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The phyllosphere microbiome

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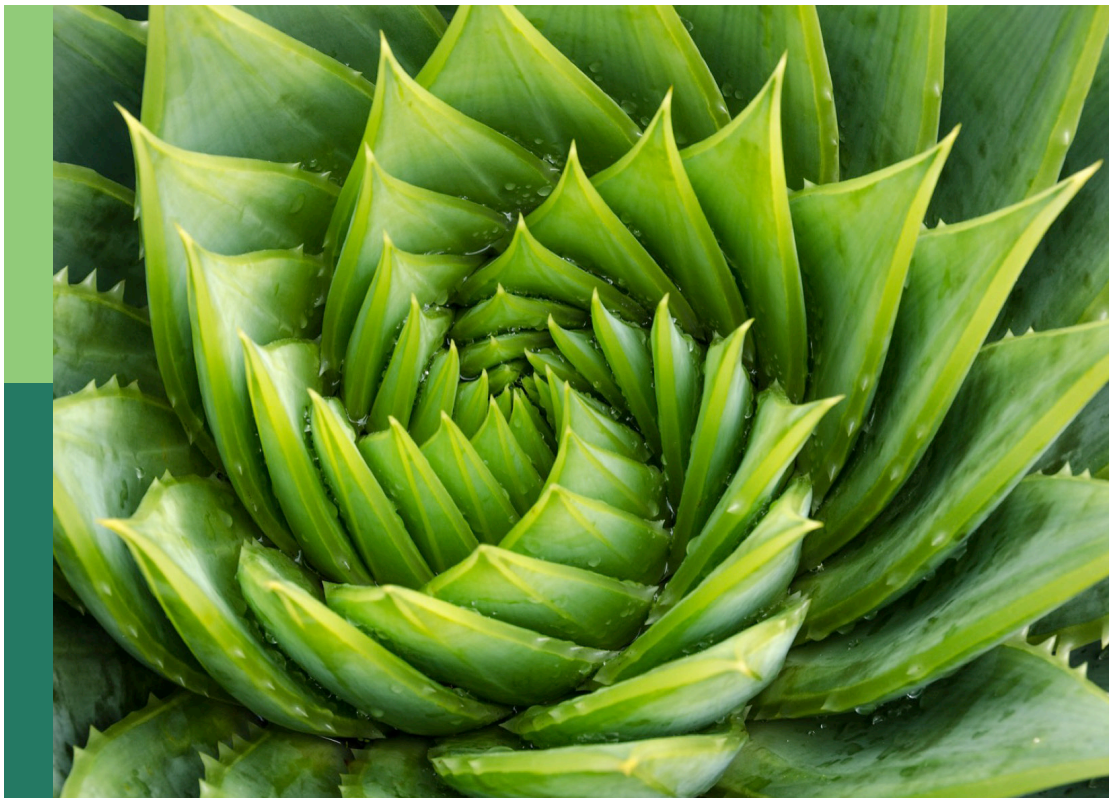

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The phyllosphere microbiome

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Editorial: The phyllosphere microbiome

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Editorial on the Research Topic
The phyllosphere microbiome

The phyllosphere microbiome is composed of microbial communities residing in the above-ground compartments of terrestrial plants, as well as the whole spectrum of biomolecules that they produce (Vorholt, 2012; Berg et al., 2020). Recent technological advances in high-throughput sequencing technologies not only have unveiled the incredible diversity of microorganisms residing on above-ground plant surfaces, including bacteria, fungi, viruses, and archaea (Trivedi et al., 2020), but also have revolutionized our understanding of phyllosphere microbial compositions and dynamics in response to host-related cues, biotic stress, environmental, and Anthropocene-linked factors (Matsumoto et al., 2021; Zhan et al., 2022). Furthermore, various studies that focused on microbial functions have shed light on the roles of phyllosphere microbes in nutrient acquisition, disease suppression, stress tolerance, and plant growth promotion (Fan et al., 2019; Liu et al., 2020; Wang and Cernava, 2020; Xu et al., 2021). By investigating the phyllosphere microbiome, we can gain insights into plant-microbe interactions, ecosystem functioning, and prepare a robust basis for sustainable agricultural practices (Wang and Cernava, 2020; Matsumoto et al., 2022; Liu et al., 2023). For the latter, disease-preventing microbes have recently emerged as a highly promising strategy (Wang and Cernava, 2023). In this Research Topic, current challenges in phyllosphere microbiome research were highlighted, including methodological issues. In addition, functional characterizations of specific microbial taxa were conducted, implications of the environment or Anthropocene on phyllosphere microbial communities were revealed, and their application potential in organic agriculture was emphasized.

Surface sterilization of various plant samples is a commonly adopted processing step for high-throughput sequencing (HTS) approaches as well as for the isolation of endophytic microorganisms. However, the potential impact of different surface sterilization techniques on the composition and diversity of endophytes has remained largely overlooked. Yu et al. investigated the influence of sodium hypochlorite (NaClO), a commonly applied disinfectant for sample pre-treatment, on the diversity of endophytic bacteria and fungi in leaves and stems of tea plants. They found that diversity assessments

of bacterial endophytes were significantly affected by certain NaClO concentrations as well as the treatment time. In contrast, the diversity of fungal endophytes was not significantly impacted according to the results obtained with HTS. Their results demonstrated that pre-treatments with NaClO should be carefully implemented in phyllosphere microbiome studies, especially in the case of precise assessments of bacterial diversity in different above-ground tissues.

The phyllosphere microbiome contributes to essential functions of host plants, such as defense against various phytopathogens. In one of the articles, the defensive function of an indigenous phyllosphere bacterium from healthy citrus plants against Huanglongbing (HLB), a devastating disease of citrus crops caused by *Candidatus* Liberibacter asiaticus (CLas), was studied by Munir et al. They found that CLas density in citrus trees respond inversely proportional to the density of the beneficial phyllosphere bacterium. Its application on diseased citrus leaves reduced pathogen density in leaf midribs by 1,000-fold *in vitro*. This disease-suppressive phyllosphere bacterium can efficiently colonize the phloem of citrus leaves, which may offer more opportunities for future management of vascular pathogens. Further explorations will be required to decipher disease-suppressive mechanisms of highly active phyllosphere bacteria.

Permafrost mounds in subarctic palsa mires are thawing due to climate change and becoming a substantial source of N_2O emissions. However, phyllosphere microbiome-related implications were not investigated so far. Nie et al. found that the N_2O emission potential in palsa bogs is increase by certain phyllosphere bacteria of two dominant *Sphagnum* mosses; especially by such that are found on *Sphagnum capillifolium*. *Pseudomonas* and members of the bacterial family Enterobacteriaceae were identified as pH-responsive hyperactive N_2O emitters. Their study revealed previously unknown hyperactive N_2O emitters associated with *Sphagnum capillifolium* that naturally occurs in melting palsa mounds, and provided novel insights into the importance of the phyllosphere microbiome in the Anthropocene.

Organic matter inputs are used as a common agricultural practice to improve soil fertility and quality, but it is sparsely understood how this practice affects the plant microbiome. Köberl et al. investigated microbial colonization of the East African highland banana cultivar "Mpologoma" under different mulch and manure treatments on three representative smallholder farms in Uganda. They found that the gammaproteobacterial community appeared stable with no significant response to organic matter inputs after 24 months of treatment. However, significant differences in the plant's carpo-, phyllo-, and rhizosphere microbial community composition and diversity were found among individual farms, independent of the added soil inputs. Their results highlight short-term microbial stability in banana cropping systems in terms of a high resilience of gammaproteobacterial communities. However, future investigations that include whole bacterial as well as multi-kingdom communities are required to provide detailed insights into potential impacts of soil amendments on the banana microbiome.

Application of conventional chemical fungicides is a common measure to counteract phytopathogens in agricultural production systems, but can also adversely affect food safety and cause environmental pollution. In the study by Sun et al., the effects of ozonated water, mancozeb, and thiophanate-methyl on the structure, composition, and diversity of strawberry phyllosphere microbial communities were investigated by high-throughput sequencing. Ozonated water was found to efficiently reduce the relative abundance of potentially pathogenic fungi and is therefore a potential biocide that could reduce environmental pollution in the future in contrast to conventional chemical agents. They also found that microbial communities in the strawberry phyllosphere showed a specific response to the employed fungicides. The observed, chemical-specific response may serve as a basis for the implementation of the phyllosphere microbiome as a highly-sensitive indicator for the use of agrochemicals and thus provide an alternative approach to available analytical approaches for residue detection.

The other six studies published in this Research Topic have provided new insights into mechanisms of phyllosphere microbiome assembly and responses in different host plants, including barley, citrus, halophytes, rice, and wheat. They not only increased our understanding of phyllosphere microbiome functions, but also provided new clues for the development of phyllosphere microbiome-based applications that could increase sustainability in agriculture. It can be expected that the required knowledge base that is needed to develop such applications will substantially grow during the next years. Continuous improvements in sequencing technologies as well as data processing will enable a more comprehensive characterization of the phyllosphere microbiome at a finer taxonomic and functional resolution. Integration of multi-omics approaches will provide a deeper understanding of microbial interactions and metabolic networks within the phyllosphere. These advancements will allow us to uncover the hidden diversity and functional potential of the phyllosphere microbiome. They will also enhance our understanding of the intricate interplay between the phyllosphere microbiota and host plants; this also includes the apparent crosstalk with the rhizosphere microbiome. Collectively, it will be important to decipher functional complementarity and synergistic effects between plants and microbes that contribute to plant health and growth, enabling the development of targeted strategies to secure global food production.

Author contributions

MW prepared the first draft of this editorial and TC conducted a critical revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Scale-Dependent Effects of Growth Stage and Elevational Gradient on Rice Phyllosphere Bacterial and Fungal Microbial Patterns in the Terrace Field

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The variation of phyllosphere bacterial and fungal communities along elevation gradients may provide a potential link with temperature, which corresponds to an elevation over short geographic distances. At the same time, the plant growth stage is also an important factor affecting phyllosphere microorganisms. Understanding microbiological diversity over changes in elevation and among plant growth stages is important for developing crop growth ecological theories. Thus, we investigated variations in the composition of the rice phyllosphere bacterial and fungal communities at five sites along an elevation gradient from 580 to 980 m above sea level (asl) in the Ziquejie Mountain at the seedling, heading, and mature stages, using high-throughput Illumina sequencing methods. The results revealed that the dominant bacterial phyla were Proteobacteria, Actinobacteria, and Bacteroidetes, and the dominant fungal phyla were Ascomycota and Basidiomycota, which varied significantly at different elevation sites and growth stages. Elevation had a greater effect on the α diversity of phyllosphere bacteria than on that phyllosphere fungi. Meanwhile, the growth stage had a great effect on the α diversity of both phyllosphere bacteria and fungi. Our results also showed that the composition of bacterial and fungal communities varied significantly along elevation within the different growth stages, in terms of both changes in the relative abundance of species, and that the variations in bacterial and fungal composition were well correlated with variations in the average elevation. A total of 18 bacterial and 24 fungal genera were significantly correlated with elevational gradient, displaying large differences at the various growth stages. Soluble protein (SP) shared a strong positive correlation with bacterial and fungal communities ($p < 0.05$) and had a strong significant negative correlation with *Serratia*, *Passalora*, unclassified_Trichosphaeriales, and antioxidant enzymes ($R > 0.5$,

$p < 0.05$), and significant positive correlation with the fungal genera *Xylaria*, *Gibberella*, and *Penicillium* ($R > 0.5$, $p < 0.05$). Therefore, it suggests that elevation and growth stage might alter both the diversity and abundance of phyllosphere bacterial and fungal populations.

Keywords: rice phyllosphere, elevational gradient, growth stage, fungi, bacteria

INTRODUCTION

The phyllosphere refers to aboveground plant surfaces as a habitat for plant associated microbes, including bacteria, fungi, protists, and viruses (Vorholt, 2012; Bringel and Couée, 2015). In comparison with coastal seawater habitats and farm soil, leaves are believed to represent one of the largest microbial habitats on Earth but is an environment of reduced bacterial complexity (Delmotte et al., 2009), dominated by a few bacterial phyla, including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. The phyllosphere is influenced by different environmental factors (Knief et al., 2010; Wellner et al., 2011; Rastogi et al., 2012; Copeland et al., 2015; Ding and Melcher, 2016), where the inhabitants face harsh environmental conditions including ultraviolet (UV) radiation, low free water availability, and nutrient limitations (Colla et al., 2017; Wang and Cernava, 2020). Phyllosphere microbial communities can affect global carbon and nitrogen cycles (Whipps et al., 2008). Individuals within this community may be beneficial, pathogenic, or antagonistic for the host plant and can strongly affect plant health (Vorholt, 2012; Brader et al., 2017; Matsumoto et al., 2021).

Elevational gradients strongly affect microbial biodiversity in the rhizosphere and bulk soil by altering plant and soil properties. The potential response of fungal assemblages to climate change has been investigated principally in soil systems (Sheik et al., 2011; Yuste et al., 2011; Bahram et al., 2015). Guo et al. (2020) suggested that bacterial and fungal α diversity were significantly higher at mid-elevation, while arbuscular mycorrhizal fungi (AMF) α diversity decreased monotonically, and that the beta diversities of the three groups were significantly affected by elevational gradients. Tang et al. (2020) highlighted that elevation was the main predictor to determine rhizosphere microbial community structure in the alpine tundra of the Changbai Mountains. Zhu et al. (2020) showed that seasonal dynamics of bacterial communities were much weaker than those imposed by elevation. However, there have been few studies on the influence of elevation on phyllosphere microorganisms. Cordier et al. (2012a) showed that the composition of fungal assemblages varied significantly over an elevational gradient, suggesting that climate warming might alter both the diversity and abundance of phyllosphere fungal species. Elevation, which is significantly related to mean annual temperature, is also known to affect the community composition of phyllosphere fungi (Qian et al., 2018). The potential responses of phyllosphere bacterial assemblages to elevational gradients have been much less thoroughly explored.

In this study, we investigated the effects of elevation and growth stage on the composition of rice phyllosphere bacterial and fungal communities. We used high-throughput Illumina sequencing to test the following two hypotheses: (1) the diversity and composition of phyllosphere bacterial and fungal communities would change with elevation at the same growth stages and (2) variation in the diversity and composition of phyllosphere bacterial and fungal communities within different growth stages at the same elevation.

