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Phyllospheric application of *Bacillus mucilaginosus* mediates the recovery of tea plants exposed to low-temperature stress by alteration of leaf endophytic community and plant physiology

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Abstract

Background In winter, tea plants are highly susceptible to low-temperature freezing damage. The rapid recovery of tea plant vigor in spring is crucial for tea yield and quality. Some studies have reported that *Bacillus mucilaginosus* could improve the stress resistance of plants. However, there were no reports on the effect of *B. mucilaginosus* on the recovery of tea plant vigor after low-temperature stress. This study firstly used different concentrations of *B. mucilaginosus* to spray tea leaves and used 16S rRNA high-throughput sequencing technology to study the impact of different treatments on tea leaf endophytic populations. Meanwhile, physiological indexes such as Soil and plant analyzer development values (SPAD), maximum photochemical quantum yield of PS II (*Fv/Fm*), and superoxide dismutase (SOD) were measured and analyzed in tea plant leaves of different treatments, and the correlation between them and the bacterial community was studied.

Results Microbial results showed that the diversity of leaf endophytic populations treated with different concentrations of *Bacillus mucilaginosus* (T1, T2, T3) was higher than that in control group (CK) leaves, and T2 treatment had the highest diversity. The dominant bacterial phyla of all samples were Proteobacteria, Actinobacteriota, Firmicutes, and Bacteroidota. At the phylum level, the relative abundance of Actinobacteriota, Firmicutes, and Bacteroidota in leaves treated with *B. mucilaginosus* was significantly higher than that in the control. At the genus level, the relative abundance of *Paenibacillus*, *Nocardioides*, and *Marmoricola* in leaves treated with *B. mucilaginosus* was significantly higher than that in the control. Different concentrations of *B. mucilaginosus* affected the distribution of leaf endophytic populations. At the level of bacterial function, abundant metabolic functional features were observed, including amino acid transport and metabolism, as well as energy production

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and conversion, indicating that bacterial metabolism in tea plant leaf samples tends to be vigorous. The treatment with *B. mucilaginosus* significantly increased the activity of antioxidant enzymes and osmolyte content, promoted the recovery of *Fv/Fm* in tea plants after low-temperature stress, and improved the resistance of tea leaves to low-temperature stress, thereby promoting recovery.

Conclusions This study showed that *B. mucilaginosus* could significantly change the community structure of leaf endophytic populations, and increase antioxidant enzyme activity and osmolyte content in tea plants after low-temperature stress, promoting the rapid recovery of photosynthesis, and thereby benefiting the recovery of tea plant leaves. This study provided a theoretical basis for the application of *B. mucilaginosus* in practical production and also provided new ideas for the recovery of tea plants exposed to low-temperature stress.

Keywords *Camellia sinensis* (L.) O. Kuntze, Low-temperature stress, *Bacillus mucilaginosus*, Leaf endophytic populations

Introduction

In the cold winter, the tea plant is vulnerable to low-temperature damage, which affects the safety overwintering of tea plants. However, many tea farmers are unable to take timely overwintering protection measures for their tea gardens in winter, resulting in low-temperature stress on tea plants. This has a negative impact on the tea yield and quality in the coming spring, which may cause irreversible losses. Therefore, reasonable measures need to be taken to promote the recovery of tea plants after low-temperature stress, to reduce unnecessary losses.

Plant growth-promoting rhizobacteria (PGPR) is a class of beneficial bacteria that can enhance plants' ability to cope with abiotic stress through direct or indirect mechanisms [1–3]. *Pseudomonas*, *Enterobacter*, and *Bacillus* all belong to PGPR, among which *Bacillus* is more typical in enhancing plant resistance to cold stress [4, 5]. Research has shown that under cold stress, inoculation with *Bacillus* could improve the cold resistance of rice. *Bacillus mucilaginosus* is a special species of *Bacillus* that is widely used as a multifunctional microbial fertilizer in agriculture and industry [6]. Meanwhile, studies have shown that foliar spraying of PGPR not only has biological control functions but also promotes plant growth [7–9]. However, there are currently no reports on foliar spraying of *B. mucilaginosus* to improve tea plant stress resistance and promote its recovery after low temperatures.

In the process of agricultural production, the artificial application of foliar fertilizers and microbial agents can have a certain impact on the microbial community structure of plant leaves. The study has shown that spraying *Bacillus subtilis* could increase the abundance of endophytic bacteria in tomato leaves, such as *Bacillales*, *Burkholderiales*, *Rhizobiales*, *Pseudomonas*, and *Actinomycetale*, and enhance metabolic pathways related to secretion, stress, and mineral nutrition [10]. Meanwhile, the application of amino acid liquid fertilizer containing *Bacillus aminoliquefaciens* SQR9 on the leaves could effectively change the microbial community of cowpea leaves, while simple amino acid liquid fertilizer cannot [11]. Similarly, inoculation with beneficial bacteria can also have an impact on the microbial community of plant leaves. For example, inoculation with beneficial bacteria such as *Funneliformis mosseae* and *Lactobacillus plantarum* was able to alter the endophytic bacterial community of wheat, increasing the abundance of *Pseudomonas*, Actinobacteria, and Bacteroides [12]. In addition, a study has shown that inoculation of *Bacillus* species into sprouted broccoli could alter the properties of its endophytic bacterial community [13, 14]. Meanwhile, *B. mucilaginosus* can enhance plant cold resistance, drought resistance, disease resistance, and stress resistance by increasing the activity of antioxidant enzymes inside the plant and reducing stress-induced lipid peroxidation, thereby improving the plant's survival ability [15, 16]. And it can produce organic acids, amino acids, polysaccharides, hormones and other substances that are beneficial for plant absorption and utilization during the growth and reproduction process [17] and produce carbonic anhydrase, which has a certain effect on the fixation of carbon dioxide.Based on the above, we speculated that spraying *B. mucilaginosus* after low-temperature stress could affect the composition of endophytic bacterial communities in tea leaves.

The growth and development process of plants is often affected by a variety of environmental stresses, such as temperature, water, and heavy metals, which affect their normal growth and development, and endophytes within plants play an important role in overcoming their survival challenges [18]. Endophytic bacteria could directly or indirectly help the host in synthesizing compounds such as proline (Pro) and soluble sugars (SS) under stress conditions, or eliminate reactive oxygen species by increasing the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), thereby improving plant tolerance to stress [19]. The study has shown that endophytic bacteria *Burkholderia phytofirmans* PsJN could effectively enhance the antioxidant enzyme activity of wheat under drought

