-knot nematode) were determined. Compared with the control group, the average dry weights of treatment groups (TF, NF, PGA, and GFL) was greater than that of CK; NF, PGA, and GFL groups respectively increased it by 38.16%, 83.24%, and 49.93% (5.14, 6.82, and 5.58 g/plant) ( $P < 0.01$ ), while TF increased only slightly, by 4.59% (Fig. 1A). The incidences of nematode disease in *P. quinquefolium* root of all treatments (TF, NF, PGA, and GFL) were lower than that of CK, which ranked as follows: GFL  $<$  PGA  $<$  TF  $<$  NF  $<$  CK. Relative to the control group, PGA and GFL significantly reduced the incidence by 57.25% and 64.35% respectively ( $P < 0.01$ ), while the TF and NF treatments decreased it less so, by 22.08% and 9.55%, respectively (Fig. 1B).

### 3.2. MIs and GFL affect content of As, Cd and Pb

The contents of As, Cd, and Pb in *P. quinquefolium* roots were determined by ICP-MS. The effects of MIs and GFL on heavy metal content differed in some degree. The contents of As were decreased by treatment groups (TF, NF, PGA and GFL) than that of CK, which ranked as follows: CK  $>$  NF  $>$  TF  $>$  PGA  $>$  GFL. In the GFL and PGA groups, the content of As were significantly reduced by 52.17% and 43.48% (0.0867 and 0.0733  $\mu\text{g/g}$ ;  $P < 0.05$ ), while TF and NF treatments decreased it by 23.91% and 19.57% (0.1167 and 0.1233  $\mu\text{g/g}$ ), respectively, when compared with the control (0.1533  $\mu\text{g/g}$ ) (Fig. 2A). The Cd content of PGA and GFL groups was lower than that



**Fig. 1.** Average weight (A) and incidence of nematode disease (B) of the roots of *Panax quinquefolium* (mean ± SE, n = 3). TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly-γ-glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group. \*P < 0.05; \*\*P < 0.01 vs CK group.

of CK, for which it was diminished by 8.33% and 6.25% (0.1467 and 0.1500 μg/g) versus the control group (0.1600 μg/g) (Fig. 2B). For Pb content, the treatments had a ranking of NF > TF > CK > PGA > GFL. Similar to As and Cd, the Pb content was lowered by most in GFL and PGA groups, by 45.66% and 35.26% (0.3733 and 0.3133 μg/g) compared with the control group (0.5767 μg/g) (Fig. 2C). In short, the least amount of As, Cd, and Pb in the medicinal root materials occurred under GFL, followed by PGA.

### 3.3. MIs and GFL affect ginsenosides content

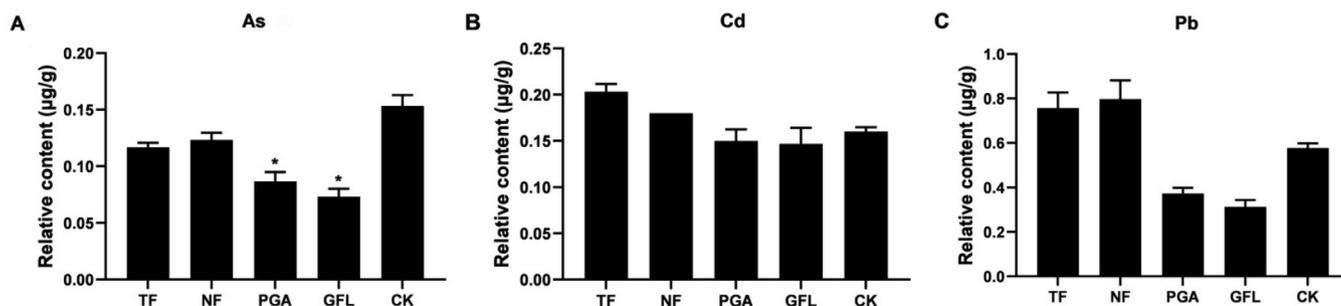
Next, UPLC was used to accurately measure the ginsenoside content in the experimental medicinal root materials. The Re content of treatment groups (TF, NF, PGA, and GFL) was greater than that of CK, which was increased by 65.05% and 67.27% (0.0149 and 0.0151 μg/g) in the TF and NF groups (P < 0.05), while PGA and GFL increased by 35.30% and 12.29% (0.0122 and 0.0101 μg/g), respectively, relative to the control (0.0090 μg/g) (Fig. 3A). The content of Rb1 of treatment groups (TF, NF, PGA, and GFL) was greater than that of CK, which was increased by 31.03%, 27.64%, 9.19%, and 9.19% (0.0138, 0.0135, 0.0119, and 0.0119 μg/g) over the control (0.0111 μg/g) (Fig. 3B). Generally, it was found that ginsenoside Re and Rb1 contents were significantly higher in the roots treated with TF and NF than those of other treatments (PGA, GFL or CK); moreover, Rg1 was only detectable in the TF group.