MATERIALS AND METHODS

Experimental Design and Site Description

The study was conducted along an elevational gradient extending from 500 to 1,200 m asl at Ziquejie Mountain, with a special terraced structure, in the County Xinhua of the Hunan province, China. We selected five sites (580, 680, 780, 880, and 980 m asl) along with this elevation range that contained a high proportion of rice (Feng Lian 1) grown on the same slope in order to avoid differences in solar exposure. At each elevation, we defined six plots located about 80 m apart. Within each plot, we selected five sub-plots that were located close together. We sampled two leaves per plant located on the outside of the stalk. The rice leaves were sampled three times [July 20 (seedling stage), August 20 (heading stage), and September 12 (mature stage)] in 2018, and each sampling process was completed within 1 day. The leaves were placed in individual plastic bags and transported back to the laboratory at 4°C and stored at that temperature until DNA extraction. Phyllosphere microorganisms were collected as previously described (Delmotte et al., 2009; Redford and Fierer, 2009; Xie et al., 2015), with slight modifications. In brief, 10 g of leaf was submerged in 100 ml of phosphate-buffered saline (PBS) with 0.01% Tween-80 in a 250-ml sterile conical flask. The flask was shaken at 250 rpm for 30 min at 28°C, and then subjected to ultrasonication for 10 min. The microbes were then collected by a 0.22-μm filter using air pump filtration, and the microfiltration membranes were stored at −80°C for subsequent DNA extraction.

Determination of Total Soluble Protein and Enzyme Activity of Rice Leaves

About 0.1 g of rice leaves was weighed and then transferred to a 1.5-ml centrifuge tube with magnetic beads, 20 mM PBS (pH = 7.4) was added at nine times the weight of the rice leaves, and the leaves were ground into a homogenate with a tissue grinder. The samples were centrifuged at 3,500 rpm for 15 min, and the supernatant of the samples was taken to obtain 10% homogenate for subsequent analysis.

The content of total soluble protein (SP) was measured by Coomassie brilliant blue method. The activity of antioxidant

enzymes (CAT: catalase, SOD: superoxide dismutase, and POD: peroxidase) of the rice leaves were measured at different growth stages and elevations in the study. The rice leaves samples were detected using the Biochemical Kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) according to the manufacturer's protocol.

DNA Extraction and Purification

The rice phyllosphere DNA samples were extracted using the MP FastDNA®SPIN Kit for soil (MP Biomedicals, Solon, OH, United States) according to the manufacturer's protocol. Before PCR amplification, the DNA concentration was measured and diluted to 30 ng/ml. The V5–V6 region of the bacterial 16S rRNA gene was amplified using the primer set 799F (AACMGGATTAGATACCCKG) and 1115R (AGGGTTGCGCTCGTTG), while the internally transcribed spacer (ITS) region of fungi was amplified with primer pair gITS7F (GTGARTCATCGARTCTTTG)/ITS4 (TCCTCCGCTTATTGATATGC), each primer pair contained a unique 12 nt barcode, and was amplified as previously described by Kong et al. (2018). The PCR products were separated by agarose gel electrophoresis, and the strong bands at ∼300 bp were collected and purified with an E.Z.N.A.®Gel Extraction Kit (Omega Bio-tek, Norcross, GA, United States). The PCR products were quantified using Qubit^{TM} 2 Fluorometer and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform by Annoroad Gene Technology Co., Ltd. (Beijing, China) according to the standard protocols.

16S rRNA Gene Amplification, Sequencing, and Processing

The 16S rDNA and ITS raw sequence data reads, in fastq format, were collected and processed with an in-house pipeline[1] containing a series of bioinformatics tools. First, samples were separated based on the 12-bp barcodes and primers. Paired end reads were combined using the FLASH program developed by Magoc and Salzberg (2011). Sequences containing ambiguous (N) bases were removed. Chimera sequences also were detected and removed using UPARSE developed by Edgar (2013)[2]. All sequences were clustered and operational taxonomic units (OTUs) were generated at the 97% similarity level, low abundance OTUs (≤1 counts) were removed from the OTU table. All chloroplast and mitochondrial sequences were removed. Representative sequences for each OTU were assigned to taxonomic groups using the RDP Classifier database (RDP training set RDP to release 11.5 database and unite database 8.2 version) (Wang et al., 2007). After accounting for sampling depth, we obtained a resampled OTU table with 12,477 and 977 sequences for bacterial and fungal samples, respectively, for the subsequent analyses. All raw bacterial sequences were deposited in the SRA database short-read archive under accession number PRJNA675674. Principal coordinate analysis (PCoA) based on the Bray–Curtis distance was performed in subsequent analyses using the "vegan" package in R (v.3.2.5). Differences in microbial community composition between two groups were evaluated by using non-parametric multi-response permutation procedure (MRPP), analysis of similarities (ANOSIM), and non-parametric permutational multivariate analysis of variance of the Adonis function (ADONIS) using the "vegan" package in R (v.3.2.5) (Anderson, 2001; Dixon, 2010). Mantel test, canonical correlation analysis (CCA), and CCA-based variance partitioning analysis (VPA) were used to measure the variations in different environmental factors.

[1]http://mem.rcees.ac.cn:8080

[2]http://drive5.com/uparse/

Statistical Analysis

The statistical significance of differences between α diversity indices of different groups and the relative abundance of the taxonomic subgroups were assessed by performing a one-way ANOVA followed by Duncan's multiple range test. Correlation analyses between elevation and the top 30 genera were also conducted with the Spearman method using the IBM SPSS software for Windows, version 22.0 (IBM Corp., Armonk, NY, United States).

RESULTS

The Physical and Chemical Properties of Rice Leaves

The content of total SP, CAT, SOD, and POD in rice leaves at different elevations and growth stages was compared (**Figures 1A–D**). The results showed that at the same elevation, except for 680 m asl, the total SP in rice leaves displayed significant differences among the different growth stages, with the seedling stage > heading stage > mature stage. There was no significant difference from 580 to 780 m asl for the total SP content at the heading stage, but it was significantly higher than at 880 and 980 m asl. The content of total SP was the highest at 680 m asl, followed by 780 and 980 m asl, and the lowest at 580 and 880 m asl.

At 580 m asl, the CAT content of rice leaves was heading stage > mature stage > seedling stage. At 680 and 880 m asl, there was no significant difference at the heading stage and mature stage, respectively, but the content of both stages was significantly higher than the seedling stage. At 780 and 980 m asl, the CAT content of rice leaves was a mature stage > heading stage > seedling stage. The CAT content in rice leaves at different elevations during the same growth stage was also compared, and no significant difference was found for the five elevations at the seedling stage. At the heading stage, the CAT content in rice leaves at higher elevations (880 and 980 m asl) was significantly higher than that at 580–780 m asl. Finally, at the mature stage, for elevations from 780 to 980 m asl, the CAT content was significantly higher than at lower elevations (from 580 to 680 m asl). The SOD content of rice leaves at the mature stage was significantly higher than at the seedling and heading stages at the elevations of 580 and 880 m asl. At the mature stage, for the elevations of 680, 780, and 980 m asl, the SOD content of rice leaves was a mature stage > heading stage > seedling stage. The SOD content of rice leaves at 580 and 880 m asl was significantly higher than at the other three elevations (680, 780,

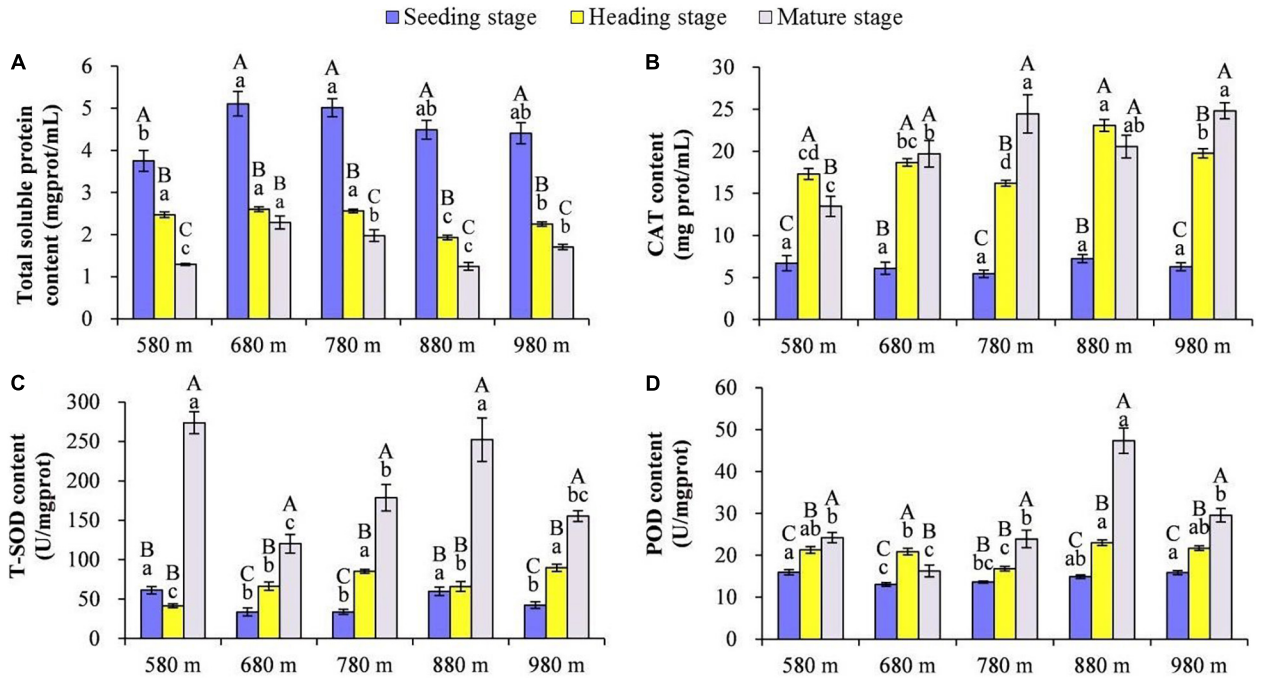

FIGURE 1 | The effect of elevation and growth stage on total soluble protein and activity of antioxidant enzymes. **(A)** Total soluble protein. **(B)** CAT content. **(C)** T-SOD content. **(D)** POD content. Capital letters indicate significant differences between different growth stages at the same elevation. Lowercase letters indicate significant differences between different elevations at the same growth stage. SP, soluble protein; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase.

and 980 m asl) at the seedling and mature stages. The SOD content of rice leaves was significantly higher at the elevations of 780 and 980 m asl than the other three elevations (580, 680, and 880 m asl) at the heading stage. At the elevations of 580, 880, and 980 m asl, the POD content of rice leaves was a mature stage > heading stage > seedling stage, and at the elevation of 680 m asl, the POD content of rice leaves was heading stage > mature stage > seedling stage. While at the elevation of 780 m asl, the POD content of rice leaves in the mature stage was significantly higher than the seedling stage and heading stage. These results showed that the POD content in rice leaves at elevations of 580, 880, and 980 m asl was significantly higher than elevations of 680 and 780 m asl at the seedling and heading stages. At the mature stage, the POD content in rice leaves had the highest value at 880 m asl, followed by 580, 780, and 980 m asl, with the lowest at 680 m asl.

The Variation of the Rice Phyllosphere Bacterial and Fungal α Diversity

In this study, rice phyllosphere bacterial and fungal α diversity (Chao1) at different growth stages along an elevational gradient was analyzed. The results of α diversity analysis for the rice phyllosphere bacterial community are shown in **Figure 2**. At 580 m asl, Chao1 first decreased and then increased with rice growth progression. There was no significant difference between the seedling stage and heading stage, but the mature stage increased significantly at 680 and 780 m asl, while decreasing significantly at 880 and 980 m asl. These results indicated that

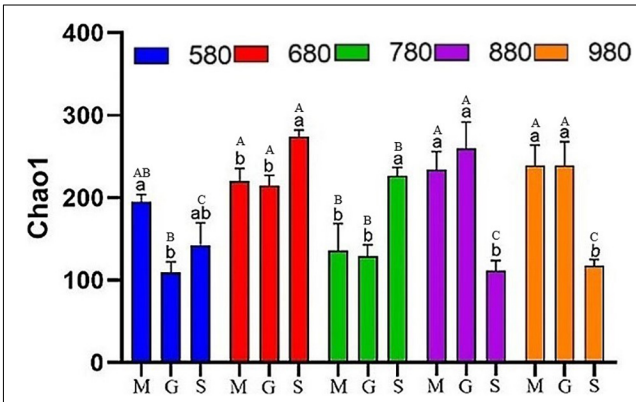

FIGURE 2 | The α diversity indices of phyllosphere bacteria among different elevations and growth stages. Comparison of α diversity of rice phyllosphere bacteria at different growth stages at the same elevation, M: seedling stage, G: heading stage, S: mature stage. Capital letters indicate significant differences between different growth stages at the same growth stage. Lowercase letters indicate significant differences at different elevations at the same elevation.

the variation trend of bacterial Chao1 differed with the growth stage at different elevations. The α diversity at different elevations during the seeding, heading, and maturity stages was compared and analyzed (**Figure 2**). At the seedling and heading stage, Chao1 first increased from 580 to 680 m asl, then decreased from 680 to 780 m asl, and then increased again gradually from 780 to 980 m asl. At the mature stage, Chao1 increased first and

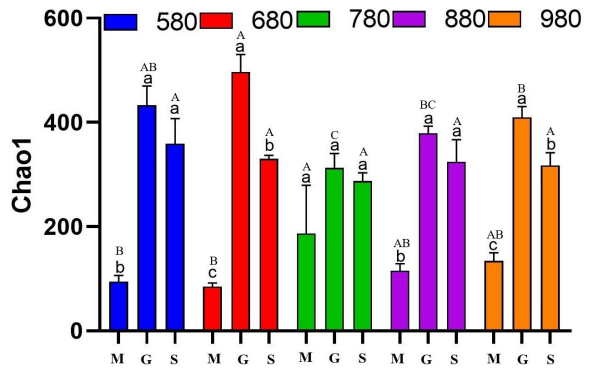

FIGURE 3 | The α diversity indices of phyllosphere fungi among different elevations and growth stages. Comparison of α diversity of rice phyllosphere fungi at different growth stages at the same elevation, M: seedling stage, G: heading stage, S: mature stage. Capital letters indicate significant differences between different growth stages at the same growth stage. Lowercase letters indicate significant differences at different elevations at the same elevation.

then decreased gradually, reaching its peak value at 680 m asl. The results showed that, from 680 to 980 m asl, there was no significant difference in rice seedling and heading stages, while diversity in the mature stage was different. In addition, the variation trend of diversity at the seedling stage and heading stage was similar at different elevations. Overall, the diversity increased gradually with elevation, while the diversity decreased gradually from 680 to 980 m asl at the mature stage.