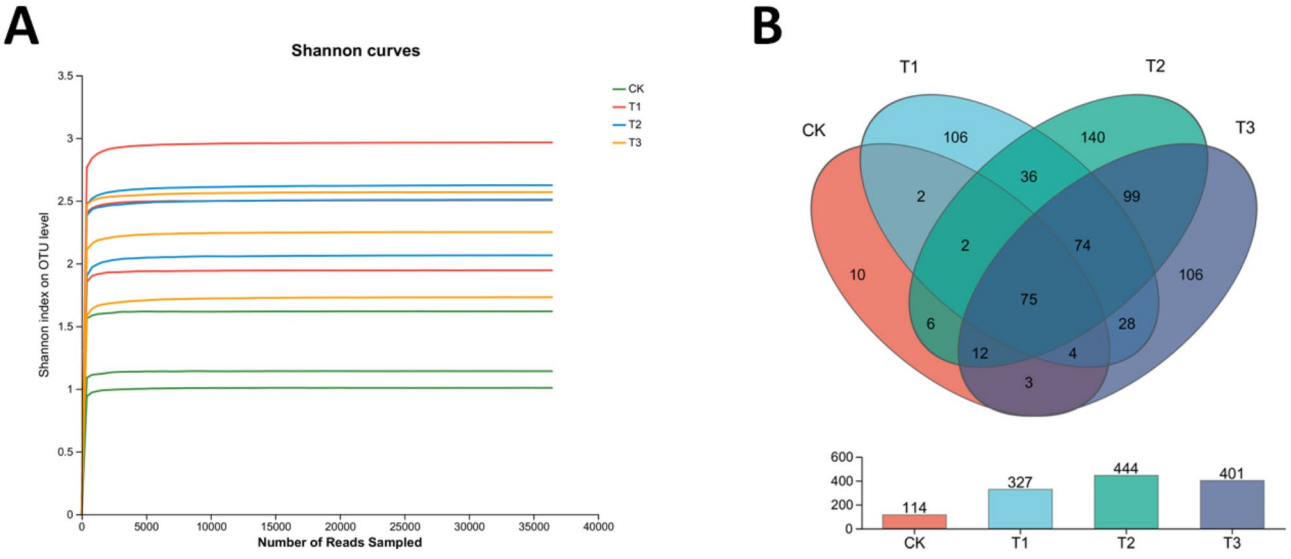

Fig. 1 Comparison of the bacterial communities in different treatments based on OTU numbers. (**A**) Rarefaction curves of bacterial communities for all treatments; (**B**) Venn diagram of OTU numbers for different treatments. CK: Spray water; T1: *Bacillus mucilaginosus* diluted 500 times; T2: *B. mucilaginosus* diluted 1000 times; T3: *B. mucilaginosus* diluted 2000 times

Table 1 Diversity index of leaf bacterial community under different treatments

Sample	Diversity index		Species richness		Coverage
	Shannon	Simpson	Ace	Chao	
CK	1.26 ± 0.26b	0.57 ± 0.10a	74.54 ± 9.51b	65.42 ± 4.49b	0.999
T1	2.47 ± 0.42a	0.19 ± 0.07b	161.06 ± 64.98ab	162.94 ± 68.02ab	0.999
T2	2.40 ± 0.24a	0.22 ± 0.09b	268.40 ± 62.32a	267.68 ± 61.80a	0.999
T3	2.18 ± 0.35a	0.25 ± 0.09b	247.02 ± 5.53a	247.87 ± 2.30a	0.999

The mean value ± standard deviation($n = 3$). Values with the same letter are not significantly different. ($P<0.05$)

stress, and improve crop yield and quality [20]. Endophytic bacterialsphingosine SaMR12 could reduce the concentration of H_2O_2 in plants by increasing the expression of glutathione reductase and related genes, thereby improving their tolerance to cadmium [21].

In this study, we used different concentrations of *Bacillus mucilaginosus* to spray tea leaves after exposure to low-temperature stress. We hypothesized that moderate application of *B. mucilaginosus* fertilizer on tea leaf surface could effectively promote the recovery of tea plant after low-temperature. And we attempted to analyze the effects of foliar spraying of *B. mucilaginosus* on the endophytic bacterial community and stress resistance indicators (SOD, CAT, and so on) of tea plants, and to identify the relationship between endophytic bacteria and stress resistance indicators.

Results

Effects of different concentrations of *Bacillus mucilaginosus* on bacterial community composition

In order to explore the effects of different concentrations of *Bacillus mucilaginosus* on the microbiome of tea plant leaves, 16S rRNA sequencing method was used to perform sequencing analysis on tea leaves treated with different concentrations (Table S1). Sparse curve analysis showed that each curve was nearly flat finally, indicating that the sequencing sample size was sufficient and the sequencing data was reasonable and acceptable (Fig. 1A). Through clustering operations, the optimized sequences were divided into Operational Taxonomic Units (OTUs) according to their similarity. With a similarity threshold of 97%, the sequences in the bacterial community were categorized into 703 OTUs using the RDP classifier Bayesian algorithm. In the bacterial community, the Venn diagram showed that the number of OTUs in T1 (327), T2 (444), and T3 (401) was significantly higher than that in CK (114), indicating that the spraying of *B. mucilaginosus* increased the number of OTUs (Fig. 1B).

In order to quantify the diversity and abundance of leaf endophytic populations under different treatments, the values of Chao, Ace, Simpson, and Shannon were calculated within a single microbial ecosystem α Diversity index (Table 1). The coverage rate of all 12 samples exceeded 0.99, indicating sufficient sequencing data. Analysis of variance (ANOVA) showed that different treatment conditions affected the leaf bacterial community. The bacterial abundance of *Bacillus mucilaginosus* treatment was significantly higher than that of the

control group (CK) ($P<0.05$). Therefore, foliar spraying of *B. mucilaginosus* could significantly affect the diversity of bacterial communities in tea leaves.

In order to intuitively study the species with higher relative abundance, we conducted taxonomic identification based on the species annotation results. The dominant bacterial phyla were Proteobacteria、Actinobacteriota、Firmicutes and Bacteroidota (Fig. 2A). The relative abundance of Proteobacteria decreased under the treatment of *Bacillus mucilaginosus*, while the relative abundance of Actinobacteriota, Firmicutes, and Bacteroidota increased under the treatment of *B. mucilaginosus*. In addition, T1 resulted in the highest relative abundance of Acidobacteriota among all treatments. Compared with CK, T3 reduced the relative abundance of Acidobacteriota (Fig. 2B). The dominant bacterial genus were *unclassified_f__Alcaligenaceae*、*Paenibacillus*、*Nocardioides*、*Marmoricola*、*Actinomycetospora*、*Pseudonocardia*、*Sphingomonas*、*Rhodococcus*、*Bacillus* and *Delftia*. Among them, *Actinomycetospora* was very close to *Pseudonocardia* in the phylogenetic tree; *Paenibacillus* was next to *Bacillus* (Fig. 2C).

Effects of different concentrations of *Bacillus mucilaginosus* on bacterial community structure

In order to study the effects of different treatments on the distribution of tea leaf endophytic populations, we calculated beta diversity based on dimensionality reduction analysis. The analysis showed that different concentrations of *Bacillus mucilaginosus* treatment affected the distribution of leaf bacterial communities (Fig. 3). The PCA diagram ($R=0.3395$, $P=0.0360$) showed differences in the bacterial communities among T2, T3, and CK (Fig. 3A). In the PCoA diagram ($R=0.3765$, $P=0.0250$), there was a significant separation of bacterial communities between the treatment with *B. mucilaginosus* (T1, T2, and T3) and CK, while there was no significant difference between the bacterial communities treated with *B. mucilaginosus* (Fig. 3B). The NMDS analysis results ($R=0.3765$, $P=0.0250$) were consistent with PCoA (Fig. 3C). In order to emphasize the differences between groups, PLS-DA was implemented using supervised algorithms. The results showed that there were significant differences in bacterial communities among different treatments. In addition, the two bacterial communities treated with CK and T1 clustered in the