### 3.4. Overall bacterial and fungal community structure in rhizosphere of *P. quinquefolium*

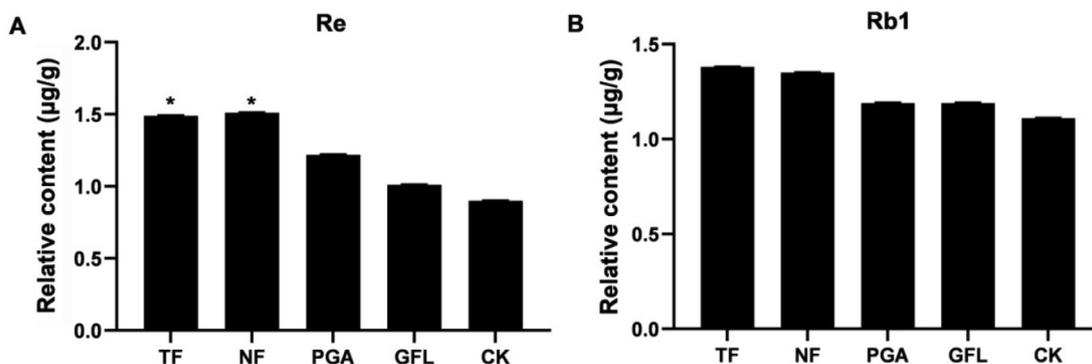
For bacteria, a total of 880 048 classified reads were obtained from the 15 soil samples, with an average of 58 670 reads per

sample. *H'* and Chao I indices were used to estimate the alpha diversity of microbial communities. The results showed that MIs and GFL led to a changed soil bacterial diversity (Fig. 4A and B). As the Venn diagram shows, the shared number of bacterial OTUs in the five groups was 3403, while the number of OTUs unique to the control, TF, NF, PGA, and GFL groups was 356, 679, 425, 351, and 268, respectively (Fig. 5A). The PCoA revealed some notable differences among bacterial communities in different treatments (Fig. 6A). Along the first principal component (35.18% contribution), the bacterial communities of TF were separated from those of NF, PGA, GFL, and CK. The bacterial communities of the NF, PGA, and GFL groups differed significantly from the control group. According to the distribution of bacterial taxa (Fig. 7A), the relative abundances of Proteobacteria and Gemmatimonadetes were lower in the treatment groups (TF, NF, PGA, and GFL) than the control, but those of Actinobacteria, Chloroflexi, Planctomyces and Firmicutes had the opposite pattern. Compared with the control group, the relative abundance of Acidobacteria was higher in both TF and GFL groups, while that of Nirosipirae was greater in the TF, NF, and PGA groups, yet lower in the GFL. These results provided crucial evidence for studying how bacterial community structure affected by different MIs and GFL.

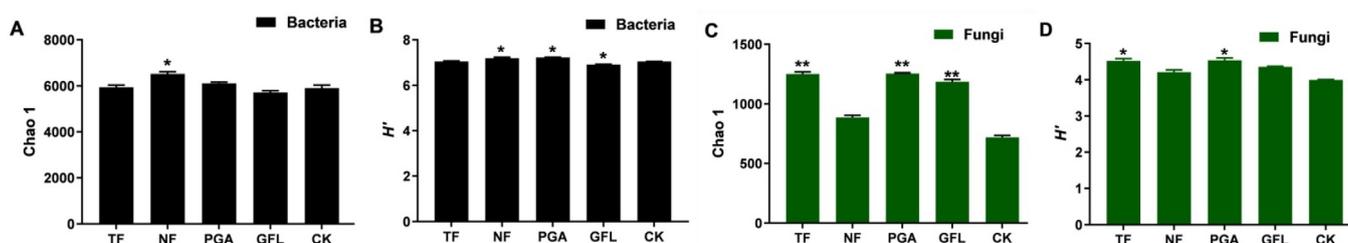
For fungi, a total of 932 347 classifiable reads were obtained from the 15 soil samples, averaging 62 156 reads per sample. Applying the MIs and GFL contributed to changed Chao I and *H'* indices (Fig. 4C and D). The Venn diagram revealed that the shared number of fungi OTUs in the five groups totaled 538, while their unique number of fungi OTUs was 83, 222, 138, 133, and 92 (Fig. 5B). Their corresponding PCoA analysis indicated that fungal communities in the control group and NF group were unlike (separated), while GFL and PGA groups were most similar (closer in



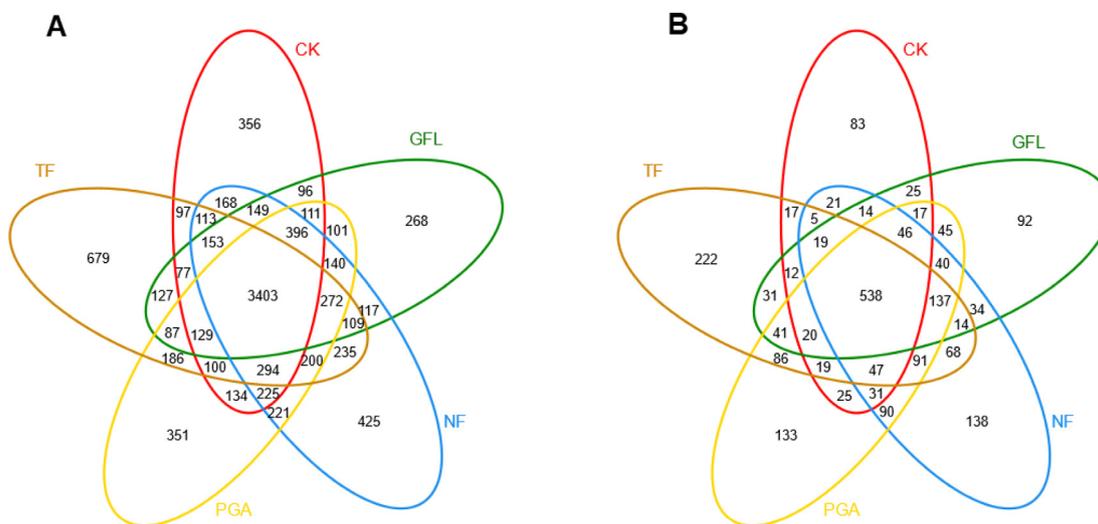
**Fig. 2.** Contents of As (A), Cd (B), and Pb (C) in roots of *Panax quinquefolium* (mean ± SE, n = 3). TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly-γ-glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group. \*P < 0.05; \*\*P < 0.01 vs CK group.