The analysis of rice phyllosphere fungal community α diversity is shown in **Figure 3**. The results indicated that fungal Chao1 at 580 and 880 m asl at the seedling stage was significantly lower than that at the heading and maturity stages, and there was no significant difference between the heading stage and maturity stage. At 680 and 980 m asl, fungal Chao1 first increased and then decreased, with significant differences among the three stages. There was no significant difference between the three stages at 780 m asl. The results showed that the phyllosphere fungal α diversity at the seedling stage increased gradually with the increase of elevation between 580 and 980 m asl. At the heading stage, the fungal α diversity first increased (580–680 m asl) and appears to have peaked at 780 m asl and then decreased. These results showed that rice phyllosphere fungal Chao1 at different elevations and stages had the same variation trend, first increasing and then decreasing. At the heading stage, the fungal α diversity of Chao1 was higher than other two stages. The fungal α diversity of rice phyllosphere in the same growth stage had no significant change with elevations.

Operational Taxonomic Unit Analysis

The shared and unique OTU numbers are shown in the Venn diagrams of **Supplementary Figure 1**. With a 97% similarity cutoff, a total of 998 and 977 OTUs were obtained for bacterial and fungal sequences, respectively. At the seedling stage, there were 88 shared bacterial OTUs among the five elevation groups. The 780 m asl (48, 22.02%) group had the highest number of unique OTUs, while the 580 m asl (25, 8.77%) group had the lowest number of unique bacterial OTUs. At the heading stage, 79 bacterial OTUs were shared among the five elevation groups. The 880 m asl (136, 30.49%) group had the highest number of unique OTUs, and the 580 m asl (8, 4.88%) group had the lowest number of unique OTUs, respectively. At the mature stage, there were 80 shared bacterial OTUs among the five elevation groups. The 680 m asl (95, 26.61%) group had the highest number of unique OTUs, and the 880 m asl (9, 5.45%) group had the lowest number of unique OTUs, respectively.

For the rice phyllosphere fungal community at the seedling stage, there were a total of 563 OTUs across the five elevation

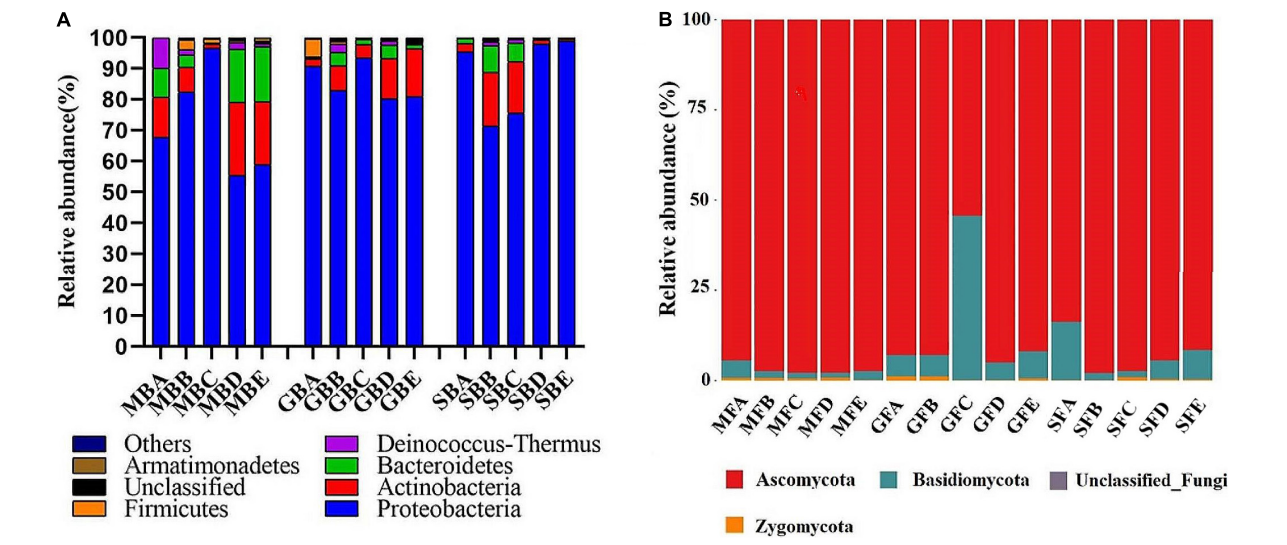

FIGURE 4 | The relative abundance of the dominant phyllosphere bacterial **(A)** and fungal **(B)** population at the phylum level. B, bacteria; F, fungi. M, seedling stage; G, heading stage; S, mature stage. (A) 580 m asl; (B) 680 m asl; (C) 780 m asl; (D) 880 m asl; (E) 980 m asl.

sample groups, with the greatest number of unique OTUs in the 780 m group (221, 39.25%), and the lowest number of unique OTUs in the 680 m group (10, 1.78%), with a total of 61 shared OTUs (**Supplementary Figure 1D**). At the heading stage, the phyllosphere fungal community was composed of 854 OTUs across the five elevation groups. The number of unique OTUs was highest in the 680 m group (70, 8.20%), and lowest in the 780 m group (13, 1.52%), with a total of 222 OTUs shared between all groups (**Supplementary Figure 1E**). At the maturity stage, there were a total of 806 OTUs in the phyllosphere fungal community across the five elevations, with the 580 m group possessing the highest number of unique OTUs (67, 8.31%), while 780 m group had the lowest number of unique OTUs (19, 2.36%), with a total of 198 OTUs shared between all groups (**Supplementary Figure 1F**).

Composition of the Rice Phyllosphere Bacterial and Fungal Community

At the phylum level, a total of 19 bacterial phyla and 1 archaeal phylum were identified. The dominant bacterial populations (relative abundance greater than 4%) were Proteobacteria, Actinobacteria, and Bacteroidetes in the seeding, heading, and mature stages of rice phyllosphere samples (**Figure 4A**). At the seedling stage, the relative abundance of Proteobacteria first increased and then decreased with elevation, while that of Actinobacteria and Bacteroidetes first decreased and then increased, with the turning point being 780 m asl. At the heading stage, the highest relative abundance of Proteobacteria was at 780 m asl, while that of Actinobacteria at 880 m and 980 m asl was higher than other elevations. At the mature stage, the relative abundance of Proteobacteria first decreased and then increased with elevation, while that of Bacteroidetes first increased and then decreased.

A total of four phyla were identified among the phyllosphere fungi (**Figure 4B**). Ascomycota and Basidiomycota were the dominant fungi in rice phyllosphere samples at the seedling, heading, and maturity stages. There were significant differences at the phylum level at different elevations during the same growth stage. With the increasing elevation at the seedling stage, the relative abundance of Ascomycota increased, while that of Basidiomycota decreased. At the heading stage, the relative abundance of Ascomycota first decreased and then increased, while Basidiomycota displayed the opposite trend with an inflection point at 780 m asl. At the mature stage, the relative abundance of Basidiomycota first decreased and then increased with the increase of elevation.

At the genus level, we analyzed the correlation between the top 30 bacterial and fungal genera and elevation for each of the three growth stages (**Table 1**). At the seedling stage, the elevation was positively correlated with the bacterial genera *Curtobacterium* and *Mucilaginibacter*, and negatively correlated with *Acinetobacter* and *Pseudomonas* ($R > 0$, $p < 0.05$), while lower correlations were observed with fungal genera. At the heading stage, the elevation was significantly positively correlated with the bacterial genera *Lapillicoccus*, *Methylobacterium*, *Mucilaginibacter*, *Acidovorax*, *Rhizobium*, *Microbacterium*, *Pedobacter*, and *Aureimonas*, and negatively correlated with *Pantoea*, *Pseudomonas*, and *Buttiauxella* ($R > 0$, $p < 0.05$), while the elevation was significantly positively correlated with the fungal genera *Alternaria*, Phaeosphaeriaceae_unclassified, and unclassified_Trichosphaeriales, and negatively correlated with *Passalora*, Mycosphaerellaceae_unclassified, and *Periconia* ($R > 0$, $p < 0.05$). While at the mature stage, the elevation

TABLE 1 | The Spearman correlation analysis between top 30 rice phyllosphere bacterial and fungal populations and elevation.

	Genus	Seeding stage	Heading stage	Mature stage
Bacteria	*Acidovorax*	−0.0109	0.738***	−0.233
	Acinetobacter	−0.565**	−0.174	−0.342
	Aureimonas	0.0695	0.601***	0.00545
	Buttiauxella	−0.0844	−0.746***	0.0381
	Curtobacterium	0.582***	−0.11	−0.38*
	Geodermatophilus	0.354	0.176	−0.525**
	Kineococcus	0.185	0.317	−0.531**
	Lapillicoccus	0.437*	0.511**	−0.719***
	Methylobacterium	0.392*	0.576***	−0.553**
	Microbacterium	−0.0735	0.733***	−0.169
	Moraxella	−0.246	−0.168	−0.722***
	Mucilaginibacter	0.502**	0.666***	−0.271
	Nakamurella	0.485**	0.254	−0.514**
	Pantoea	0.485**	−0.692***	−0.484**
	Pedobacter	−0.142	0.615***	−0.0558
	Pseudomonas	−0.584***	−0.515**	−0.0926
	Rhizobium	−0.46*	0.674***	0.225
	Spirosoma	0.473**	0.106	−0.622***
Fungi	Phaeosphaeriaceae_unclassified	−0.187	0.792**	−0.902**
	Passalora	−0.0963	−0.945**	0.847**
	Dothideomycetes_unclassified	−0.166	−0.18	−0.812**
	Alternaria	0.182	0.604**	−0.792**
	Mycosphaerellaceae_unclassified	0.421**	−0.824**	0.743**
	Pleosporales_unclassified	−0.154	0.267	−0.69**
	Unclassified_Trichosphaeriales	−0.412**	0.812**	−0.682**
	Periconia	0.362*	−0.698**	0.635**
	Ophiosphaerella	−0.296	0.188	−0.612**
	Nectriaceae_unclassified	−0.207	−0.349	−0.584**
	Unclassified_Montagnulaceae	0.217	−0.859**	0.549**
	Acremonium	−0.347*	0.0941	−0.518**
	Phoma	0.196	−0.361	0.475*
	Ascomycota_unclassified	0.0385	−0.588**	0.459*
	Unclassified_Pleosporales	0.121	0.0471	−0.424*
	Monographella	0.449**	−0.188	0.345
	Nigrospora	−0.17	−0.514**	0.337
	Fusarium	0.196	−0.502*	−0.286
	Cryptococcus	−0.327*	−0.749**	0.188
	Paraphaeosphaeria	−0.326*	−0.404*	−0.106
	Aspergillus	−0.24	−0.498*	−0.0981
	Pyrenochaetopsis	0.291	0.494*	0.0942
	Penicillium	0.00342	−0.651**	0.00784
	Cladosporium	−0.456**	−0.0941	−0.00392

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 2 | The relative abundance of genera was significantly correlated with elevation among the top 30 phyllosphere bacterial genera.

Growth stage	Genus	580 m	680 m	780 m	880 m	980 m
Seeding stage	*Acinetobacter*	4.15 ± 1.81a	0.47 ± 0.19b	0.12 ± 0.03b	1.76 ± 1.65ab	0.08 ± 0.02b
	Curtobacterium	1.51 ± 0.34b	1.68 ± 0.72b	0.48 ± 0.25b	4.82 ± 0.43a	5.14 ± 0.63a
	Mucilaginibacter	0.21 ± 0.06b	0.27 ± 0.18b	0.10 ± 0.10b	1.55 ± 0.34a	1.72 ± 0.42a
	Pseudomonas	27.34 ± 8.89a	8.96 ± 2.6b	0.19 ± 0.03b	1.26 ± 0.36b	1.14 ± 0.81b
Heading stage	*Acidovorax*	0.02 ± 0.01b	0.07 ± 0.02b	0.06 ± 0.01b	0.12 ± 0.05b	1.56 ± 0.32a
	Aureimonas	0.02 ± 0.01c	0.61 ± 0.02b	0.36 ± 0.02bc	0.76 ± 0.22ab	1.23 ± 0.36a
	Buttiauxella	70.01 ± 3.45a	51.7 ± 6.8b	63.16 ± 6.1ab	18.02 ± 4.97c	21.28 ± 1.98c
	Methylobacterium	0.43 ± 0.12b	3.59 ± 0.87ab	1.12 ± 0.09b	7.04 ± 2.61ab	3.22 ± 0.48a
	Microbacterium	0.30 ± 0.08b	2.52 ± 0.38b	1.95 ± 0.17b	2.19 ± 0.78b	11.17 ± 1.69a
	Mucilaginibacter	0.06 ± 0.03b	0.04 ± 0.01b	0.06 ± 0.03b	0.30 ± 0.10a	0.26 ± 0.03a
	Pantoea	4.57 ± 2.46a	2.47 ± 0.93ab	0.77 ± 0.17b	0.23 ± 0.09b	0.14 ± 0.02b
	Pedobacter	0.01 ± 0.01b	0.02 ± 0.01b	0.25 ± 0.08a	0.11 ± 0.08ab	0.13 ± 0.03ab
	Pseudomonas	1.27 ± 0.52c	6.43 ± 0.52a	2.8 ± 0.35b	0.46 ± 0.24c	0.36 ± 0.04c
	Rhizobium	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.02 ± 0.01a	1.90 ± 1.63a
	Lapillicoccus	0.01 ± 0.00a	0.02 ± 0.00a	0.00 ± 0.00a	0.44 ± 0.30a	0.12 ± 0.04a
Mature stage	*Geodermatophilus*	0.10 ± 0.03b	0.76 ± 0.14a	0.57 ± 0.16a	0.05 ± 0.01b	0.02 ± 0.01b
	Kineococcus	0.50 ± 0.17c	3.57 ± 0.22a	2.05 ± 0.25b	0.09 ± 0.03c	0.09 ± 0.01c
	Methylobacterium	1.20 ± 0.39c	8.48 ± 0.77a	3.76 ± 0.42b	0.77 ± 0.33c	0.28 ± 0.06c
	Moraxella	0.10 ± 0.04b	5.57 ± 1.11a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
	Nakamurella	0.14 ± 0.07b	0.79 ± 0.45a	0.2 ± 0.07ab	0.05 ± 0.01b	0.01 ± 0.01b
	Spirosoma	1.49 ± 0.55b	7.37 ± 2.12a	1.96 ± 0.82b	0.21 ± 0.04b	0.11 ± 0.04b
	Lapillicoccus	0.28 ± 0.12b	1.14 ± 0.55a	0.06 ± 0.04b	0.02 ± 0.00b	0.01 ± 0.00b

Different letter indicated significant difference among different groups.

was negatively correlated with the bacterial genera *Moraxella*, *Lapillicoccus*, *Spirosoma*, *Methylobacterium*, *Kineococcus*, *Geodermatophilus*, and *Nakamurella* ($R > 0$, $p < 0.05$), and positively correlated with the fungal genera *Passalora*, *Periconia*, and Mycosphaerellaceae_unclassified, and negatively correlated with the fungal genera Phaeosphaeriaceae_unclassified, Dothideomycetes_unclassified, and *Alternaria*.