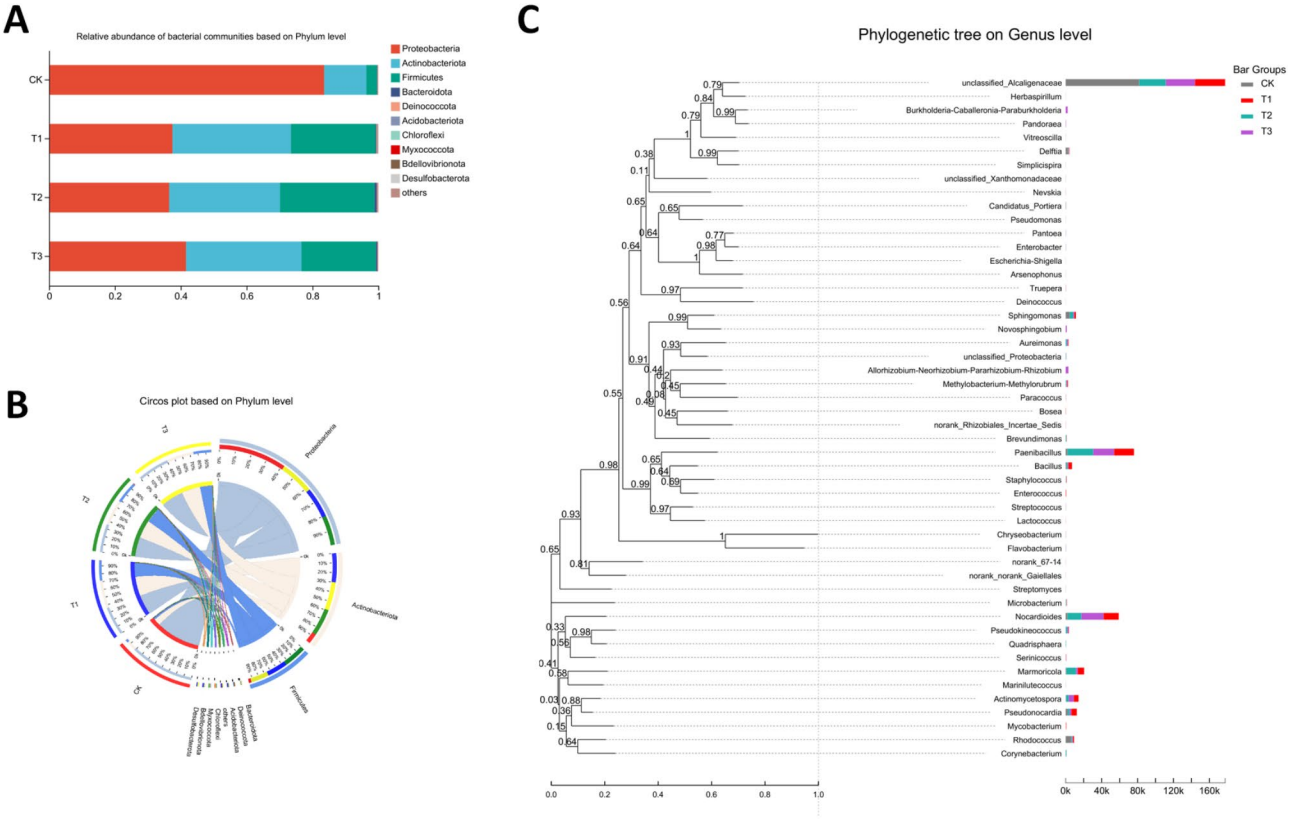

Fig. 2 Bacterial community composition in different treatments. (**A**) Bar plot of relative abundance of bacterial communities based on phylum level; (**B**) Circos plot of different treatments based on phylum level; (**C**) Composition and phylogenetic tree of bacterial communities with different treatments based on genus level

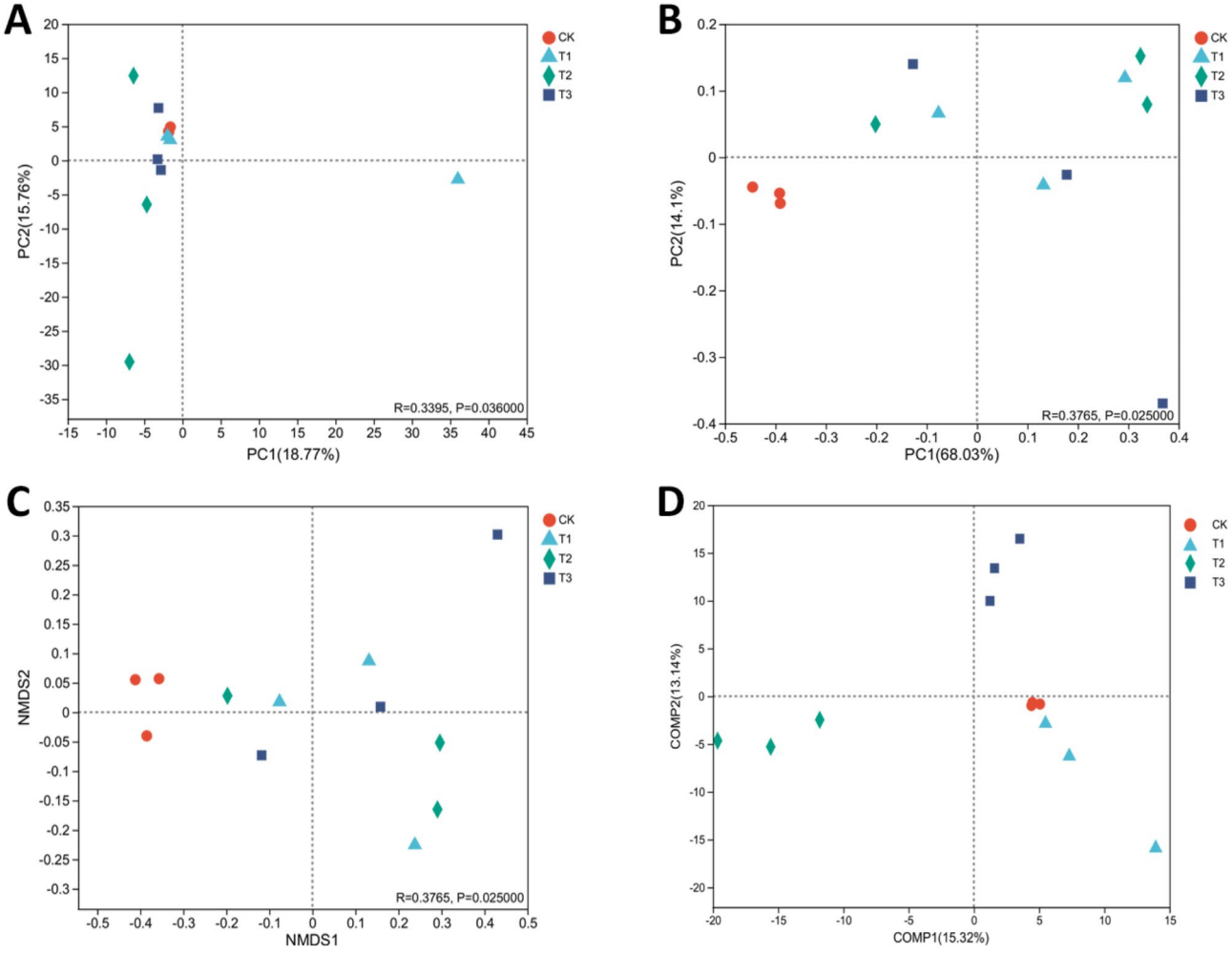

Fig. 3 Combined plot integrated with the PCA (**A**), PCoA (**B**), NMDS (**C**), and PLS-DA (**D**) plots of the bacterial communities corresponding to beta diversity

same quadrant, while the bacterial communities of T2 and T3 were located in the other two different quadrants (Fig. 3D).

In order to further explore the differences in bacterial community structure among different treatments, we conducted corresponding studies on different genus. The top 50 genera from all treatments were scanned and the heatmap was drawn based on their relative abundance. The results showed that there were differences in bacterial composition among different treatments, and the difference in bacterial community composition between the treatments of *Bacillus mucilaginosus* (T1, T2, T3) and CK was greater than the difference between T1 and T2, which was consistent with the results of PCoA, NMDS, and PLS-DA. The 50 genera shown in the Heatmap belong to the five main phyla: Proteobacteria, Firmicutes, Actinobacteriota, Bacteroidota, and Deinococcota. Most of the dominant genus of CK treatment belong to Actinobacteriota, while most of the dominant genus of *B. mucilaginosus* treatment belong to Proteobacteria (Fig. 4A).