**Fig. 3.** Ginsenosides Re (A) and Rb1 (B) contents in roots of *P. quinquefolium* (mean ± SE, n = 3). TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly-γ-glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group. \*P < 0.05; \*\*P < 0.01 vs CK group



**Fig. 4.** Chao 1 (A) and  $H'$  values (B) for bacterial and Chao 1 (C) and  $H'$  values (D) for fungal diversity in TF, NF, PGA, GFL, and CK soils (mean ± SE, n = 3). TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly-γ-glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group. \*P < 0.05; \*\*P < 0.01 vs CK group.



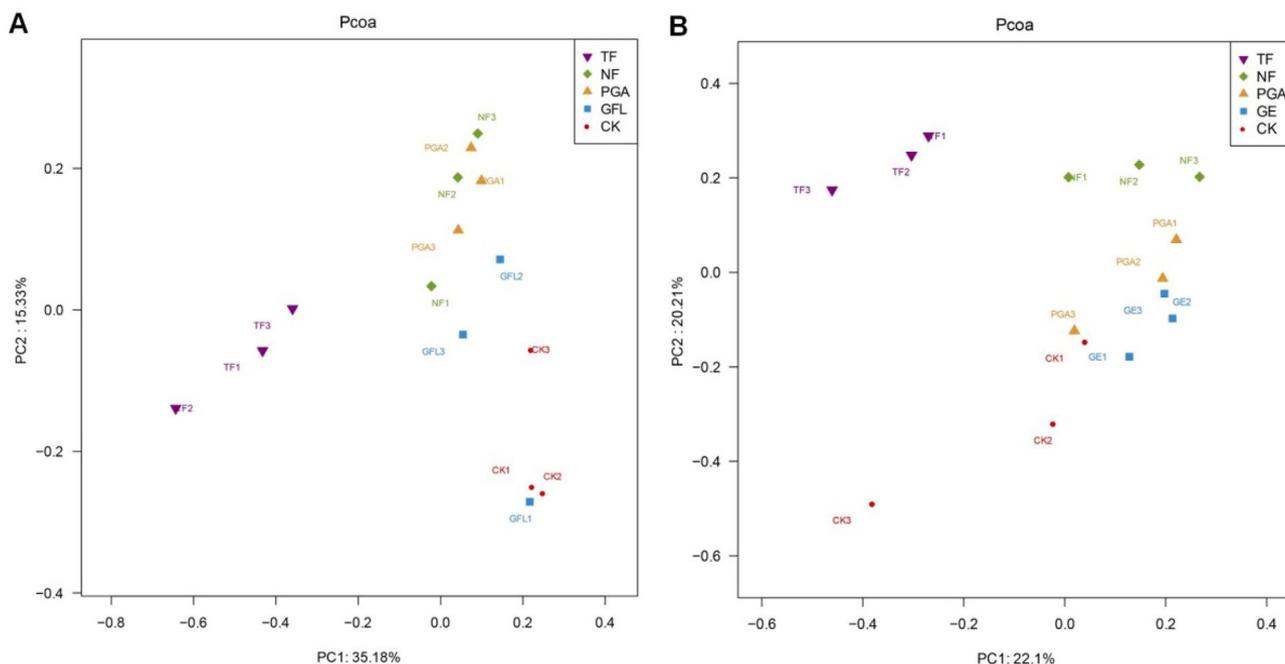
**Fig. 5.** Venn diagrams showing the core rhizosphere microbiome (OTUs) of bacterial (A) and fungal (B) communities among the TF, NF, PGA, GFL and CK groups. TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly-γ-glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group.

ordination space). Along the first principal component (22.1% contribution), the TF's fungal communities were positioned apart from the other treatment groups, suggesting their dissimilarity (Fig. 6B). The distribution of fungal taxa is shown in Fig. 7B, Ascomycota, Mucoromycota and Basidiomycota are the dominant phyla of all rhizosphere soil samples, accounting for 73.60%, 14.67%, and 5.62% of all OTUs, on average. Compared with the control group, the abundance of Ascomycota in the treatment groups (TF, NF, PGA, and GFL) increased by 14.62%, 5.45%, 6.92% and 1.83%, respectively. The relative abundances of Chytridiomycota and

Mucoromycota in all four treatment groups (TF, NF, PGA, and GFL) were lower than in the control group.

### 3.5. Special bacterial and fungal communities

The LefSe analysis was used to identify those bacteria with significant differences between different treatments (Fig. 8A). At the genus level, four characteristic species were significantly more abundant in the NF samples, where *Nitrospira* showed the highest abundance, followed by *Ktedonobacter*, *Streptacidiphilus*, and *Nor-*



**Fig. 6.** Principal coordinate analysis (PCoA) plot based on bacterial (A) and fungal (B) community scores from the TF, NF, PGA, GFL, and CK soil samples. The scatterplot is of principal coordinate 1 (PC1) vs. principal coordinate 2 (PC2). TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly- $\gamma$ -glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group.

della. In particular, the relative abundances of *Nordella* and *Nitrospira* were significantly increased, by 75.68% and 158.10%, compared with the control; in the PGA group, the abundance of *Hyphomicrobium* was significantly increased (by 23.61%). *Chujaibacter* was most abundant in the GFL treatment, while *Rhizobacter*, *Rhodanobacter*, *Dokdonella*, *Aeromicrobium*, *Rhodopseudomonas*, and *Shimazuella* were richer in the control than any treatments. Importantly, the relative abundance of *Bacillus* under three MI treatments (TF, NE, PGA) exceeded that under the control. *Lactobacillus* microorganisms were detected in the GFL treatment, but not in the control group.