We analyzed the dynamic changes of bacterial genera that displayed a significant correlation with elevation at the different sites during the seedling, heading, and mature stages (**Table 2**). At the seedling stage, the relative abundances of *Acinetobacter* and *Pseudomonas* at 580 m asl were significantly higher than other elevations, but there was no significant difference among the other four elevations. The relative abundances of *Curtobacterium* and *Mucilaginibacter* at 580, 680, and 780 m asl were significantly lower than at 880 and 980 m asl. At the heading stage, the relative abundances of *Acidovorax* and *Microbacterium* were significantly higher at 980 m asl than at the other four elevations. While the relative abundance of *Buttiauxella* at 580, 680, and 780 m asl was significantly higher than at 880 and 980 m asl, and the relative abundance of *Mucilaginibacter* at 580, 680, and 780 m asl was significantly lower than at 880 and 980 m asl. The relative abundance of *Pantoea* at 580 and 680 m asl was higher than that between 780 and 980 m, while *Rhizobium* and *Lapillicoccus* displayed no significant difference among the five elevations. At the mature stage, the relative abundances of *Moraxella*, *Lapillicoccus*, *Spirosoma*, *Methylobacterium*, *Kineococcus*, *Geodermatophilus*, and *Nakamurella* first increased and then decreased with elevation, reaching a maximum value at 680 m asl, which was consistent with the trend of α diversity.

We also analyzed the dynamic changes of fungal genera that displayed a significant correlation with elevation at the different sites during the seedling, heading, and mature stages (**Table 3**). At the seedling stage, the relative abundance of the top 30 fungal genera had a low correlation with elevation. At the heading stage, the relative abundance of most fungal genera was not significantly different at the five elevations, the exception being unclassified_Trichosphaeriales, which was significantly higher at 680 m than at 980 m asl, and no significant difference was found between the other elevations. At the mature stage, the relative abundance of most genera showed no significant difference between the five elevations, while the relative abundance of *Acremonium* at 580 m asl was significantly higher than that at 880 m, but there was no significant difference at the other elevations. The relative abundance of *Ophiosphaerella* at 680 m asl was significantly higher than that at 780, 880, and 980 m asl, while no significant difference was found with the other elevations. The relative abundance of unclassified_Trichosphaeriales was significantly higher at 680 m asl than that at 980 m asl, and no significant difference was found at the other elevations.

Bacterial and Fungal Community Structure in Rice Phyllosphere

The results of bacterial PCoA showed that bacteria PCoA1 and PCoA2 accounted for 46.3 and 22.3% of the variation, respectively (**Figure 5A**). The phyllosphere bacterial communities of rice at the seedling stage and heading stage were relatively similar, while the bacterial community at the mature stage was significantly different from either of the two earlier stages. Fungal PCoA1 and PCoA2 explained 43.667

TABLE 3 | The relative abundance of genera was significantly correlated with elevation among the top 30 phyllosphere fungal genera.

Growth stage	Genus	580 m	680 m	780 m	880 m	980 m
Seeding stage	Mycosphaerellaceae_unclassified	0.01 ± 0c	0.02 ± 0c	0.02 ± 0.01c	0.08 ± 0.02b	0.19 ± 0.01a
	Acremonium	0.18 ± 0.03a	0.07 ± 0.01a	0.04 ± 0.01a	0.34 ± 0.24a	0.04 ± 0.03a
	Cladosporium	1.95 ± 0.37c	5.98 ± 0.24a	1.28 ± 0.05c	3.94 ± 0.82b	1.95 ± 0.18c
	Cryptococcus	0.39 ± 0.2b	0.32 ± 0.08b	0.03 ± 0.02b	1.66 ± 0.65a	0.49 ± 0.21b
	Monographella	0.06 ± 0.01a	0.03 ± 0.01a	0.06 ± 0.02a	0.15 ± 0.08a	0.09 ± 0.04a
	Paraphaeosphaeria	0.34 ± 0.11b	1.37 ± 0.11a	0.58 ± 0.11b	1.04 ± 0.19a	0.3 ± 0.04b
	Periconia	0.2 ± 0.03b	0.2 ± 0.03b	0.5 ± 0.14a	0.45 ± 0.08ab	0.53 ± 0.11a
	Unclassified_Trichosphaeriales	16.13 ± 1.11b	32.69 ± 1.18a	17.11 ± 1.51b	11.4 ± 2.14c	5.77 ± 1.6d
Heading stage	Mycosphaerellaceae_unclassified	0.06 ± 0.01a	0.11 ± 0.03a	0.01 ± 0a	0.01 ± 0a	0 ± 0a
	Phaeosphaeriaceae_unclassified	0.14 ± 0.07a	0.21 ± 0.05a	0.33 ± 0.02a	0.77 ± 0.27a	1.45 ± 0.18a
	Alternaria	0.47 ± 0.13a	0.39 ± 0.04a	0.34 ± 0.02a	0.82 ± 0.19a	1.78 ± 0.24a
	Aspergillus	0.34 ± 0.11a	1.03 ± 0.6a	0 ± 0a	0 ± 0a	0.02 ± 0.01a
	Cryptococcus	3.59 ± 2.74a	2.33 ± 1.29a	0.16 ± 0a	0.08 ± 0.02a	0.01 ± 0a
	Fusarium	0.49 ± 0.16a	0.76 ± 0.36a	0.03 ± 0a	0.06 ± 0.02a	0.11 ± 0.06a
	Nigrospora	0.14 ± 0.02a	0.38 ± 0.1a	0.14 ± 0.01a	0.09 ± 0.02a	0.12 ± 0.02a
	Paraphaeosphaeria	0.45 ± 0.2ab	0.96 ± 0.24ab	0.33 ± 0.02a	0.24 ± 0.04ab	0.19 ± 0.04b
	Passalora	58.83 ± 4.98a	34.86 ± 4.01a	19.97 ± 0.37a	16.96 ± 1.35a	13.78 ± 1.23a
	Penicillium	0.33 ± 0.17a	0.43 ± 0.12a	0.02 ± 0a	0.02 ± 0.01a	0.02 ± 0.01a
	Periconia	0.53 ± 0.08a	0.4 ± 0.09a	0.06 ± 0.01a	0.03 ± 0.01a	0.08 ± 0.02a
	Pyrenochaetopsis	0.02 ± 0.01ab	0.04 ± 0.01b	0.01 ± 0ab	0.04 ± 0.01a	0.26 ± 0.1ab
	Unclassified_Montagnulaceae	0.08 ± 0.02a	0.04 ± 0.01a	0.01 ± 0a	0.01 ± 0a	0.01 ± 0.01a
	Unclassified_Trichosphaeriales	11.79 ± 2.41ab	29.02 ± 4.43a	25.01 ± 0.38ab	63.17 ± 3.58ab	45.67 ± 2.96b
	Ascomycota_unclassified	0.09 ± 0.04a	0.09 ± 0.02a	0 ± 0a	0.01 ± 0a	0.01 ± 0a
Mature stage	Dothideomycetes_unclassified	1.03 ± 0.29a	1.16 ± 0.32a	0.61 ± 0.42a	0.92 ± 0.33a	0.53 ± 0.16a
	Mycosphaerellaceae_unclassified	0.05 ± 0.05a	0 ± 0a	0.08 ± 0.08a	0.27 ± 0.22a	0.7 ± 0.4a
	Nectriaceae_unclassified	6.75 ± 0.84a	4.63 ± 0.81a	8.15 ± 1.87a	2.3 ± 0.57a	7.98 ± 2.52a
	Phaeosphaeriaceae_unclassified	0.18 ± 0.09a	0.23 ± 0.1a	0.02 ± 0.02a	0.06 ± 0.04a	0.01 ± 0.01a
	Acremonium	1.27 ± 0.3a	0.66 ± 0.29ab	0.96 ± 0.51ab	0.33 ± 0.14b	0.42 ± 0.22ab
	Alternaria	0.39 ± 0.1a	0.86 ± 0.23a	0.73 ± 0.24a	0.52 ± 0.22a	1.15 ± 0.42a
	Ophiosphaerella	0.18 ± 0.08ab	0.33 ± 0.15a	0.01 ± 0.01b	0 ± 0b	0.03 ± 0.02b
	Passalora	8.3 ± 1.35a	15.96 ± 4.7a	8.17 ± 3.25a	11.84 ± 2.55a	8.37 ± 3.34a
	Periconia	0.14 ± 0.07a	0.23 ± 0.15a	0.84 ± 0.3a	0.61 ± 0.34a	0.74 ± 0.3a
	Phoma	0.87 ± 0.19a	2.23 ± 0.78a	1.05 ± 0.51a	3.33 ± 0.88a	2.92 ± 1.5a
	Unclassified_Montagnulaceae	0.08 ± 0.08a	0.12 ± 0.12a	0.07 ± 0.05a	0 ± 0a	0.15 ± 0.09a
	Unclassified_Pleosporales	0.11 ± 0.08a	0.21 ± 0.21a	0.09 ± 0.06a	0.12 ± 0.08a	0.06 ± 0.03a
	Unclassified_Trichosphaeriales	1.59 ± 0.38ab	2.39 ± 0.56a	1.14 ± 0.54ab	1.6 ± 0.49ab	0.27 ± 0.1b
	Pleosporales_unclassified	3.46 ± 0.92a	3.22 ± 1.04a	3.13 ± 1.77a	2.95 ± 0.79a	2.14 ± 0.59a
	Ascomycota_unclassified	0.15 ± 0.09a	0.33 ± 0.2a	1.94 ± 1.89a	0.24 ± 0.16a	0.33 ± 0.28a

The different letters indicated a significant difference among different groups.

and 13.631% variation, respectively (**Figure 5B**). Different from bacteria, there were significant differences among the phyllosphere fungal community at all three stages of rice growth.

To further verify the above results, the differences between groups were compared in the rice bacterial and fungal community structure at different elevations for the three growth stages (**Tables 4**, **5**). The results showed that there was no significant difference in the rice bacterial community between 880 and 980 m asl at the seedling stage, but there were significant differences between the samples of the other elevations. There were significant differences among the five elevations at the heading stage. At the mature stage, 880 m asl was not observed to be significantly different from 580 to 980 m asl, but there were significant differences between other elevations. We also analyzed the community changes in the three growth stages at different elevations, and the results showed that there were significant differences between different growth stages at the same elevation, except for the seedling and heading stages at 680 m asl.

At the seedling stage, the phyllosphere fungal communities at 980 m asl were significantly different from those at 580 and 680 m asl, respectively, and no significant differences were observed between the samples at other elevations (**Table 5**). At the heading stage, the phyllosphere fungal community at 680 m asl was not significantly different from those at 580 and 980 m asl, while significant differences were observed between other elevations. At the mature stage, the phyllosphere fungal

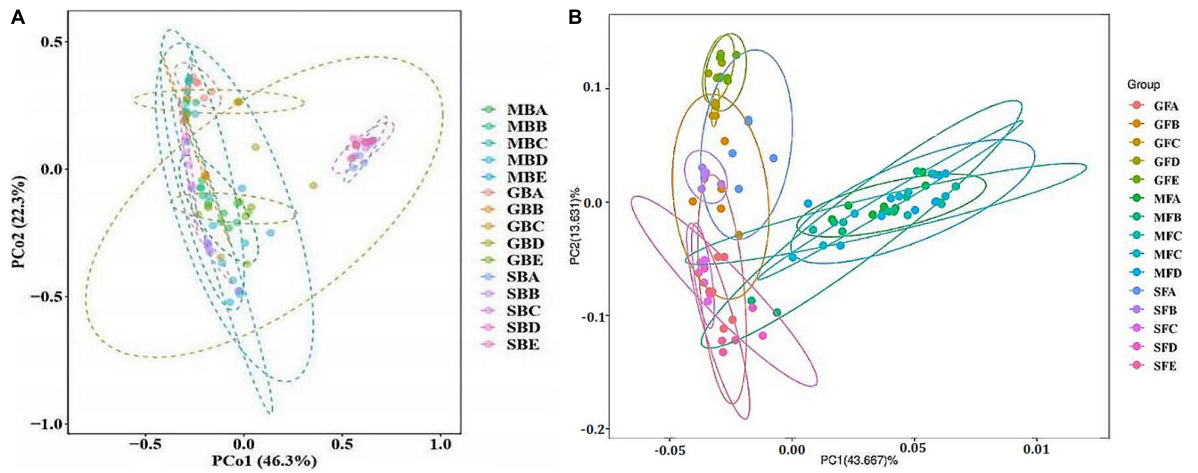

FIGURE 5 | Principal coordinate analysis (PCoA) of rice phyllosphere bacteria **(A)** and fungi **(B)** among different elevational sites and growth stages. **(A)** PCoA of rice phyllosphere bacteria. **(B)** PCoA of rice phyllosphere fungi. B, bacteria; F, fungi. M, seedling stage; G, heading stage; S, mature stage. (A) 580 m asl; (B) 680 m asl; (C) 780 m asl; (D) 880 m asl; (E) 980 m asl.

communities at 580 m asl were significantly different from those at 680 and 780 m asl; the phyllosphere fungal communities at 680 m were significantly different from those at 780, 880, and 980 m asl; the phyllosphere fungal communities of rice samples at 780 m asl were significantly different from those at 880 m asl; and there were significant differences between the other elevations.