In order to identify the bacterial communities with significant differences in leaves between different treatments, the LEfSe tool was used to further analyze the bacterial communities from phylum to genus, and a total of 34 taxa were analyzed as biomarkers for corresponding treatments (Fig. 4B). Among them, 19 taxa were identified at the genus level, of which 2, 1, 15, and 1 were considered biomarkers for CK, T1, T2, and T3 (Fig. 4B). Considering the relative abundance of each bacterial genus, investigated the top 10 genera. The results showed significant differences in species composition at the genus level among different treatments, with both *Marmoricola* and the *Corynebacterium* significantly enriched in the *Bacillus mucilaginosus* treatment, with their relative abundances both being highest in T2. In addition, the relative abundance of *Sphingomonas* decreased in the *B. mucilaginosus* treatment (Fig. 4C).

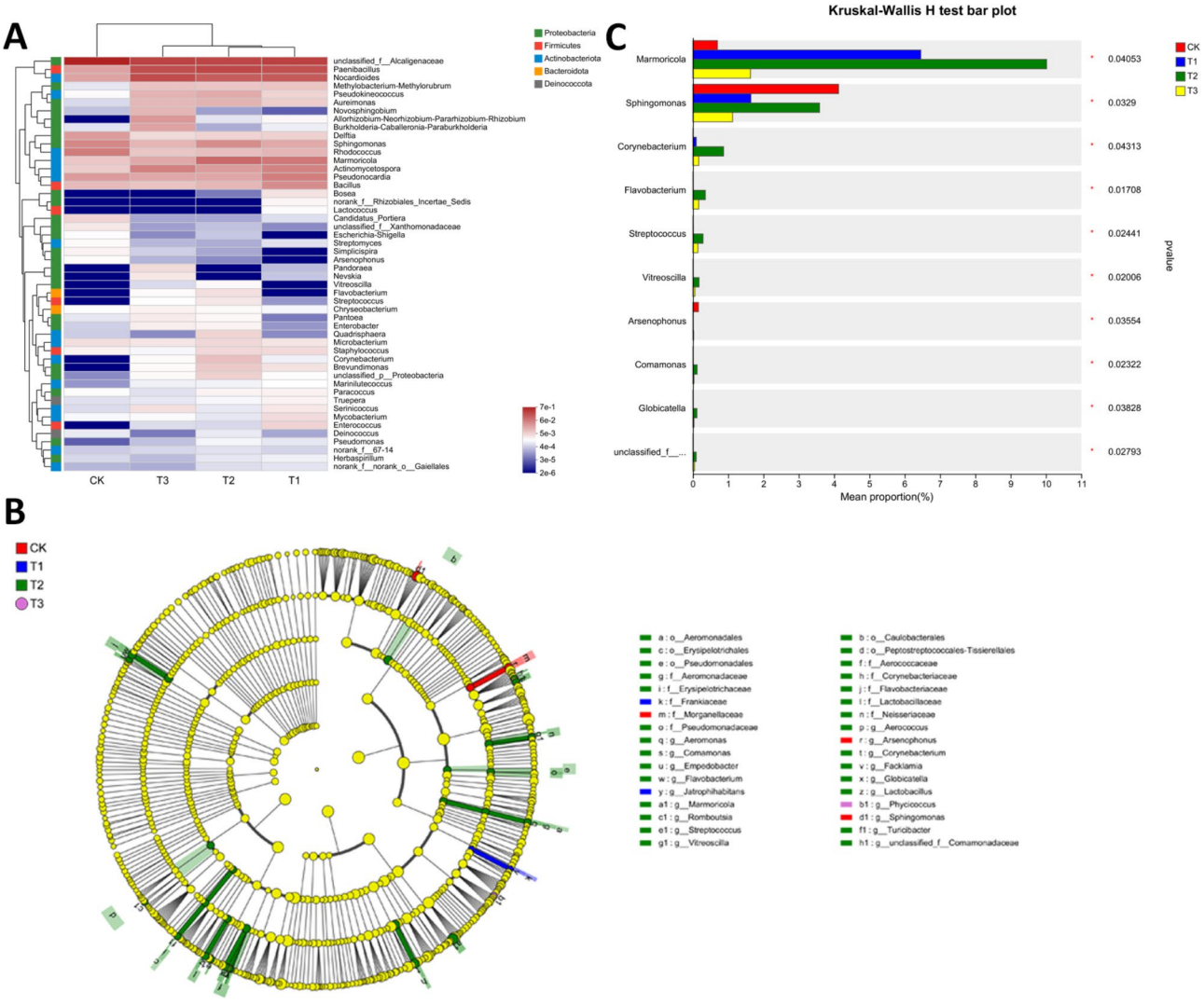

Fig. 4 Variation of the bacterial communities in different treatments based on genus. (**A**) Heatmap of the top 50 genera based on logarithmic statistics of identified OTUs among different treatments; (**B**) LDA Effect Size (LEfSe) analysis based on the genus among different treatments; (**C**) Bar plot of relative abundance of the top 10 genera among different treatments based on the Kruskal-Wallis test

Prediction of leaf bacterial community function

In order to better study the effect of the application of *Bacillus mucilaginosus* on the function of the bacterial community in tea plant leaves, PICRUSt was used to analyze the bacterial community in the Cluster of Orthologous Groups (COG) database. The results showed that different treatments had certain effects on the functional features of bacteria. It can be observed that the metabolic function is abundant in our samples, which indicates that the bacterial metabolism in the tea leaf samples tends to be vigorous. These functional features include amino acid transport and metabolism; inorganic ion transport and metabolism; energy production and conversion; transcription; carbohydrate transport and metabolism; lipid transport and metabolism; coenzyme transport and metabolism; and nucleotide transport and metabolism. It is worth noting that these functions are most relatively abundant in CK. Additionally, in T1, T2, and T3, these functions have the lowest relative abundance in T2, such as amino acid transport and metabolism, organic transport and metabolism, energy production and conservation, and so on (Fig. 5).

Effects of different concentrations of *Bacillus mucilaginosus* on activity of antioxidant enzymes and the content of osmolyte

In order to evaluate the effects of different concentrations of *Bacillus mucilaginosus* sprayed on leaf surface on leaf physiological function, relevant leaf physiological indexes were measured (Table 2). The results showed that the content of malondialdehyde (MDA) was CK > T1 > T3 > T2, and the relative conductivity (REC)