Likewise, the LEfSe analysis was used to detect differential fungi occurring in the 15 soil samples (Fig. 8B). The *Conlarium*, *Corynascus*, *Endophragmiella*, *Helicoma* and *Sarocladium* genera were significantly more abundant in the TF group, while *Chaetomium*, *Gymnascella*, and *Pseudaleuria* dominated the NF soil samples. In this NF group, the relative abundance of *Chaetomium* increased substantially, by 153.82%, over the control. GFL sustained a greater abundance of *Arachnomyces*, *Crassicarpon*, and *Arcopilus* than the control group, and this treatment also significantly augmented the relative abundance of *Xylaria*, by 219.39%. Additionally, the control group harbored a community richer in *Neurospora*, *Plectosphaerella*, and *Phialemonium*. Notably, the *Helicoma*, *Halenospora*, *Pithoascu*, and *Microascus* genera were all detected in every treatment group (TF, NF, PGA, GFL), yet not in the control group. The respective relative abundances of *Helicoma* under TF, of *Halenospora*, *Pithoascu* under NF, and of *Microascus* under PGA group surpassed those in the other groups. Finally, the relative abundance of *Candida* increased by 44.96% in the GFL compared to the control group.

### 3.6. Predictive functions of bacteria community

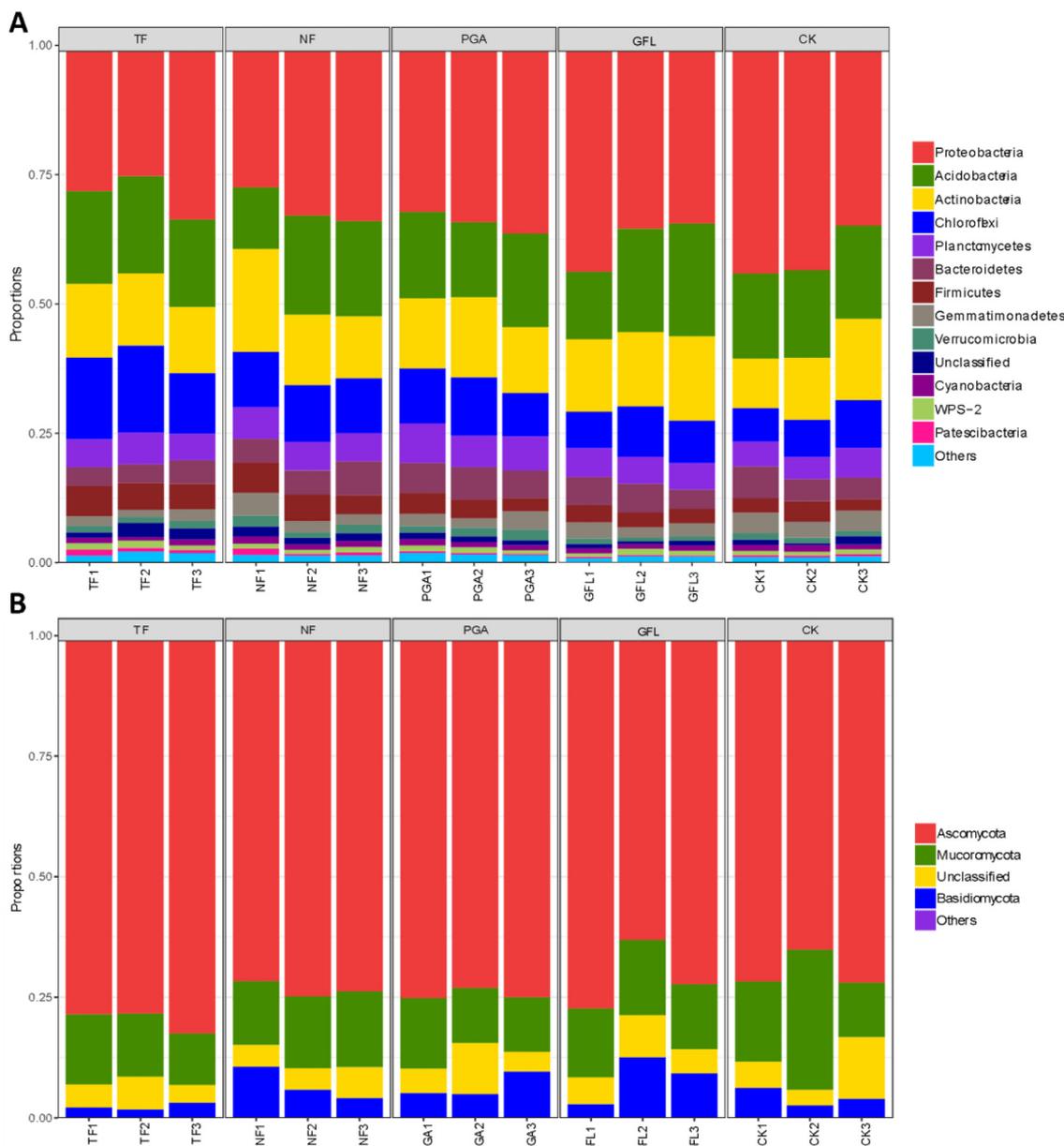
To verify the direct effect of MIs and GFL on soil microbial function, PICRUSt was used to analyze the sequence of 16S rRNA marker genes. The results showed that the gene copy number related