Relationships Among Physicochemical Properties and Microbial Communities

Canonical correlation analysis was used to investigate the relationships between microbial communities and physicochemical properties (**Figure 6**). CCA1 and CCA2 accounted for 56.84 and 17.80%, respectively, of the total variation in the bacterial communities (**Figure 6A**), while accounting for 53.73 and 16.61%, respectively, of the total variation in the fungal communities (**Figure 6B**). The results of VPA showed that SP and antioxidant enzymes (CAT, SOD, and POD) accounted for 2.21 and 9.99%, respectively, of the total variation of phyllosphere bacterial populations (**Figure 6**). In addition, SP and physicochemical properties accounted for 7.15 and 10.34% of the total variation of phyllosphere fungal populations, respectively. These physicochemical properties also, respectively, accounted for 12.20 and 17.49% of the total variation in the bacterial and fungal communities. Among these physicochemical properties, SP shared a strong positive correlation with bacterial and fungal communities ($p < 0.05$) based on the Bray–Curtis distance and Jaccard distance matrix (**Supplementary Table 1**).

Correlation analysis of SP, antioxidant enzymes (SOD, POD, and CAT), and the top 30 dominant microbial genera (**Figure 7**) showed that SP showed a strong significantly negative correlation with *Serratia*, *Passalora*, unclassified_Trichosphaeriales, antioxidant enzyme ($R > 0.5$, $p < 0.05$), and significantly positive correlation with the fungal genera *Xylaria*, *Gibberella*, and *Penicillium* ($R > 0.5$, $p < 0.05$). Among the top 30 bacterial genera, *Mucilaginibacter* and *Nakamurella* had significant negative correlations with antioxidant enzymes, but weak correlations ($R < 0.5$, $p < 0.05$). *Serratia* had a strong significant positive correlation with antioxidant enzymes ($R < 0.5$, $p < 0.05$). Among the top 30 fungal genera, *Fusarium*, *Gibberella*, *Monographella*, *Paraphaeosphaeria*, *Penicillium*, and *Xylaria* had significant negative correlations with antioxidant enzymes ($R < 0.5$, $p < 0.05$), while *Passalora* had a strong significant positive correlation with antioxidant enzymes ($R > 0.5$, $p < 0.05$).

DISCUSSION

The leaves of plants present an area that is available for potential microbial colonization and growth, and the microbial populations of this phyllosphere can be abundant and taxonomically diverse (Bodenhausen et al., 2014). These organisms are crucial for leaf biological processes and ecosystem functions (Ortega et al., 2016), and have distinct impacts on their host plants by serving as plant pathogens or growth-promoting bacteria (Kinkel, 1997; Whipps et al., 2008). Microbial colonization of the phyllosphere is controlled by natural environmental factors. The variation of phyllosphere bacterial communities along elevational gradients may provide a potential link with temperature, which corresponds to elevation gradients over short geographic distances. At the same time, the plant growth stage is also an important factor in affecting phyllosphere microorganisms. Therefore, it is crucial to study the influence of both elevation gradients and growth stages on phyllosphere microorganisms, especially the core microbiome. In our study, we observed the dynamic change of phyllosphere microbial communities using an Illumina MiSeq-based approach, at different growth stages and elevations.

At all elevations, the rice leaf SP content decreased gradually with the rice growth stage. But at the same growth stage, there were slight differences in the SP between different elevations

TABLE 4 | Dissimilarity test of phyllosphere bacterial community between two different sample groups among different elevations at the seedling, heading, and mature stages.

Growth stage		MRPP		ANOSIM		PERMANOVA	
		Delta	*p*	*R*	*p*	*F*	*p*
Seeding stage	580 vs. 680 m	0.4783	0.003	0.4962	0.003	4.0604	0.003
	580 vs. 780 m	0.3007	0.002	0.9574	0.004	23.4574	0.005
	580 vs. 880 m	0.4709	0.01	0.6796	0.005	6.2416	0.007
	580 vs. 980 m	0.4769	0.002	0.7444	0.002	6.4354	0.005
	680 vs. 780 m	0.333	0.002	0.5129	0.003	6.1696	0.002
	680 vs. 880 m	0.5031	0.009	0.5092	0.011	4.69	0.016
	680 vs. 980 m	0.5092	0.007	0.5342	0.012	4.1205	0.004
	780 vs. 880 m	0.3255	0.004	0.8277	0.004	19.1718	0.003
	780 vs. 980 m	0.3316	0.003	0.7518	0.001	15.9034	0.002
	880 vs. 980 m	0.5017	0.058	0.2000	0.067	2.0966	0.079
Heading stage	580 vs. 680 m	0.2341	0.004	0.5564	0.003	10.9207	0.004
	580 vs. 780 m	0.2511	0.044	0.3074	0.056	3.2379	0.048
	580 vs. 880 m	0.4157	0.002	0.7314	0.003	9.8282	0.003
	580 vs. 980 m	0.2342	0.007	1.0000	0.002	39.8444	0.005
	680 vs. 780 m	0.243	0.005	0.7000	0.003	6.8864	0.002
	680 vs. 880 m	0.4075	0.005	0.5685	0.005	6.2698	0.004
	680 vs. 980 m	0.2261	0.004	1.0000	0.003	29.5623	0.003
	780 vs. 880 m	0.4245	0.004	0.5981	0.002	6.5610	0.005
	780 vs. 980 m	0.2431	0.002	1.0000	0.003	26.1789	0.005
	880 vs. 980 m	0.4076	0.005	0.4462	0.002	4.4366	0.003
Mature stage	580 vs. 680 m	0.1401	0.004	1.0000	0.002	116.8755	0.001
	580 vs. 780 m	0.1739	0.002	1.0000	0.001	119.5832	0.002
	580 vs. 880 m	0.0965	0.264	0.0935	0.163	1.3831	0.247
	580 vs. 980 m	0.075	0.015	0.3722	0.016	3.6047	0.019
	680 vs. 780 m	0.2264	0.003	0.8407	0.008	16.1691	0.001
	680 vs. 880 m	0.1491	0.002	1.0000	0.003	112.7412	0.002
	680 vs. 980 m	0.1276	0.004	1.0000	0.003	122.2524	0.002
	780 vs. 880 m	0.1828	0.002	1.0000	0.002	112.2833	0.002
	780 vs. 980 m	0.1614	0.004	1.0000	0.003	121.7608	0.005
	880 vs. 980 m	0.084	0.175	0.0453	0.263	1.1243	0.325

TABLE 5 | Dissimilarity test of phyllosphere fungal community between two different sample groups among different elevations at the seedling, heading, and mature stages.

Growth stage		MRPP		ANOSIM		PERMANOVA	
		Delta	*p*	*R*	*p*	*F*	*p*
Seeding stage	580 vs. 680 m	−0.007	0.613	0.024	0.311	0.061	0.517
	580 vs. 780 m	0.003	0.357	0.234	0.110	0.106	0.194
	580 vs. 880 m	0.010	0.233	0.062	0.210	0.090	0.160
	580 vs. 980 m	0.039	0.032	0.220	0.031	0.131	0.044
	680 vs. 780 m	0.011	0.197	0.201	0.104	0.128	0.120
	680 vs. 880 m	0.016	0.156	0.105	0.130	0.108	0.144
	680 vs. 980 m	0.045	0.026	0.267	0.017	0.155	0.026
	780 vs. 880 m	0.022	0.146	0.259	0.081	0.147	0.061
	780 vs. 980 m	0.000	0.407	0.114	0.231	0.107	0.348
	880 vs. 980 m	0.004	0.349	0.049	0.229	0.089	0.293
Heading stage	580 vs. 680 m	0.121	0.069	0.500	0.029	0.267	0.119
	580 vs. 780 m	0.630	0.031	1.000	0.028	0.861	0.030
	580 vs. 880 m	0.383	0.029	0.979	0.025	0.667	0.027
	580 vs. 980 m	0.524	0.024	1.000	0.031	0.827	0.035
	680 vs. 780 m	0.412	0.032	0.583	0.039	0.558	0.032
	680 vs. 880 m	0.191	0.059	0.552	0.057	0.413	0.064
	680 vs. 980 m	0.276	0.029	0.583	0.026	0.474	0.023
	780 vs. 880 m	0.505	0.032	0.885	0.034	0.689	0.029
	780 vs. 980 m	0.677	0.030	0.458	0.019	0.904	0.027
	880 vs. 980 m	0.182	0.031	0.500	0.029	0.357	0.058
Mature stage	580 vs. 680 m	0.269	0.023	0.375	0.024	0.360	0.020
	580 vs. 780 m	0.308	0.026	0.531	0.031	0.440	0.023
	580 vs. 880 m	0.124	0.157	0.125	0.278	0.232	0.182
	580 vs. 980 m	0.145	0.064	0.448	0.057	0.298	0.042
	680 vs. 780 m	0.529	0.025	1.000	0.023	0.822	0.029
	680 vs. 880 m	0.253	0.028	0.542	0.019	0.296	0.029
	680 vs. 980 m	0.301	0.023	0.417	0.034	0.404	0.032
	780 vs. 880 m	0.163	0.037	0.427	0.020	0.226	0.052
	780 vs. 980 m	0.114	0.122	0.292	0.110	0.146	0.378
	880 vs. 980 m	−0.004	0.447	−0.021	0.481	0.103	0.575

(**Figure 1A**). The results indicated that SP was less affected by elevation than it was more affected by the growth stage. The SP content was also an important factor affecting rice phyllosphere microorganisms (**Figure 6**). In addition, SP had a strong significant negative correlation with antioxidant enzymes ($R > 0.5, p < 0.05$).

The CAT, POD, and SOD are important antioxidant enzymes that cooperate to remove excess reactive oxygen species (ROS), thereby protecting the structures and functions of cellular components (Zhang et al., 2014). POD is widely distributed in plant tissues and is involved in diverse growth, development, and senescence processes in plants. CAT catalyzes the dismutation of excess H_2O_2 into oxygen and water, maintaining H_2O_2 at a low level, while SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Zhang et al., 2014). There was little difference in the activity of antioxidant enzymes at the seedling and heading stages; however, there was a great difference at the mature stage in the activity of these enzymes at different elevations (**Figures 1B–D**). This indicated that the activity of antioxidant enzymes in rice leaves was less affected by elevation at the seedling stage and heading stages, but was more affected by elevation at the mature stage. At the same elevation, the activity of antioxidant enzymes at the heading and mature stages was significantly higher than that at the seedling stage. These results indicated that the activity of antioxidant enzymes increased gradually during the growth process of rice and could be affected by elevation at the mature stage. In addition, *Serratia* and *Passalora* have strong significant positive correlations with antioxidant enzymes, which may be one of the causes of the changes in antioxidant enzymes.

There is a link between biodiversity and stability, as high biodiversity is generally associated with greater stability in an

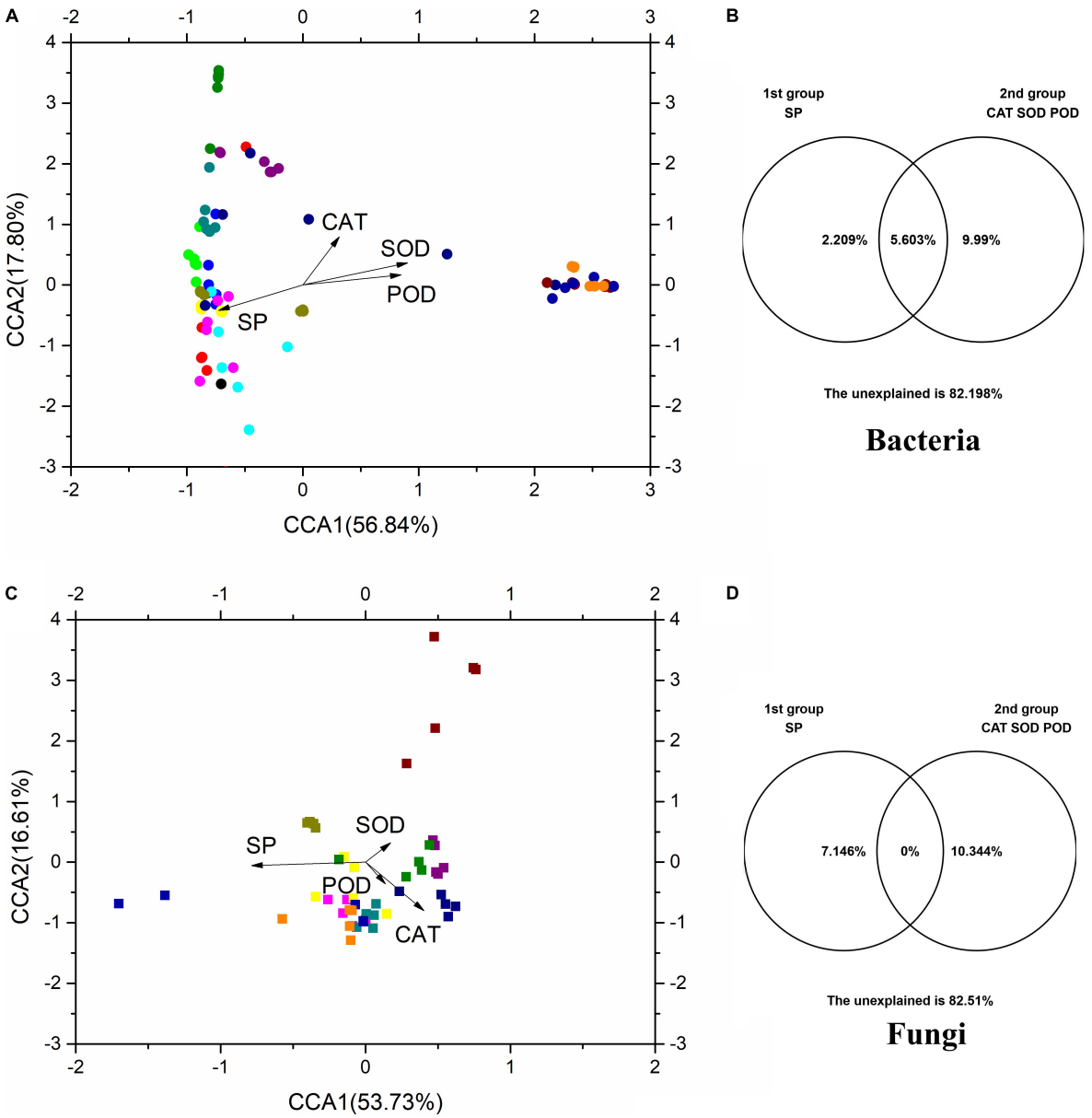

FIGURE 6 | The CCA and VPA of rice phyllosphere bacteria **(A,B)** and fungi **(C,D)** among different elevational sites and growth stages. CCA, canonical correspondence analysis; VPA, variance partitioning analysis. SP, soluble protein; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase.