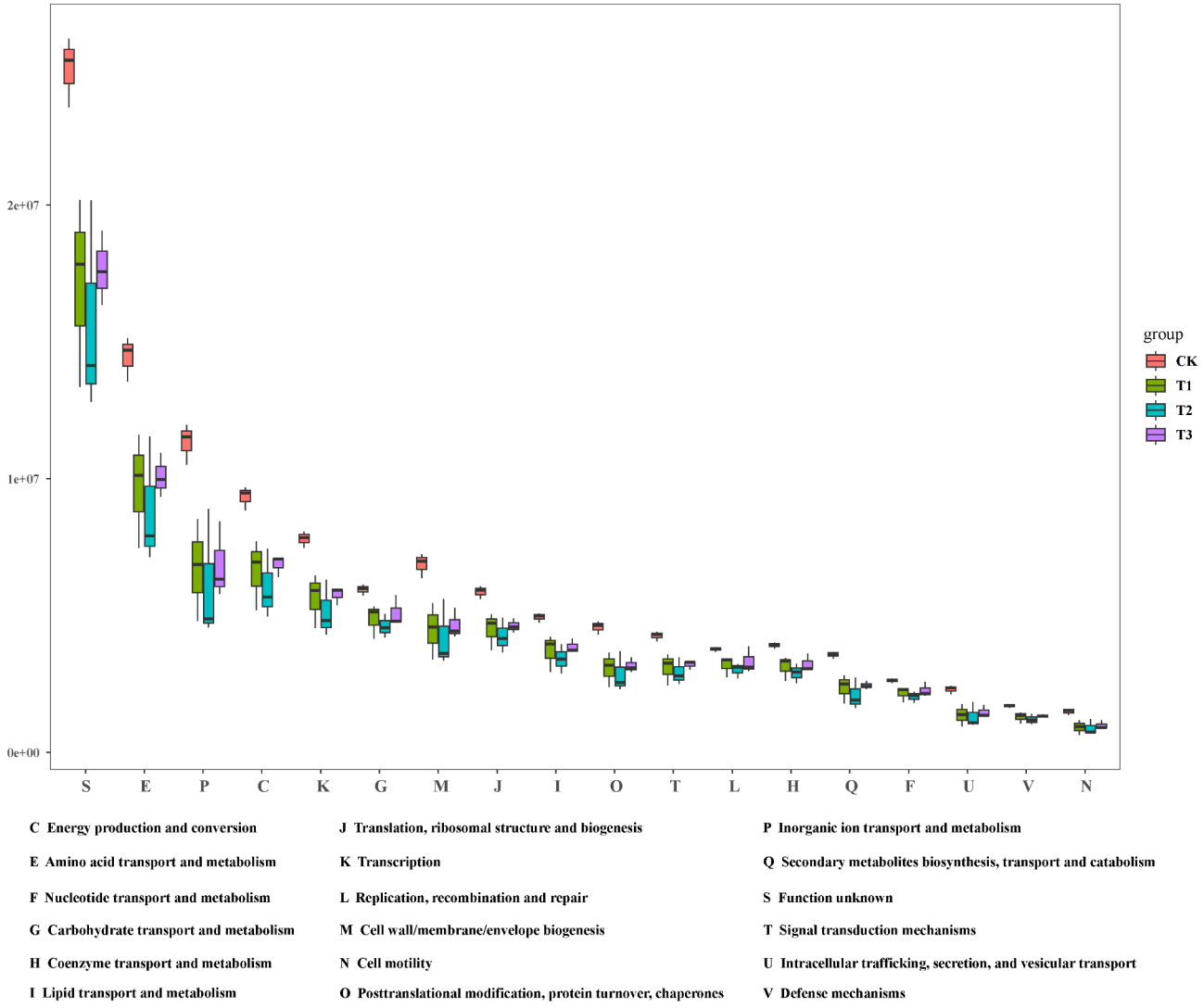

Fig. 5 The bacterial communities functional features of leaf under different treatments

Table 2 Physiological indexes of leaves under different treatments

	MDA (nmol/g)	REC	SP (mg/g)	SS (mg/g)	SOD (U/g)	POD(ΔOD_{470}/min/g)	CAT(μmol/min/g)
CK	18.59 ± 2.07a	0.33 ± 0.01a	0.16 ± 0.01c	32.61 ± 1.34b	134.22 ± 7.89b	110.20 ± 23.51b	9.81 ± 0.74b
T1	17.75 ± 0.54ab	0.30 ± 0.01bc	0.18 ± 0.01c	35.84 ± 2.22ab	136.95 ± 6.63b	133.78 ± 20.77b	11.14 ± 0.74b
T2	14.22 ± 0.74c	0.31 ± 0.01b	0.27 ± 0.01a	42.20 ± 6.39a	138.85 ± 1.89ab	459.19 ± 94.53a	13.48 ± 1.17a
T3	15.53 ± 0.31bc	0.29 ± 0.01c	0.22 ± 0.00b	35.12 ± 0.51ab	150.34 ± 1.14a	346.43 ± 58.91a	10.30 ± 0.86b

The mean value ± standard deviation($n = 3$). Experimental data were analyzed by the Duncan multiple-range test, with $P < 0.05$ considered statistically significant, and multiple comparison results were marked by the letter-marking method, Values with the same letter are not significantly different. MDA malondialdehyde, REC relative conductivity, SP soluble protein, SS soluble sugar, SOD superoxide dismutase, POD peroxidase, CAT catalase

content was CK > T2 > T1 > T3, indicating that spraying *B. mucilaginosus* on the leaf surface could alleviate the degree of damage to leaf membranous caused by low-temperature stress. Soluble protein (SP): T2 > T3 > T1 > CK, soluble sugar (SS): T2 > T1 ≈ T3 > CK, indicating that *B. mucilaginosus* could promote the accumulation of carbon and nitrogen substances. The content of superoxide dismutase (SOD) was T3 > T2 > T1 > CK, the content of peroxidase (POD) was T2 > T3 > T1 > CK, and the content of catalase (CAT) was T2 > T1 > T3 > CK. The enzyme activity of the treatment group (T1, T2, T3) was significantly higher than that of the control group (CK), indicating that *B. mucilaginosus* could improve antioxidant enzyme activity and play a role in low-temperature recovery.

Effects of different concentrations of *Bacillus mucilaginosus* on soil and plant analyzer development values (SPAD) and maximum photochemical quantum yield of PS II (Fv/Fm) in tea plants

The SPAD value is a parameter that evaluates the plant's relative photosynthetic pigments or indicates the plant's degree of greenness. As shown in Fig. 6A, the SPAD value under CK (70.583) was significantly lower than that under *Bacillus mucilaginosus* treatment T1 (74.550), T2 (78.950), and T3 (75.583). Among them, the SPAD value with T2 was the highest.

In order to understand the effect of different concentrations of *B. mucilaginosus* on the potential quantum efficiency of tea plant leaves, *Fv/Fm* (maximum photochemical quantum yield of PS II) values were measured. We measured the *Fv/Fm* of tea seedlings during the low-temperature process, and the results showed that during the low-temperature treatment process, the *Fv/Fm* value continued to decrease with the increase of low-temperature time, and ultimately decreased to around 0.55 (Fig. 6B). During the low-temperature recovery period, with the continuous increase of spraying frequency, *Fv/Fm* values under CK and *B. mucilaginosus* treatments (T1, T2, and T3) as the number of sprays increased. However, the *Fv/Fm* values of *B. mucilaginosus* treatments were significantly higher than those of the control, indicating that foliar spraying of *B. mucilaginosus* after low-temperature stress could improve the light energy utilization efficiency of tea leaves, thereby promoting the growth and recovery of tea leaves. Among them, the *Fv/Fm* values of T2 were consistently higher than those of other treatments (Fig. 6C), indicating that foliar spraying of *B. mucilaginosus* diluted 1000 times had the best recovery effect.

The Relationship between the dominant phyla of leaf bacterial community and physiological indexes

We used the Spearman correlation heatmap to analyze the relationship between bacterial dominant phyla and the main physiological indexes of leaves (Fig. 7). The results showed that the abundance of Bacteroidota was significantly negatively correlated with the MDA content in leaves, while the abundance of Abdidibacterota was positively correlated with the SOD content in leaves. At the same time, the abundance of Firmicutes was positively correlated with the contents of SP, SS, POD, CAT, *Fv/Fm*, and SPAD in leaves. The abundance of Bacteroidota was positively correlated with SP content, *Fv/Fm*, and SPAD in leaves. The abundance of Fusobacteriota was positively correlated with the contents of SS and CAT in leaves. However, Proteobacteria showed a significant negative correlation with SS content, *Fv/Fm*, and SPAD in leaves. The results indicated that the bacterial community structure of leaves might have a significant impact on physiological indexes.