to mineral absorption, plant-pathogen interaction, photosynthesis, oxidative phosphorylation and nitrogen metabolism in the rhizosphere soil of *P. quinquefolium* increased significantly after applying MIs and GFL. As shown in Fig. 9, the numbers of gene copies involved to mineral absorption from TF (8187), NF (8238), PGA (7023) and GFL (8199) were higher than that of CK (6468); Importantly, compared with control (79140), the gene copy number of involved to plant-pathogen interaction in response to TF (101717), NF (104137), PGA (98165) and GFL (109319) increased significantly, which was 28.53%, 31.59% and 24.04% respectively; TF, NF, PGA and GFL treatments also increased the photosynthetic gene copy number by 28.55%, 4.93%, 22.74% and 33.90%, respectively; For oxidative phosphorylation, the gene copy numbers of TF (1224097), NF (1244360), PGA (1187971) and GFL (1309243) were higher than those of CK (946360); the gene copy number of nitrogen metabolism from TF (574229), NF (589035), PGA (55168) and GFL (61600) were all higher than CK (450829).

## 4. Discussion

### 4.1. MIs and GFL could modify microbial community structure and composition in rhizosphere of *P. quinquefolium*

PGPM can play a direct or indirect role in plant growth. Some genera of PGPM are extensively studied, such as *Bacillus*, *Lactobacillus*, *Chaetomium*, and *Trichoderma*. On the one hand, PGPM can improve soil fertility by fixing nitrogen and dissolving potassium and phosphorus, thus contributing to improve the plant growth (Bononi, Chiamonte, Pansa, Moitinho, & Melo, 2020; Ghadam Khani, Enayatizamir, & Norouzi Masir, 2019; Liu, Li, Li, Guan, & Chen, 2019). On the other hand, they can release IAA, ACC deaminase, and other bioactive substances into the environment to stimulate plant growth (Barnawal et al., 2017; Khan et al., 2020). Moreover, some strains show strong and stable activity against plant diseases or pathogenic organisms (Dong et al., 2018; Luo,

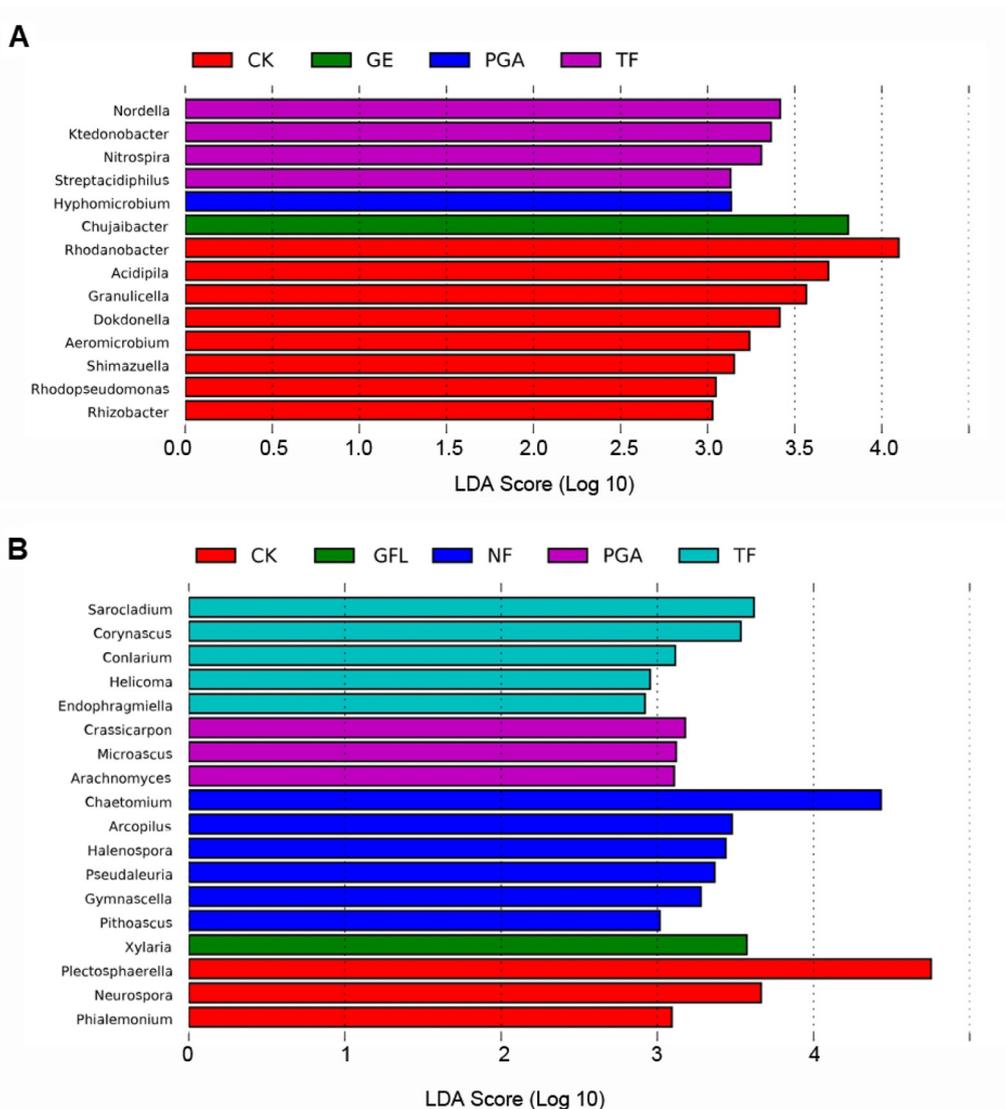


**Fig. 7.** Taxonomic classification of bacterial (A) and fungal (B) reads retrieved from the TF, NF, PGA, GFL and CK soil samples (pooled) at the phylum level. TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly- $\gamma$ -glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group.