ecosystem. Some studies have suggested that the strength of species interaction determines the biodiversity and stability of the microbial community (Ratzke et al., 2020). In addition, other studies found that the biodiversity of keystone phylotypes determines crop production (Fan et al., 2021). In our study, from 680 to 980 m asl, there was no significant difference between the seedling and heading stages of rice, while diversity at the mature stage was significantly different from the two proceeding stages (**Figure 2**). Rice phyllosphere bacterial community α diversity showed different changes during the growth period at low (580 m), medium (680 and 780 m), and high (880 and 980 m) elevations (**Figure 2**). In addition, the variation trend of diversity at the seedling and heading stages were similar for different elevations. On the whole, diversity increased gradually with elevation, while diversity decreased gradually from 680 to 980 m asl at the mature stage (**Figure 2**). The fungal α diversity of rice phyllosphere first increased and then decreased with growth, with the highest α diversity observed at the heading stage, while the elevation had only a small effect on the α diversity of the rice phyllosphere fungal community (**Figure 3**). This indicated that the elevation had a greater effect on the α-diversity of phyllosphere bacteria than on fungi, and the growth stage had a great effect on both bacteria and fungi. The present results are in agreement with previous studies (Cordier et al., 2012a; Qian et al., 2018) that showed microbial diversity was affected by elevation and

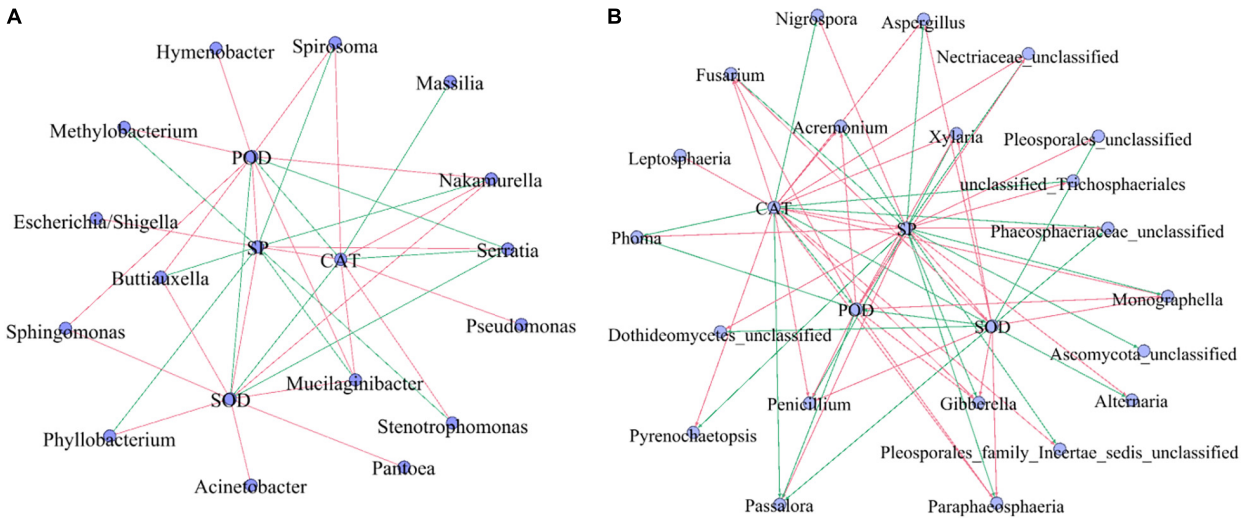

FIGURE 7 | The correlation analysis among SP, antioxidant enzyme (SOD, POD, and CAT), and top 30 bacterial **(A)** and fungal **(B)** populations. SP, soluble protein; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase. Red line: negative correlation, green line: a positive correlation. The correlation among SP, antioxidant enzyme (SOD, POD, and CAT), and top 30 microbial populations (genera) by the Pearson methods at $p < 0.05$. Standardization method: standardize environmental data only (scale each factor to zero mean and unit variance).

growth stages. Plant phyllosphere microorganisms associated with plant growth are very important to plant health and are closely related to important issues such as efficient utilization of nutrients, continuous cropping, and crop rotation. In general, the α diversity of rice phyllosphere bacteria displayed a more significant variation with elevation, which indicated that the influence of elevations on the α diversity of rice phyllosphere bacteria was higher than that of the growth stage. However, the variation of α diversity of rice phyllosphere fungi was not significant with elevation. On the contrary, at the same elevation, the variation of α diversity of rice phyllosphere fungi at different growth stages was more significant, which indicated that the growth stage had a greater effect on α diversity of rice phyllosphere fungi.

Phyllosphere microorganisms are strongly influenced by various biological and abiotic factors (Bulgari et al., 2011; Rastogi et al., 2012; Trivedi et al., 2012). However, phyllosphere-adapted bacteria may modify their local environment and increase the absorption of nutrients from the external environment (Bodenhausen et al., 2014). Previous research has indicated that phyllosphere fungal assemblages have a spatial structure that might be shaped by abiotic factors, especially temperature (Jumpponen and Jones, 2009; Cordier et al., 2012b). The variation of phyllosphere bacterial communities along elevation gradients may provide a potential link with temperature, which corresponds to elevation gradients (Cordier et al., 2012a). Our results showed that the composition of the phyllosphere bacterial and fungal communities varied considerably over a gradient at the same stage of rice growth, the most significant change occurred at 780 m asl (**Figure 4**). Previous studies have shown that the composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*) also vary significantly along an elevation gradient, and the variations in the assemblage composition were well correlated with variations in the average temperature (Cordier et al., 2012a). In our study, the variation of phyllosphere bacterial and fungal communities during the same growth period may be due to the change in temperature caused by elevation. In addition, we also analyzed the composition structure of the phyllosphere microflora in the seedling, heading, and mature stages of rice growth at the same elevation by dissimilarity and PCoA. There was a significant difference in bacterial and fungal community composition at different growth stages. The bacterial community structure at the seedling and heading stages was similar but different from that at the maturity stage. However, there were significant differences in fungal community structure between the seedling stage, heading stage, and mature stage. The phyllosphere microbiome has been known to be affected by the host genotype, where the plant host genetic factors shape the associated microbiota (Bodenhausen et al., 2014), and the host genotype is an important determinant of crop health (Rumakanta et al., 2015).

Among the five elevations during the three growth stages, Proteobacteria, Actinobacteria, and Bacteroidetes were the dominant bacterial phyla, and Ascomycota and Basidiomycota were the dominant fungal phyla (**Figure 4**). However, the relative abundance and composition of the dominant bacterial phyla were affected differently at the various elevations, indicating that the main rice phyllosphere bacterial population was affected by elevation. Therefore, we further studied the relationship between the main phyllosphere bacterial genera and elevation, discovering that some bacterial genera displayed significant correlations. The results suggested that elevation was significantly and positively correlated with *Lapillicoccus* and *Methylobacterium* at the seedling and heading stages and negatively correlated with these two genera at the mature

stage. Elevation was also significantly positively correlated with *Pantoea*, which can act as a pathogen-causing infection in plants and humans, at the seedling stage, but negatively correlated at the heading and mature stages. As the most common community members of the plant phyllosphere, *Methylobacterium* plays a critical role in protecting the host plants from various pathogens (Madhaiyan et al., 2006; Ardanov et al., 2012). *Alternaria* is a strong pathogenic fungus, which can cause a variety of plant diseases (Maiti et al., 2007; Logrieco et al., 2009; Kgatle et al., 2018). The relative abundance of *Alternaria* was positively correlated with elevation, which indicated that the higher the elevation, the greater the risk of disease in rice. At the heading stage, elevation was significantly negatively correlated with *Passalora*, Mycosphaerellaceae_unclassified, *Periconia*, etc., while at the mature stage the elevation was significantly positively correlated with *Passalora*, Mycosphaerellaceae_unclassified, *Periconia*, and so on, indicating that the elevation at the maturity stage and the heading stage had opposing effects on the phyllosphere fungal community.

CONCLUSION

In summary, using high-throughput sequencing methods, this study demonstrated a significant shift in the diversity and community composition of phyllosphere bacteria and fungi at the rice seedling, heading, and mature stages along an elevational gradient from 580 to 980 m asl. The results showed that the elevation had a greater effect on the phyllosphere bacterial α diversity, but less on the α diversity of phyllosphere fungi. The growth stage had a great effect on the α diversity of both phyllosphere bacteria and fungi. The results also showed that the diversity and composition of phyllosphere bacterial and fungal communities varied significantly between elevations at different growth stages, in terms of both the relative abundance of species and the variations in bacterial and fungal composition were well correlated with variations in the average elevation. SP had a strong significant negative correlation with antioxidant enzymes ($R > 0.5, p < 0.05$). In conclusion, elevation and growth stage had significant effects on the diversity, composition, and abundance of rice phyllosphere bacterial and fungal communities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA675674.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.766128/full#supplementary-material

Supplementary Figure 1 | The shared and united operational taxonomic unit (OUT) among different gradients and growth stages. B, bacteria; F, fungi. M, seedling stage; G, heading stage; S, mature stage. (A) 580 m asl; (B) 680 m asl; (C) 780 m asl; (D) 880 m asl; (E) 980 m asl.

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Defeating Huanglongbing Pathogen *Candidatus* Liberibacter asiaticus With Indigenous Citrus Endophyte *Bacillus subtilis* L1-21

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Huanglongbing (HLB) has turned into a devastating botanical pandemic of citrus crops, caused by *Candidatus* Liberibacter asiaticus (*C*Las). However, until now the disease has remained incurable with very limited control strategies available. Restoration of the affected microbiomes in the diseased host through the introduction of an indigenous endophyte *Bacillus subtilis* L1-21 isolated from healthy citrus may provide an innovative approach for disease management. A novel half-leaf method was developed *in vitro* to test the efficacy of the endophyte L1-21 against *C*Las. Application of *B. subtilis* L1-21 at 10^4 colony forming unit (cfu ml^{-1}) resulted in a 1,000-fold reduction in the *C*Las copies per gram of leaf midrib (10^7 to 10^4) in 4 days. In HLB-affected citrus orchards over a period of 2 years, the *C*Las incidence was reduced to < 3%, and *C*Las copies declined from 10^9 to 10^4 g^{-1} of diseased leaf midribs in the endophyte L1-21 treated trees. Reduction in disease incidence may corroborate a direct or an indirect biocontrol effect of the endophytes as red fluorescent protein-labeled *B. subtilis* L1-21 colonized and shared niche (phloem) with *C*Las. This is the first large-scale study for establishing a sustainable HLB control strategy through citrus endophytic microbiome restructuring using an indigenous endophyte.

Keywords: Citrus, *Bacillus subtilis*, endophyte, pathogen, restructuring, microbiome

INTRODUCTION

Huanglongbing (HLB) disease, a major uncontrollable disease of citrus trees resulting in significant yield losses, is caused by one or a combination of the phloem-inhabiting α-proteobacteria *Candidatus* Liberibacter asiaticus (*C*Las), *Ca.* Liberibacter africanus (*C*Laf), and *Ca.* Liberibacter americanus (*C*Lam) (Bové, 2006). The bacterium *C*Las may cause imbalances in host metabolism due to consumption, competition, and depletion of host nutrients (Duan et al., 2009). The main vector of *C*Las is Asian citrus psyllid (ACP), *Diaphorina citri* (Narouei-Khandan et al., 2016),

and understanding the mechanisms involved in plant-pathogen interaction is essential for the development of novel HLB management strategies (Albrecht and Bowman, 2008; Fan et al., 2011).

The characteristics of HLB disease symptoms include blotchy mottled and pale yellow leaves, followed by distinct yellow shoots, corky veins, stunting, lopsided fruits with color inversion, and twig dieback (Ajene et al., 2020). Roots are also observed with a dramatic reduction in the fibrous root mass of infected plants, which leads to poorly developed root systems (Johnson et al., 2014; Li et al., 2019). Citrus-infected plants with HLB are not always but confused easily with symptoms of nutrient deficiency and without professional training (Tian et al., 2014) because the pathogen distribution is always highly patchy, as leaves and stems contain most of the bacterial titers (Li et al., 2006). In the beginning, the titer may be the highest in the roots (Johnson et al., 2014; da Rocha et al., 2019). The symptoms are clearer and more apparent in the cooler season compared to warmer months. Shimwela et al. (2019) estimated that the incubation period can be several months to years. It is still unknown that how long the trees have been infected before the appearance of symptoms, but symptomatic citrus trees eventually show disease symptoms (Dala-Paula et al., 2019).

So far, there are no curative methods to control this pathogen; although, scientists have used a few control measures to eliminate or reduce the pathogen growth. Approaches to control HLB include frequent use of antibiotics such as penicillin (Shin et al., 2016; Ascunce et al., 2019; McVay et al., 2019) and oxytetracycline (Blaustein et al., 2018), screening for small molecule inhibitors (Pagliai et al., 2015), combinations of stock and scion (Albrecht et al., 2016), graft-based chemotherapy (Zhang et al., 2012; Yang et al., 2016) and transgenic technology (Hao et al., 2016; Zou et al., 2017), control of psyllid vector (Tomaseto et al., 2019), and, finally, the most extreme measure is chopping off and burning down the diseased trees, which results in polluting environment even more. However, this method may slow down the disease, but with huge losses.