Discussion

Bacillus mucilaginosus increased the diversity of endophytic bacteria in tea plants after low-temperature stress

The diversity of plant endophytes plays an important role in promoting plant adaptation to adverse environments (low temperature, drought, etc.) as well as plant growth and development [22–28]. The previous study has shown that foliar spraying could increase the biodiversity of plant microbiota [29]. The results of this study indicated that the treatment with *Bacillus mucilaginosus* significantly increased the diversity and richness of bacterial communities in tea leaves. Therefore, we speculated that *B. mucilaginosus* could increase the low-temperature resistance of tea plants by increasing the diversity of bacterial communities, thereby promoting the low-temperature recovery of tea plants. On the other hand, we analyzed the distribution of bacterial communities in tea leaves treated with different concentrations of *B. mucilaginosus*. The results of the bacterial community further revealed a clear separation between the *B. mucilaginosus* treatment and the control treatment,

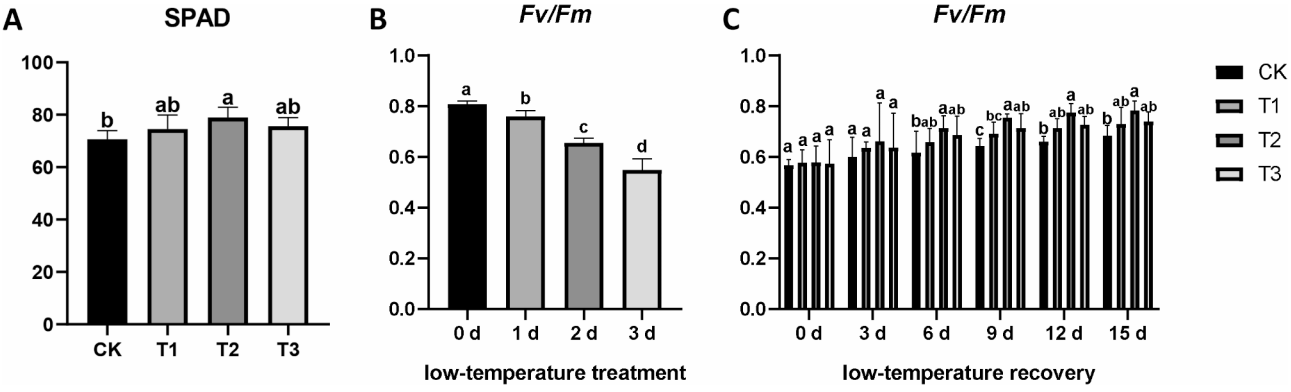

Fig. 6 (**A**) SPAD value of the day of sample collection; (**B**) Changes in *Fv/Fm* under low-temperature treatment conditions; (**C**) Changes in *Fv/Fm* under different treatments during low-temperature recovery. Lowercase letters represent significant differences. ($P < 0.05$)

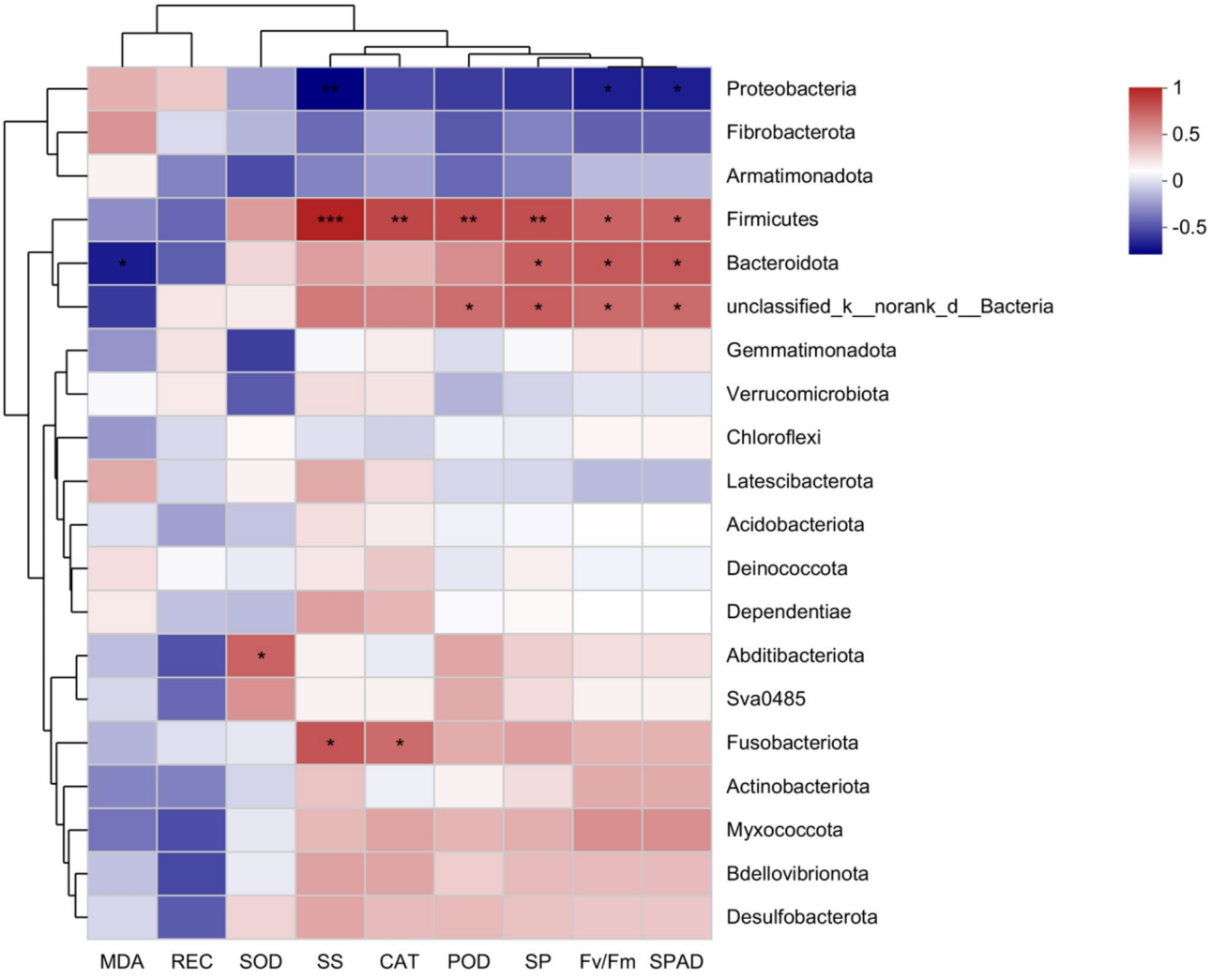

Fig. 7 The Spearman correlation heatmap between leaf physiological indexes and leaf bacterial communities under different treatments

indicating differences in the structure of the leaf bacterial community under the *B. mucilaginosus* treatment and the control treatment (Fig. 3). It can be seen that different and relatively independent micro-ecosystems were constructed between the treatment of *B. mucilaginosus* and the control treatment. Compared with the control, a more abundant and diverse microecological environment could be obtained by spraying *B. mucilaginosus* on the leaf surface.

The treatment with different concentrations of *Bacillus mucilaginosus* had an impact on the bacterial community structure of tea plant leaves. In this study, the composition of the tea leaf bacterial community was analyzed at the phylum level, and the results showed that the dominant phyla of tea leaf bacteria were Proteobacteria, Actinobacteriota, Firmicutes, and Bacteroidota (Fig. 2A). The study has shown that endophytic bacteria could promote plant growth, improve nutrient absorption, and enhance adaptability to climate change [30]. Proteobacteria and Firmicutes were common plant endophytic bacteria with relative abundances ranging from 39 to 97% and 14–44%, respectively [31, 32]. Firmicutes have thick cell walls, mostly spherical or rod-shaped, and most can produce spores, which can resist extreme environments such as drought and low temperatures. Some of these genera could promote photosynthesis [33–35]. If the plant is subjected to adverse factors such as stress that affect its normal growth, its Actinobacteriota will also be affected. At this time, Actinobacteriota will directly or indirectly affect the stress response of plants, enhance their tolerance to stress, and promote plant growth [36]. Our results showed that the relative abundance of Actinobacteriota and Firmicutes in tea leaves treated with *B. mucilaginosus* was higher than that in water treatment, indicating that the use of *B. mucilaginosus* might increase the abundance of Firmicutes to produce more spores to resist low-temperature environment. At the same time, increasing the abundance of Actinobacteriota could

stimulate the stress response of tea plant leaves, thereby improving the resistance of tea plants, and thus promoting the recovery and growth of tea plants after low temperatures. The relative abundance of Bacteroidota in tea leaves treated with *B. mucilaginosus* was higher than that in water treatment. The study has shown that Bacteroidota plays an important role in plant tolerance in response to adversity [37]. This indicated that Bacteroidota might play an important role in promoting the recovery of tea plants after low temperatures by *B. mucilaginosus*. In addition, we further explored the significant accumulation of *Marmoricola* and *Paenibacillus* at the genus level (Fig. 2C). *Marmoricola* has been shown to promote plant growth [38] and resist abiotic stresses [39]. In this study, treatment with *B. mucilaginosus* significantly increased the relative abundance of *Marmoricola*. Studies have shown that *Paenibacillus* isolated from plant internal tissues could directly or indirectly promote the growth of host plants [40–45]and have the potential to promote plant growth at low temperatures [46]. Foliar spraying of *B. mucilaginosus* has the potential advantage of exogenous *Bacillus* intervention, significantly improving the competitive advantage of the *Paenibacillus*. These results suggested that *B. mucilaginosus* might promote the recovery of tea plants after low temperatures by increasing the abundance of *Marmoricola* and *Paenibacillus*.