Hou, Yang, Qi, & Zhao, 2018). Furthermore, some microorganisms have shown good application prospects for the bioremediation of heavy metal-contaminated land (Banerjee, Hazra, Das, & Sengupta, 2020; Pandey, Manjunath, & Sahu, 2019). However, one thing cannot be ignored: the influence of some beneficial microorganisms upon plants is not necessarily directly caused by its inoculation, but may be related to inducement or inhibition facilitated by other resident microbial populations (Trabelsi & Mhamdi, 2013). Because of these special traits, many microorganisms are sought after and used as active strains of a microbial inoculant, and now widely utilized in agricultural production and environmental management.

In this study, MIs and GFL modified the microbial structure and composition in the rhizosphere of *P. quinquefolium*. Firstly, they changed the microbial diversity index and the number of specific OTUs (Figs. 4, 5). Importantly, MIs and GFL clearly contributed to the structural changes in rhizosphere microbial communities of

*P. quinquefolium*. At the phylum level, the relative abundance of Proteobacteria was reduced by 2.93%–11.76%, while that of Ascomycota was increased by 1.83%–14.62%. Additionally, treating the plant cultivation with TF, NF, and PGA promoted the relative abundance of Nitrospirae in rhizosphere soil (Fig. 6). Our LEfSe analysis uncovered significant changes in the genera forming under different treatments. Among these, some microorganisms can participate in the biological nitrogen fixation process, control of plant diseases, and degradation of contaminated soil. The relative abundance of *Nordella* and *Nitrospira* in the TF group was significantly increased by 75.68% and 158.10%. Recently, Yu et al. (2019) reported *Nordella* and *Rhizobium* were increased significantly after using legumes for bioremediation of soil, and *Nitrospira* spp. are known to play a key role in the global nitrogen cycle via their nitrification activity (Koch, van Kessel, & Lucker, 2019). Moreover, NF increased the relative abundance of *Chaetomium* by 153.81%; this genus and their metabolites can generally be used



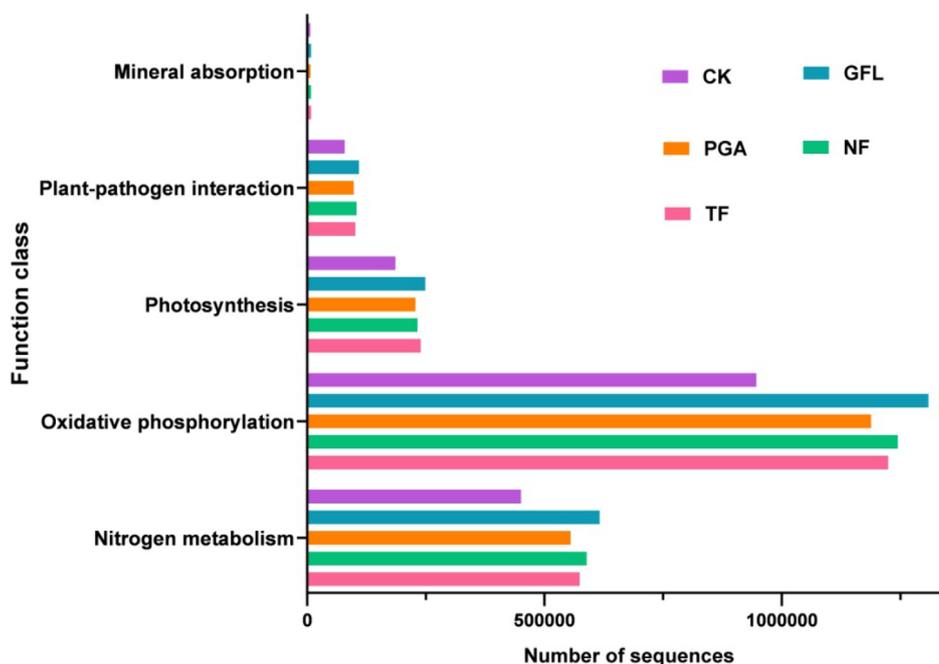
**Fig. 8.** Linear discriminant analysis effect size analysis of bacterial (A) and fungal (B) taxa for TF, NF, PGA, GFL and CK. Significance was defined as a *P*-value < 0.05 and an LDA score greater than 2.0. TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly- $\gamma$ -glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group.

for the biological control of plant pathogenic organisms (Khan et al., 2019; Zhou, Piao, Yan, & Wang, 2019). Furthermore, we found that PGA significantly increased the relative abundance of *Hyphomicrobium* by 23.61%. It was reported that *Hyphomicrobium* spp. harbored bioremediation potential for degrading organic reagents (methamidophos and dimethylsulfoxide) in the environment, and they can be applied to remediate polluted environments (Fukushima et al., 2013; Wang et al., 2010). In our experiment, *Microascus* was also detected in four treatment groups (TF, NF, PGA, and GFL), but not in the control group. Species of *Microascus* are usually used as plant biocontrol strains to control pathogens (Fotso et al., 2018). Additionally, GFL increased the relative abundance of *Xylaria* by a staggering 219.39%; in this respect, a number of previous studies have reported that *Xylaria* spp. may be used to control plant root-knot nematodes (Kim et al., 2018). Furthermore, the results of our LEfSe analysis revealed a significantly greater abundance of *Plectosphaerella* and *Rhizobacter* in the control group than in the treatment groups (i.e., TF, NF, PGA, and GFL). Interestingly, these two genera are often linked to plant diseases of crops (Hu, Strom, Haarith, Chen, & Bushley, 2019; Song, Wu, Yin, Lian,

& Yin, 2017). In summary, MIs and GFL promoted the accumulation of potential beneficial microorganisms and the reduction of potential harmful microorganisms, thus modifying the microbial structure and composition in the rhizosphere of *P. quinquefolium*.