However, biocontrol of plant diseases can be the most promising alternative to continuously failing treatments and endophyte-mediated biocontrol offers consistent results due to intimate association with its host and shared niche with the pathogen where endophyte could fully manifest their antagonistic potential, whereas the non-endophytic biocontrol agents often fail under field conditions due to fierce competition in the rhizosphere and harsh environmental conditions (Ahmed et al., 2020; Blacutt et al., 2020; Trivedi et al., 2020). In biocontrol programs, native endophytes could be used to maintain long-term colonization inside the citrus host and eliminate *C*Las through niche and nutrient competition (Munir et al., 2018b). Biocontrol of plant pathogens has yielded effective results using bacteria under laboratory conditions (Herschkovitz et al., 2005; Andreote et al., 2010; Dematheis et al., 2013) and moderate effects under field conditions (Mattos-Jr et al., 2020), but the potential of indigenous endophytic bacteria as economically useful biocontrol agents against citrus pathogens has not been investigated, especially for HLB control (Munir et al., 2021). This approach may bring a revolution in managing citrus disease caused by phloem-limited α-proteobacteria (Bové, 2006).

Plant diseases and various plant protection measures lead to perturbations of the host microbiome (Irigoyen et al., 2020); it is also evident that *C*Las is associated with changes in microbial population dynamics during the HLB disease progression (Zhang et al., 2013a, 2016; Munir et al., 2019); therefore, it is of immense importance to understand the impact of key endophytic bacteria in the citrus health and defense against HLB. It is suggested that the key inhibitors of *C*Las play an important role in pathogen suppression (Barnett et al., 2019).

In an effort to gain in-depth insight, the aim of this study was to quantify the effects of regular applications of an indigenous endophyte, *Bacillus subtilis* L1-21 isolated from healthy citrus trees (selected based on potential biocontrol activities against several bacterial and fungal pathogens) to diseased trees for *C*Las reduction. This study highlights the promising research findings of using a novel citrus half-leaf method to reduce the *C*Las in the presence of *B. subtilis* L1-21. Most importantly, the evidence was provided from the two field experiments in the presence of endophyte L1-21, where citrus was diseased for more than 5 and 2 years, respectively. Finally, we point out that the red fluorescent protein (RFP)-tagged endophyte L1-21 shared the same niche with *C*Las inside citrus phloem. To the best of our knowledge, this is the first study undertaking a citrus endophyte-mediated management against HLB disease on a large field scale with consistent control.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The endophytic strain *B. subtilis* L1-21, an indigenous endophyte isolated from a healthy citrus host (Munir et al., 2020a) and deposited in the Chinese Culture Collection Bank, Beijing (Accession number: CGMCC15726), was selected for the greenhouse and citrus field experiments (Binchuan, Yunnan Province, China). The endophyte *B. subtilis* L1-21 and its RFP-tagged strain were stored in 40% glycerol (v/v) at −80°C; stock culture was renewed every 4 months on Luria Bertani (LB) agar and cultured for 24–48 h to check the stability of the potential endophyte. A pure culture of endophyte L1-21 was grown until the late log phase in LB broth for 24–48 h at 37°C in a shaking incubator (150 rpm). The colony forming units (cfu ml^{-1}) of the endophyte were checked using contrasting dilutions in sterile distilled water with 0.1% Tween 20 and plated on LB agar before foliar spraying on the citrus trees.

Novel Citrus Half-Leaf Method

We designed a novel citrus half-leaf method to quantify *C*Las copies in leaves prior to and after treatment based on conventional and quantitative PCR (qPCR). Diseased citrus leaves [*Citrus reticulata* (*C. reticulata*)] were collected randomly from 16 years old citrus grove and brought to the laboratory in ice and immediately processed for experiment or stored in 4°C till further use. Firstly, DNA was extracted from one-half of a diseased leaf using the cetyltrimethylammonium bromide (CTAB) method (Araújo et al., 2002; Munir et al., 2019) and conventional PCR analysis, using the primer set Cal-R/Cal-F

(Jagoueix et al., 1996), was followed by nested PCR analysis, using the primer set CG03F/CG05R (Zhou et al., 2007), for confirmation of "*Ca.* Liberibacter asiaticus." Primers used in this study are given in **Supplementary Table S1**. Once confirmed as positive for *C*Las, the other half of the citrus leaf (4–5 cm) was suspended with 10^4-10^6 cfu ml^{-1} of endophytes in a 5-ml Eppendorf tube and the different antibiotics, i.e., penicillin (100 $\mu g\ \mu l^{-1}$) dissolved in distilled water, spinosad (100 $\mu g\ \mu l^{-1}$) dissolved in hexane solution, and shenqimycin (100 $\mu g\ \mu l^{-1}$) dissolved in distilled water as chemical control (positive). LB broth and sterilized distilled water were used as uninfected control (negative). The Eppendorf tubes were kept for 96 h at room temperature, followed by extraction of DNA from the leaf midribs. The *C*Las 16S ribosomal RNA (16S rRNA) gene was amplified as discussed previously (Munir et al., 2019). The weight of each half leaf was recorded prior to the experiment. Each treatment comprised three replicate Eppendorf tubes that each contained six diseased citrus leaves midribs. The experiment was repeated five times. Treatment effects on *C*Las were tested by loading the amplified product on 1% agarose gel. In another experiment testing for *C*Las and endophytes abundance, diseased leaves were placed in Petri dishes (180 mm) and different treatments were performed. DNA was extracted from one-half of a diseased leaf using the CTAB method (Araújo et al., 2002) and amplified with the same primers as described previously. Once the leaf was confirmed as infected with *C*Las, the other half of the leaf was kept in a 180-mm Petri dish with 10^4 and 10^6 cfu ml^{-1} or penicillin (100 $\mu g\ \mu l^{-1}$) dissolved in distilled water. LB and distilled water were used as controls. The Petri dishes were kept at room temperature for an interval of 1 day for collected leaves to be checked for pathogen concentration and endophytes for 4–5 days; then, DNA was extracted from the midribs and present endophytes were isolated (Araújo et al., 2002; Munir et al., 2018b). The weight of each half leaf was recorded prior to the experiment. Each treatment comprised three replicates of 12 diseased citrus leaves (six random top and bottom half leaves, each) and the experiment was repeated five times. The presence of the pathogen was confirmed using standard qPCR analysis and density (copies g^{-1}) was calculated based on a standard curve generated by cloning 382 bp of a specific DNA fragment located in the ribosomal protein (*rplJ*) (Munir et al., 2020a). The PCR reaction was performed in a 25-μl reaction mixture containing 1 × PCR buffer (SYBR Green Master Mix; Bio-Rad, United States), 0.8 μl of each primer (CQULA04R/CQULA04F), and the appropriate amount of DNA template in an RT-PCR system (StepOne Real-Time PCR System, Applied Biosystems, United States) as reported previously (Wang et al., 2006). Changes in *C*Las copies were tested from 0 to 4 days, based on CT values of pathogen copies g^{-1} of the citrus leaf that were calculated using the standard curve generated previously; changes in endophyte density were recorded daily as log cfu g^{-1} of citrus leaf midrib.

Phloem Colonization of *Bacillus subtilis* L1-21 RFP

For confirmation of phloem colonization of the endophyte, the RFP-tagged strain of *B. subtilis* L1-21 was generated using genomic DNA by amplifying the *mKate2* coding sequence with ribosome-binding site. *Bam* H1 and *Hin*d III enzymes were used for product digestion and ligated with the plasmid pYC127 as discussed previously (Chai et al., 2008). The resulting plasmid designated as PY69 was used to make overexpression RFP strain of *B. subtilis* L1-21. A strain expressing red fluorescent reporter gene (*mKate2*) was grown until late log phase in LB medium with 10 $\mu g\ \mu l^{-1}$ chloramphenicol in shaking incubator at 37°C. Citrus plants (2 years old) in the greenhouse were foliar sprayed with RFP-tagged endophytic strain L1-21 and its colonization was visualized by confocal laser scanning microscope (CLSM) after 2 days. Citrus leaves were detached and gently washed for surface sterilization as performed previously to remove unattached cells from the citrus leaves (Munir et al., 2021). Leaves midribs were separated and put on the specimen holder by adding optimal cutting temperature (OCT) compound (Sakura, Europe). Specimen holder containing citrus leaves midribs were sliced briefly on the microtome-cryostat (Leica Biosystems, United States). Slice midribs were examined using CLSM (Olympus, FV10-ASW). The emission of RFP was measured at 405 to 635 nm after excitation at 559 nm, while citrus plant autofluorescence was measured after excitation at 405 and 635 nm for blue and green fluorescence, respectively. Images were captured using an automatic picture program FV1000 Viewer. The experiment was repeated three times, each time with three replicates.

Study Site and Sample Processing From HLB-Affected Citrus Groves

The experimental field site was located at Binchuan, Dali City, Yunnan Province, China (100.5754° E, 25.8272° N), with an average annual temperature of 33–35°C and total annual rainfall of about 1,000 mm. Foliar symptoms in one citrus grove (*C. reticulata*), 16 years old, indicated > 90% *C*Las disease incidence, and the second citrus grove (*C. reticulata*), 4 years old, which was located 2 km away from the first citrus grove, exhibited 100% *C*Las infection as confirmed through conventional PCR and qPCR before the start of experiments. Two field trials were conducted from 2017 to 2019: in one citrus grove, 162 trees (**Supplementary Figure S1**) were treated with contrasting treatments and in the second citrus grove, 93 of 525 trees were selected (three replicates of 31 trees) for *C*Las titer test. Prior to the start of each trial, a field survey was carried out to visually determine individual tree HLB severity and detected the *C*Las pathogen using *C*Las-specific primers in PCR and qPCR analyses. The soil treatments at the citrus groves comprised fertilizer 1 (rapeseed cape with *Bacillus amyloliquefaciens* Y2, as a root growth promoting agent); fertilizer 2 (rapeseed cape without *Bacillus amyloliquefaciens* Y2); and no rapeseed cape as a control (F_0). Antibiotic penicillin (100 $\mu g\ \mu l^{-1}$) dissolved in distilled water was applied one time through trunk injection at the start of the experiment as shown in **Supplementary Table S2**. In addition, we applied 5 kg of fertilizer to each tree around a water drip of about 20 cm deep. The antibiotics and endophytes used in this study are given in **Supplementary Table S2**.

Field Endophyte Treatment Application

The endophyte application was carried in the field experiments. Two injection ports per tree were made by drilling 30 mm

into the xylem tissue using a 7.14-cm drill bit, located at the opposite sides of the trunk at ∼20 cm above the union bud. Antibiotic and endophyte (*B. subtilis* L1-21) treatments were injected from a pressurized bottle into each port using a micro drip infusioner at the recommended pressure (<50 psi). The first injection was done with penicillin (0.1%) for 4 days on 54 trees, with 27 trees as a control. The second injection was done 2 weeks after the antibiotic treatment with the endophyte (10^6 cfu ml^{-1}) on 54 trees. We also applied 10^6 cfu ml^{-1} of the endophyte or penicillin as a monthly foliar spray on 27 trees for each treatment, until all leaves were wet, in the early morning or late evening. All the experiments were arranged as three replicates of three trees in a randomized complete block design.

Isolation of Endophytes From Citrus Trees

Candidatus Liberibacter asiaticus causing HLB disease negatively affected the indigenous endophytic microbes. Therefore, we estimated the density of endophytic bacteria from three replicates of six citrus tree leaves that were sampled from the trees and transported to the laboratory in a cooler with ice. Endophytes were isolated from three replicates of treatment samples collected on the first week of each month in 2017–2018 as isolated previously (Araújo et al., 2002).

Candidatus Liberibacter asiaticus DNA Extraction and PCR Amplification

Leaf samples from all the treatments were collected monthly from the citrus groves to quantify *C*Las titer. Leaves were treated as above before DNA extraction. Methods for DNA extraction, PCR, and qPCR were those described previously.

Statistical Analysis

Endophytic bacteria populations were calculated, based on average logarithm (base 10) cfu values, and analyzed using the GraphPad Prism version 8 (San Diego, California, USA). Pathogen copies g^{-1} of diseased citrus leaf material were calculated using a standard curve value (Munir et al., 2020a). Treatment effects were tested using the ANOVA in the SPSS Statistics version 22.0 (IBM Corporation, Armonk, New York, USA) and treatment means were compared using Duncan's multiple range test at $p < 0.05$.

RESULTS

Bacillus subtilis L1-21 Suppression of *C*Las in the Laboratory

We used the indigenous endophyte *B. subtilis* L1-21 against *C*Las in the leaf midrib by means of citrus half-leaf method (**Figures 1A,B**) maintained under room temperature in Eppendorf tube. *C*Las copies in leaf material were reduced by 5 days after treatment with the endophyte at 10^4 and 10^6 cfu ml^{-1} (**Figure 1C**); similarly, the antibiotics used in this experiment also reduced *C*Las copies (**Figure 1D**). Subsequent dilutions of the endophyte validated the results, confirming that *C*Las copies inside the citrus leaf midrib were reduced by a single application of the endophyte. Since the leaves midribs were cut and put on shaking in water, we suggested that pathogen ooze out of the midribs and no positive band was observed.

To further confirm the efficacy of the endophyte against *C*Las, we used the half-leaf method to test pathogen copies in one half of the leaf present in Petri dish treated with a single application of the *B. subtilis* L1-21 (10^4 and 10^6 cfu ml^{-1}) or penicillin antibiotic (as a positive/chemical control) and LB broth and water (as negative/uninfected controls). The presence of the pathogen was confirmed using standard qPCR analysis and density (copies g^{-1}) was calculated based on a standard curve (Munir et al., 2020a). CLas pathogen copies in the leaf midribs treated with the endophyte L1-21 (10^4 and 10^6 cfu ml^{-1}), penicillin, LB, and water were 1.12×10^7, 1.23×10^6, 4.71×10^5, 2.38×10^5, and 1.51×10^6, respectively (**Figure 1E**; **Supplementary Table S3**). Application of the endophyte reduced *C*Las copies in the leaf midribs 1,000-fold (1.12×10^7 to 3.72×10^4) and 100-fold (1.23×10^6 to 3.93×10^4) by 4 days after a single treatment of 10^6cfu ml^{-1} and 10^4 cfu ml^{-1} (**Figure 1F**; **Supplementary Table S3**) of the endophyte, respectively. Application of penicillin reduced *C*Las in diseased citrus leaves 10-fold (4.71×10^5 to 1.64×10^4) and there was no effect of LB or water controls by 4 days after treatment. These results clearly indicated that application of the endophytes reduced CLas pathogen in diseased citrus leaves. The half-leaf method was also employed to check the pathogen reduction in individual leaves using top and bottom parts to confirm validation of results and we showed that *C*Las could be reduced to more than 90% after 3–4 days (**Supplementary Table S4**).