In addition, the PICRUSt functional prediction tool was used to conduct preliminary functional prediction of endophytic bacterial communities in tea leaves. The results showed that our samples had abundant metabolic functions, mainly concentrated on amino acids, inorganic ions, and lipid metabolism. Amino acid metabolism and lipid metabolism are metabolic processes that are essential for the survival of microorganisms [47]. Among them, amino acid transport and metabolism are mainly involved in amino acid decomposition and transformation and protein synthesis, providing essential energy for microorganisms [48, 49]. This indicated that during the low-temperature recovery process, microorganisms could continuously synthesize organic matter and various functional proteins through limited metabolic pathways to maintain cell activity and enhance their adaptability to the external environment.

Bacillus mucilaginosus increased the activity of antioxidant enzymes and the content of osmolyte in tea plants after low-temperature stress

After being subjected to low-temperature stress, plants will produce excessive reactive oxygen species (ROS) to damage lipid membranes and cause damage to plants [50, 51]. Malondialdehyde (MDA) is a common product of lipid peroxidation, reflecting the degree of oxidative damage [52]. In this study, the MDA content was significantly lower under the treatment of *Bacillus mucilaginosus* than under the treatment of water, indicating that *B. mucilaginosus* could alleviate oxidative damage caused by low-temperature stress. In addition, protective enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the antioxidant enzyme system can eliminate excess ROS, thereby reducing the oxidative damage caused by reactive oxygen species to plants under low-temperature stress. Among them, SOD can eliminate superoxide anion radicals through disproportionation reactions, while POT and CAT can effectively decompose H_2O_2 produced by stress [53]. When plants respond to the excessive accumulation of ROS under low temperatures, the activities of SOD, POD, and CAT will significantly increase [54, 55]. The accumulation of osmolytes such as soluble proteins (SP) and soluble sugars (SS) is another adaptation mechanism of plants to adverse environments [56]. In this study, the activities of SOD, POD, and CAT, as well as the content of SP and SS were significantly higher under the treatment of *B. mucilaginosus* than those under the treatment of water (Table 2), indicating that *B. mucilaginosus* could increase the activity of antioxidant enzymes and the content of osmolyte, effectively alleviate oxidative damage induced by low-temperature stress, and promote the low-temperature recovery of tea plants.

Previous studies have shown that endophytic bacteria could induce antioxidant enzyme activity by reducing the degradation of unsaturated fatty acids, thereby removing reactive oxygen species and improving plant resistance [57]. In this study, the spraying of *B. mucilaginosus* changed the composition of the bacterial community in tea leaves. In addition, Spearman correlation heatmap analysis showed a significant positive correlation between Firmicutes and POD, CAT, SP, and SS (Fig. 7). Some studies have shown that the total chlorophyll content and SOD activity of wheat [58] and CAT activity of *Medicago truncatula* seedlings increased significantly after inoculation with *Paenibacillus* [59]. Meanwhile, *Bacillus* strain could significantly increase the activities of SOD, CAT and POD in rice [60], and significantly promote the growth of tomato plant and the activities of SOD, POD and CAT [61]. Both *Paenibaclillus* and *Bacillus* belong to Firmicutes. In this study, CAT and POD are highly positively correlated with Firmicutes, indicating that Firmicutes plays an important role in improving the activity of related antioxidant enzymes in tea tree during low-temperature recovery. Therefore, spraying *B. mucilaginosus* might regulate leaf physiological activities by altering the bacterial community in the leaves.

Bacillus mucilaginosus increased SPAD value and *Fv/Fm* in tea plants after low-temperature stress

The chlorophyll content of higher plants directly determines the intensity of photosynthesis and indirectly

reflects the plant's resilience [62]. Low-temperature stress can cause a decrease in chlorophyll content, inhibiting the absorption and utilization of light energy by plants [63]. Therefore, leaf chlorophyll content is usually used as an indicator to measure the overall health status of plants after stress. There is a positive correlation between SPAD value and chlorophyll content [64]. Therefore, we determined the SPAD value of tea leaves under different treatments. In this study, the SPAD value of the control treatment was significantly lower than that of the *Bacillus mucilaginosus* treatment, indicating that spraying *B. mucilaginosus* on the leaves after low-temperature stress could significantly increase the SPAD value of tea leaves and increase the chlorophyll content of tea leaves, thus effectively alleviating the damage of low temperature on the photosynthetic system of tea leaves, and enhancing the adaptability and recovery of photosynthetic system to low temperature.

The *Fv/Fm* (maximum photochemical quantum yield of PS II) value of plants reflects the potential quantum efficiency of PS II and is used as a sensitive index of plant photosynthetic performance. The optimal *Fv/Fm* value for most plant species is around 0.83 [65–67]. When the plant is grown in a suitable environment, *Fv/Fm* remains in a stable range (around 0.83), but when the plant is subjected to environmental stress (low-temperature, drought, etc.), the *Fv/Fm* value decreases [68]. In this study, during low-temperature treatment, the *Fv/Fm* value gradually decreased and finally dropped to about 0.55 (Fig. 6B), indicating that low-temperature caused great stress to tea plants. During the low-temperature recovery period, *Fv/Fm* values increased under all treatments, but the *Fv/Fm* values of CK were lower than those of T1, T2, and T3, and the *Fv/Fm* values of T2 were the highest. At 15 d, the *Fv/Fm* value of CK was about 0.68, while T1, T2, and T3 were above 0.73, among which T2 could reach 0.8 (Fig. 6C). These indicated that the tea plant of CK had not yet returned to its normal state, while the tea plant of T1, T2, and T3 had recovered to a greater extent, and T2 had returned to its normal state. The above results indicated that foliar spraying of *B. mucilaginosus* was beneficial for the recovery of tea plants after low temperatures, with T2 (diluted 1000 times) having the best effect.

In addition, spearman correlation heatmap analysis showed a significant positive correlation between Firmicutes, Byacteroidota, and *Fv/Fm*, SPAD. *Bacillus* belongs to the Firmicutes. Studies have shown that inoculation with *Bacillus* could increase the content of chlorophyll, soluble sugars, and carotenoids in maize seedlings [69]. Similarly, the endophytic bacterium *Bacillus pumliu* significantly increased the *Fv/Fm* value and total chlorophyll content of sugar beets [70]. The above results indicated that spraying *B. mucilaginosus* on tea leaves after low-temperature stress might increase the chlorophyll content by increasing the abundance of the genera in the Firmicutes phylum, thereby promoting the recovery of *Fv/Fm*.