#### 4.2. MIs and GFL could increase yield of *P. quinquefolium*

In recent years, MIs have been widely applied in crop cultivation management, for example, to bananas, tea, and rice (Jha et al., 2020; Shen et al., 2018; Zhou et al., 2020). This formulation not only can reduce the application of chemical fertilizers, but it also plays a certain role in the prevention and control of plant diseases. Besides the use in environmental management, GFL also has potential advantages for improving the quality of medicinal herbs, such as *Salvia miltiorrhiza* (Wei, Cao, et al., 2020). Our research results suggested that MIs and GFL are beneficial for increasing the root dry weight of *P. quinquefolius*, in that these applications promoted a 4.59%–83.24% greater root biomass accumulation. We also found that some special microbial taxa were changed by the introduction of MIs and GFL, such as *Xylaria* in GFL (an increase



**Fig. 9.** Relative abundance of predictive metagenome functions from samples. TF, NF, PGA, and GFL represent the rhizosphere soil of *P. quinquefolium* treated by TuXiu microbial inoculum + Fifty potassium, No. 1 microbial inoculum + Fifty potassium, Gulefeng poly- $\gamma$ -glutamic acid microbial inoculum, and garbage fermentation liquid, respectively, with CK as the control group.

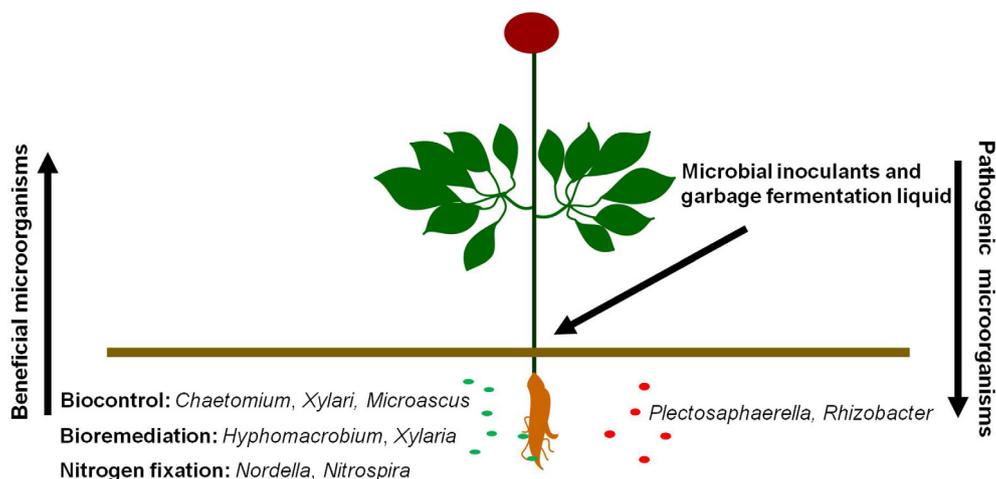
of 219.39%) and *Chaetomium* in NF (an increase of 153.82%), which may contribute to the growth promotion of plants. According to previous reports, inoculation with *X. regalis* can significantly increase the stem length, root length, and stem root dry matter yield of pepper seedlings (Adnan et al., 2018). The inoculation of *C. cuprum* in the rhizosphere of *Eucalyptus globulus* promoted this herb to express auxin biosynthesis gene (Ortiz et al., 2019). Moreover, *Bacillus* sp. and *Lactobacillus* sp. existed in the MIs and GFL, that are beneficial to plant biomass accumulation. *B. megaterium*, which can be used as a zinc-dissolving agent to enhance the yield of pepper (Bhatt & Maheshwari, 2020). Under 100 and 150 mmol/L NaCl stress, *Lactobacilli* sp. can promote the germination of lettuce seeds by 25% and 15%, respectively (Hussein & Joo, 2018). Collectively, these studies underscore the indispensable role of beneficial microorganisms in plant growth, and also point to why MIs and GFL can offer environmentally friendly and novel strategies for improving the yield of *P. quinquefolium*.

Our study also proved that MIs and GFL lessen the severity of nematode disease in *P. quinquefolium*, especially those grown in GFL-treated plots, where the disease was inhibited by 64.35%. According to our LEfSe analysis results, GFL could increase the relative abundance of *Xylaria* by 219.39%. It has been reported that lectin produced by *X. hypoxylon* can inhibit the plant parasitic nematode *Ditylenchus dipsaci* (Zhao, Guo, Liu, Wang, & Ng, 2009). So, we speculate this certain genus may work as potential biocontrol agent against plant nematode diseases. Moreover, *Bacillus* and *Lactobacillus* are already in use as biocontrol agents of plant parasitic nematodes. The lethal concentration (LC<sub>50</sub>) of dichloromethane extract of *B. subtilis* and *B. cereus* was 204 g/ml, being far superior to that of furantol, a professional nematicide (Oliveira et al., 2014). The *L. brevis* WiKim0069 isolated from Kimchi has strong nematicidal activity against the second-stage juveniles of *M. incognita*, *M. arenaria*, and *M. hapla*, as well as *M. incognita* eggs. Moreover, the fermentation of WiKim0069 can also reduce the gall formation of melons under field conditions, and its control effect (62.8%) exceeds that of fosthiazate (32.8%) (Seo et al., 2019). These studies implied that MIs and GFL are effective at

alleviating the nematode disease of *P. quinquefolium*. To our best knowledge, this study is the first to report on the successful biological control of plant root-knot nematode disease by GFL.

#### 4.3. MIs and GFL could increase content of Rb1 and Re in *P. quinquefolium* roots

In our study, ginsenoside (Rb1 and Re) content increased by 9.05%–42.14% in the *P. quinquefolium* roots treated with different MIs and GFL. This result is consistent with previous findings that microbial inoculum and garbage fermentation liquid can improve the quality of Chinese medicinal herbs (Bao, Bu, Liu, & Ha, 2019; Dong et al., 2019). Additionally, many studies have reported on endophytic fungi promoting the accumulation of metabolites in medicinal plants (Park et al., 2019; Song et al., 2017). *Chaetomium* spp. are among those taxa beneficial to the biosynthesis of active ingredients in medicinal plants. It has been reported that *C. globosum* can significantly stimulate the secondary metabolism of *Salvia miltiorrhiza* (Zhai et al., 2017), *Anoectochilus roxburghii* (Ye et al., 2020), and *Chrysanthemum morifolium* (Song, Dai, Liu, & Cai, 2010). In our study, the abundance of *Chaetomium* was increased by 66.30%–153.82% in the four treatment groups. Although no study has yet to directly report that *Chaetomium* is associated with ginsenoside synthesis, the above studies do imply that *Chaetomium* can promote the accumulation of secondary metabolites in medicinal plants. Therefore, it could be possible to regulate the microflora related to metabolite accumulation of medicinal plants via the appropriate use of MIs and GFL, to stimulate metabolites' accumulation. In this study, under the TF and NF treatments, the relative abundances of *Nitrospira*, *Ktedonobacter*, *Conlarium*, *Corynascus*, and *Pithoascus* were significantly increased. It is estimated that only about 0.1%–10% of soil microorganisms have been studied well (Berg & Smalla, 2009), so we boldly speculate that some of these microorganisms we detected may be related to the accumulation of metabolites of *P. quinquefolium*, but more experimental studies are needed to test this hypothesis.



**Fig. 10.** Potential mechanism of microbial inoculants and garbage fermentation liquid regulating the soil microecology to enhance yield and reduced As content of *P. quinquefolium*. The green and red circles represent the beneficial and pathogenic microorganisms, respectively.

#### 4.4. MIs and GFL could reduce content of as in *P. quinquefolium* roots

In this study, MIs and GFL contributed to a decrease in the content of the heavy metal As in *P. quinquefolium*, especially under GFL (52.17%) and the PGA (43.48%) microbial inoculant. Furthermore, lower Cd and Pb contents were also found in the PGA and GFL groups. *Xylaria* is a genus that responds positively to the introduction of GFL, which involves the biodegradation of heavy metals (Wong et al., 2018). *Hyphomicrobium* is a genus whose members were significantly increased by the PGA microbial inoculant in our field experiment. *Hyphomicrobium* spp. may be used as Mn-oxidizing bacteria in biofilters, and they can participate in the removal of both As and Mn metallic elements (Yang, Li, Chu, Ren, & Zhang, 2014). These studies jointly suggest that these taxa of beneficial microorganisms may contribute to the reduction of heavy metal content in plant roots. Often *Bacillus* and *Lactobacillus* are used to bioremediate heavy metal-contaminated soils. *B. nealsonii* ARP2 can adsorb 93% of As (V) from an arsenic-containing medium, while *B. tequilensis* ART2 can adsorb 77% of As (III), and this bacterial strain's cell membrane can interact with As and accumulate in its cells (Pandey et al., 2019). *L. casei* DSM 20,011 is capable of removing As(V) (Halttunen, Finell, & Salminen, 2007). Overall, beneficial microorganisms related to the introduction of MIs and GFL, figured prominently in enabling the biological removal of heavy metals in this study. Therefore, both MIs and GFL should be considered as effective microbial remediation means for soil contaminated by heavy metals.

PICRUSt analysis based on 16S rRNA showed that the introduction of MIs and GFL provided a suitable rhizosphere environment for the bacteria which were related to nitrogen fixation, phosphorus solubilization, plant photosynthesis, mineral element absorption and plant pathogen interaction. Taken together, we speculated that application of microbial inoculants and garbage fermentation liquid, promoted *P. quinquefolium* yield and reduced the As content by improving the beneficial microorganisms and inhibiting pathogenic microbes in soil, as shown in Fig. 10, thus the introducing microbial inoculants and garbage fermentation liquid into *P. quinquefolium* cultivation has a broad application prospect.

## 5. Conclusion

In this study, field applications of microbial inoculants and garbage fermentation liquid were able to reduce the occurrence of

nematode diseases, promote the yield and effective components of *P. quinquefolium*, and reduce the As content in the medicinal materials. Metagenomics analysis confirmed the application of microbial inoculants and garbage fermentation liquid resulted in more potentially beneficial soil microorganisms—namely *Nordella*, *Nitrospira*, *Chaetomium*, *Hyphomicrobium*, *Microascus*, and *Xylaria*—and fewer potential harmful microorganisms, thus changing the structure and composition of the rhizosphere community. These results suggest microbial inoculants and garbage fermentation liquid could improve the structure and composition of the rhizosphere microbiota, and have positive effects on the quality of *P. quinquefolium*, both of them are beneficial to the development of ecological cultivation of *P. quinquefolium*.

## Authors' Contributions

XW and JH designed the research. PC and XW performed the research. PC and XW analyzed the data. PC wrote the manuscript. All authors have read and approved the final manuscript.

## 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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