Conclusion

This study revealed the influence of the Phyllospheric application of *Bacillus mucilaginosus* on bacterial diversity and community structure in tea leaves after low-temperature stress. Compared with clean water, *B. mucilaginosus* significantly increased the diversity of leaf bacteria and effectively regulated the bacterial community structure. Meanwhile, the use of *B. mucilaginosus* improved antioxidant enzyme activities and increased the chlorophyll content of leaves, which promoted the rapid recovery of photosynthesis in tea leaves after low-temperature stress. In summary, this study provided a new idea for the recovery of tea plants after low-temperature stress, which has important significance for the application of *B. mucilaginosus* in tea gardens.

Materials and methods

Plant materials and treatment

This study was conducted at Qingdao Agricultural University (Qingdao, Shandong, China). One-year-old *C. sinensis* '*Longjingchangye*' tea seedlings (Purchased from Yarun Tea Co., Ltd. (Nanjing, China)) were used as test materials and were subjected to low-temperature treatment (daytime 4℃, 16 h/nighttime 0℃, 8 h) for three consecutive days. Different concentrations of *Bacillus mucilaginosus* (this bacterial fertilizer came from Xingtai Sinobest Biotechnology Co., Ltd, Hebei, China, the effective number of viable bacteria: 5.0×10^{10}CFU/g; the registration number of this product is "Microbial Fertilizer (2020) approval number (8593)) were sprayed on the leaf surface of tea seedlings after low-temperature stress. The experiment was divided into four groups, with 48 tea seedlings in each group. Distilled water (control group, CK), diluted 500 times (T1, 1×10^8 CFU/mL), diluted 1000 times (T2, 5×10^7 CFU/mL), and diluted 2000 times (T3, 2.5×10^7 CFU/mL) of *B. mucilaginosus* were sprayed on the surface of tea seedling leaves in equal amounts, respectively. The spraying standard was based on the presence of water droplets dripping down, spraying once every 3 d, a total of 5 times. Environmental conditions: day 16 h (12 ℃)/ night 8 h (10 ℃), light intensity 11000 Lux, air humidity 80 ± 5%. We used 48 tea seedlings for each treatment, which is a sufficient sample size.

Sample collection

On the third day after the fifth spraying treatment, samples were taken from the 2-4th mature leaves at the top of the tea seedling. Sixty leaves of tea plants were collected from each of the four groups of treatments, and each

group of leaves was randomly divided into six replicates with 10 leaves each. Three replicates were used for the determination of leaf physicochemical indexes, and the other three replicates were used for the determination of the microbiome. The samples used for physiological index determination were frozen in liquid nitrogen and stored in an ultra-low temperature refrigerator at -80 ℃. The microbiome determination samples were disinfected on the surface, washed with distilled water to remove surface dust and other substances, then put into test tubes, 10mL of 75% alcohol was added and soaked for 3 min. Then, the samples were rinsed with sterile distilled water three times and soaked in 5% sodium hypochlorite for 5 min. After being rinsed with sterile distilled water three times, and then absorbed the water with sterile filter paper, the samples were wrapped in aluminum foil and stored in an ultra-low temperature refrigerator at -80℃ for DNA extraction.

Determination of soil and plant analyzer development values (SPAD) and maximum photochemical quantum yield of PS II (Fv/Fm)

On the day of sample collection, the soil and plant analyzer development values (SPAD) of the mature leaves under different treatments were measured using the TYS-4 N plant nutrient meter (Zhejiang Topo Yunnong Technology Co., Ltd., China). The third mature leaf at the top of the tea plant was randomly selected, avoiding the leaf veins, and the upper, middle, and lower parts of the leaf were measured once, and the average value was calculated. Each treatment had six replicates.

The leaves were fully dark acclimated for 20 min using a dark acclimation clip, and the maximum photochemical quantum yield of PS II (*Fv/Fm*) of tea leaves was determined using the FP110-LM/D instrument (PS I, Czech Republic). Each treatment had six replicates.

Determination of soluble sugar (SS) and soluble protein (SP) content

The contents of soluble sugar and soluble protein were determined with the kit of Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). Each treatment had three replicates.

Determination of malondialdehyde (MDA) content and relative electrical conductivity (REC)

The content of malondialdehyde was determined with the kit of Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). Relative electrical conductivity (REC) was measured using a conductivity meter (DDSJ-308 A, China). Each treatment had three replicates.

Determination of antioxidant enzyme activity

The activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) were determined according to the kit of Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). Each treatment had three replicates.

DNA extraction and PCR amplification

Microbial DNA was extracted from 12 tea plant leaf samples under four treatments using the DNeasy® PowerSoil® Pro Kit (QIAGEN, USA) according to the manufacturer's protocol. Subsequently, the extracted genomic DNA was detected by 1% agarose gel electrophoresis. Specific primer 799F_1193R (5-AACMGGATTAGATACCCKG-3\5-ACGTCATCCCCACCTTCC-3) was used to amplify the V5-V7 variable region of bacterial 16S rRNA gene. Amplification products were specifically detected by 2% agarose gel electrophoresis and further purified and quantified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and QuantiFluor™ -ST Blue Fluorescence Quantification System (Promega, USA), respectively.

Illumina sequencing and sequencing data processing

The library construction kit TruSeqTM DNA Sample Prep Kit (Illumina, San Diego, USA) was used for library construction. After the library was successfully constructed, quantification was performed using Qubit®2.0 fluorometer (Life Technologies, Carlsbad, USA) and qPCR. After quantitative detection, subsequent sequencing was performed on the Illumina MiSeq platform. The sequenced raw data were spliced through FLASH (V1.2.11, https://ccb.jhu.edu/software/FLASH/index.shtml) to obtain the original sequence. Then used the QIIME software (V1.9.1, http://qiime.org/install/index.html) for bioinformatics analysis. Uparse (V11.0, http://www.drive5.com/uparse/) was used to Cluster the operational classification units (OTUs). The classification of each 16 S rRNA gene sequence was analyzed by the RDP classifier algorithm (70% confidence threshold) (http://rdp.cme.msu.edu/) with the SILVA database (https://www.arb-silva.de/) as a control.

Data analysis

Microbial diversity was analyzed using Alpha diversity analysis, including Chao, Shannon, Ace, Simpson, and sparse curves, and calculated using Mothur software (V1.30.2, https://www.mothur.org/wiki/Download_mothur). Based on the Bray-Curtis distance of R3.3.1, principal coordinate analysis (PCoA) was applied to reduce the dimensionality of the original variables. The Circos diagram was built using Circos – 0.67-7 software (http://circos.ca/). Linear discriminant analysis (LDA) combined with effect quantity measurement (LEfSe) analysis, used

LEfSe software (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload) to Search for biomarkers with statistical differences between different treatments. The relationship between leaf bacterial community structure and leaf physiological indexes was analyzed by the Spearman correlation heat map using the pheatmap package. Based on the interactive Meiji microbial diversity analysis cloud platform (https://cloud.majorbio.com/page/tools/, Majorbio Co., Ltd, Shanghai, China), fur ther comparative analysis of species annotation results. The original sequence data was stored in the sequence reading archive and can be obtained for free at NCBI (PRJNA1004548).

The physiological indexes of tea leaves were statistically analyzed by SPSS 18.0 software (SPSS Inc., Chicago, USA). One-way ANOVA and Duncan multiple-range test were used to analyze significant differences between the physiological data at different concentration treatments, and differences were considered statistically significant when the P-value < 0.05. Create graphics using Adobe Photoshop 2020.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03880-1.

Supplementary Material 1: **Table S1**: Sequencing quality

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Author contributions

XH conducted the experiment, analyzed the data, and wrote a manuscript. YZ S participated in manuscript writing and picture production. YW, ZT D, and K F participated in the experimental design and revised the manuscript. LT S, JZ S, YL M, and SS W revised the manuscript. All authors contributed to the article and approved the submission.

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Data availability

The raw sequencing data have been deposited in NCBI Sequence Read Archive (SRA) under accession number PRJNA1004548 for bacteria.

Declarations

Ethics approval and consent to participate

We confirmed that all methods were in compliance with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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