Bacillus subtilis L1-21-Derived Indigenous Endophytes Inside Citrus Leaves

Prior to application of the endophyte, indigenous citrus endophyte density in the citrus leaves was 10^5 cfu g^{-1}; following a single treatment with endophytic strain L1-21, the density of other endophytes present inside leaves increased to 10^9 cfu g^{-1} (**Figure 2A**). Treated endophyte L1-21, which displayed pinkish color on LB media, was recovered from the diseased citrus leaves during different time intervals with different treatments mentioned (**Figure 2B**). The application of penicillin initially decreased leaf midrib microbe density, but this effect subsided with time as the endophyte density returned to pretreatment levels. The endophyte density was unaffected by the LB and water treatments.

Bacillus subtilis L1-21 Colonization in Citrus Phloem

To prove the hypothesis that the indigenous endophyte *B. subtilis* L1-21 colonization inside citrus midribs phloem helps in excluding the *C*Las, which resides in citrus phloem, an RFP-tagged L1-21 strain was constructed that can express *mKate2* genes. After observation with CLSM, we found that RFP-tagged L1-21 successfully colonizes the citrus midribs (**Figure 3**). Two days after inoculation, we observed that a lot of cells are present inside the phloem. These results indicated that the endophyte not only colonizes the citrus phloem completely but also reduces the pathogen load present in the citrus phloem as proved above.

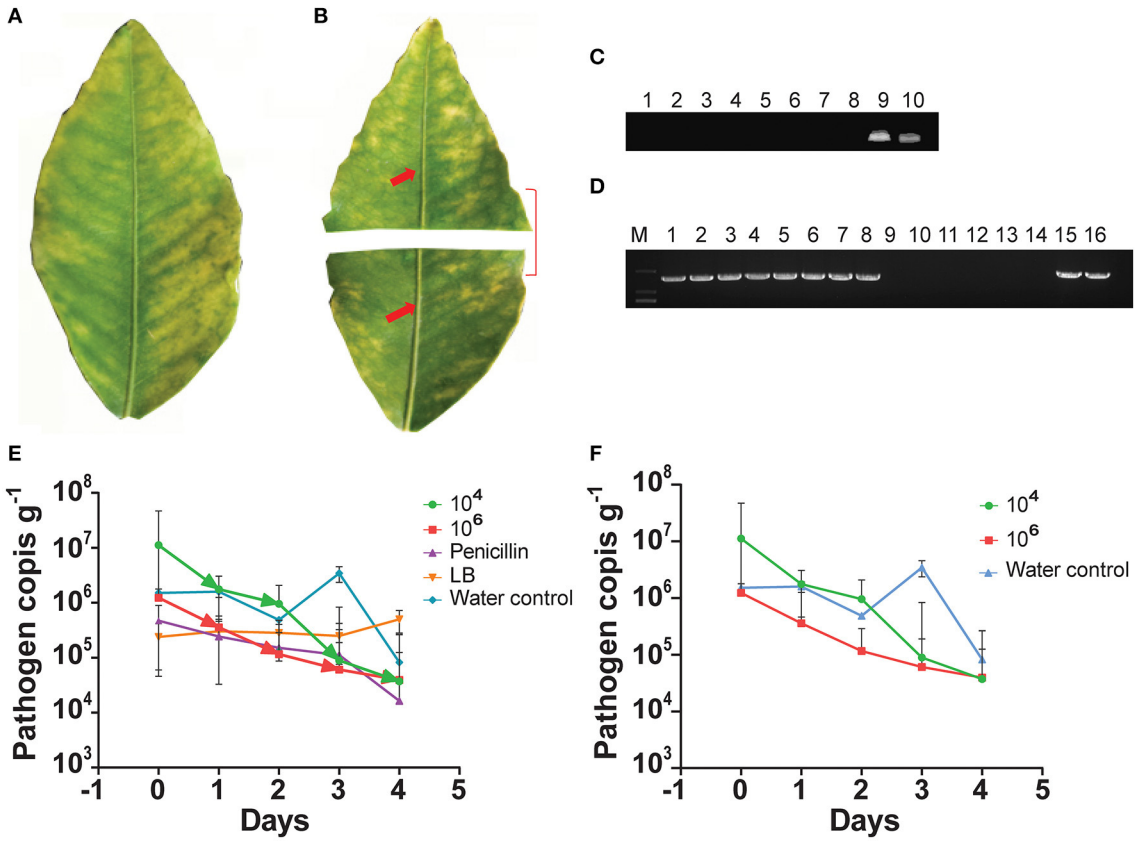

FIGURE 1 | Citrus half-leaf method for reduction of *Candidatus* Liberibacter asiaticus (CLas) in the diseased citrus leaves from 0 to 4 days. Sketch of citrus leaf used in novel citrus half-leaf experiment. **(A)** Whole citrus leaves showing Huanglongbing (HLB) symptoms; **(B)** Leaf was parted into two parts; the first part was used to detect the CLas pathogen using conventional and quantitative PCR (qPCR); after confirmation, the second part was used in the experiment for *Bacillus subtilis* (*B. subtilis*) L1-21 treatment. Red arrows indicate leaf midribs used for extraction of DNA for conventional and qPCR; **(C)** Conventional PCR and diseased citrus leaves were treated with endophytes (10^3-10^6 cfu ml^{-1}) and antibiotics penicillin (100 μg μl^{-1} and 500 μg μl^{-1}), shenqinmycin (100 μg μl^{-1}), and spinosad (100 μg μl^{-1}); lane 9, water control; lane 10, positive control; **(D)** Conventional PCR, before application; Lane 1, citrus leaf sample treated with penicillin; Lane 2, leaf samples treated with spinosad; Lane 3, citrus leaves treated with shenqinmycin; Lane 4, sample in Luria Broth (LB) medium treated with endophytes without shaking; Lanes 5 and 6, samples in LB medium and water, respectively, on shaker; Lanes 7 and 15, diseased citrus leaf in water only; Lanes 8 and 16, positive control; after application: Lane 9, indicated reduction of CLas pathogen with different treatments including penicillin; Lane 10, spinosad; Lane 11, shenqinmycin; Lane 12, endophyte treatment in LB medium without shaking; Lane 13, endophyte treatment in LB medium with shaking; Lane 14, samples in water with shaking; **(E)** qPCR, pathogen copies per gram of leaves midrib using 10^4 and 10^6 colony forming unit (cfu) ml^{-1} of endophyte treatment one time. Pathogen copies g^{-1} was calculated based on the standard curve of recombinant plasmid pUC18-382-HLB generated through qPCR. Penicillin was used as a positive control. LB medium and water were used as negative control treatments. Three biological replicates were used for each treatment and each replicate consisted of 12 diseased leaves (including 6 top and bottom half leaves each). The experiment was repeated five times; and **(F)** Pathogen copies g^{-1} of leaves using 10^4 and 10^6 cfu ml^{-1} of endophyte and water treatment one time. The values are means ± SD of five replicated experiments.

Efficacy of Short-Term Field Applications of *Bacillus subtilis* L1-21 Against CLas

In 2017–2018, the efficacy of potential endophyte *B. subtilis* L1-21 was tested against CLas in two diseased citrus groves (>90% and 100% HLB prevalence, respectively) containing 16- and 4-year-old citrus trees, respectively. We treated the CLas-infected mandarin groves with contrasting applications of the endophyte, antibiotic, and biofertilizers to determine the most effective management strategy (**Supplementary Figure S1**). HLB-affected citrus grove was completely recovered after 6–7 months of successful treatment with the indigenous endophyte (**Supplementary Figures S1B,C**). Antibiotic and biofertilizers were applied once at the start of the experiment and endophytes were applied monthly, following leaf sampling to check densities of CLas and the endophyte. Prior to the start of the experiment, all the citrus trees were tested for the total number of endophytes present inside each tree (**Figure 4A**). *Bacillus subtilis* L1-21 applied to the citrus trees spreads to neighboring, untreated trees (**Supplementary Figure S2**); therefore, the endophytic density of all the citrus trees were presented on monthly basis. The density of CLas and endophytes was measured monthly in leaf samples collected from each of the 162 citrus trees using conventional and nested PCR techniques (**Figures 4B–D**). The health of leaves was improved following treatment with the endophyte for 1 year,

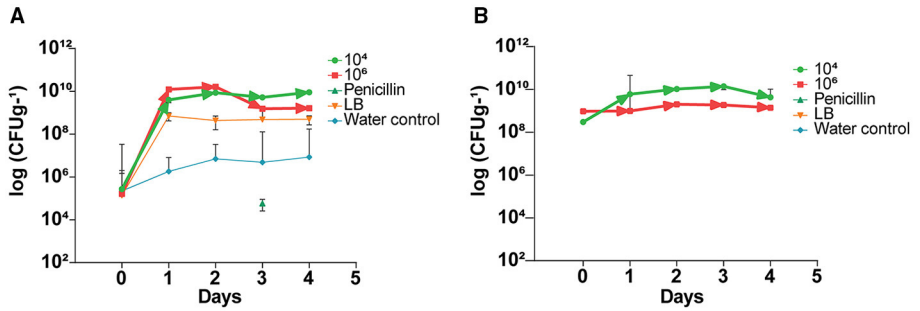

FIGURE 2 | Elevation of indigenous endophytes derived after application of *B. subtilis* L1-21 in citrus half-leaf method. **(A)** Native endophytes density in the diseased citrus leaves after application of endophytes and penicillin and negative control. **(B)** Recovery of treated endophytes from the diseased citrus leaves during different time intervals with different treatments. The populations of endophytic bacteria were calculated based on the average logarithm (base 10) of bacteria recovered from the plant leaves. The detection limit (1×10^2 cfu leaves^{-1}) was treated as log 0 for mean calculation. The log cfu values were analyzed with the GraphPad Prism version 8 (San Diego, California, USA). The values are means ± SD of five replicated experiments.

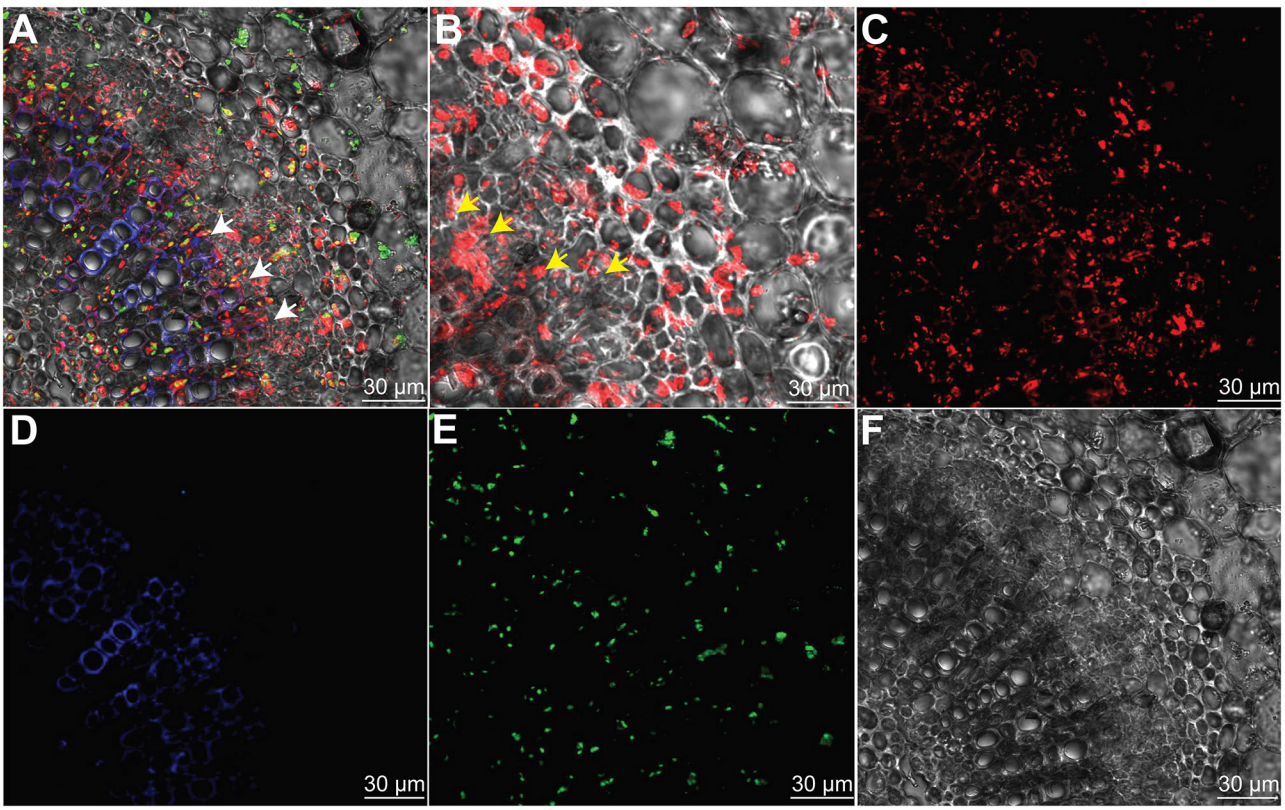

FIGURE 3 | *Bacillus subtilis* L1-21 colonization in the phloem of citrus leaves midribs. **(A–E)** Indigenous citrus endophyte expressing *mKate2* was grown until the late log phase and foliar sprayed on citrus plants in the greenhouse. Confocal laser scanning microscope was carried for visualization of *B. subtilis* L1-21 red fluorescent protein (*RFP*) in phloem; **(A)** Transverse section of merged citrus leaves midrib displaying red fluorescence (phloem); green autofluorescence (chlorophyll); blue fluorescence depicted the xylem; **(B)** Enlarge part from panel A observed for details colonization of endophyte inside citrus leaves phloem; **(C)** Individual RFP of *B. subtilis* L1-21; **(D)** Xylem with blue autofluorescence; **(E)** Green autofluorescence generated by chlorophyll; and **(F)** Dark field showing citrus phloem. The scale represents the 30 µM. The white and yellow arrows represent the localization of RFP-tagged *B. subtilis* L1-21 inside phloem.

where they changed from yellow to green, and we found negative effects of CLas on the endophytic microbiomes of the citrus tree. Citrus trees were monitored monthly to assess visual disease symptoms and following 1 year of monthly applications of *B. subtilis* L1-21, CLas density in the trees reduced significantly. In the first quarter of the experiment, the number of diseased trees reduced to <100, where trees that had been characterized by yellow and mottled leaves began to develop more robust shoots

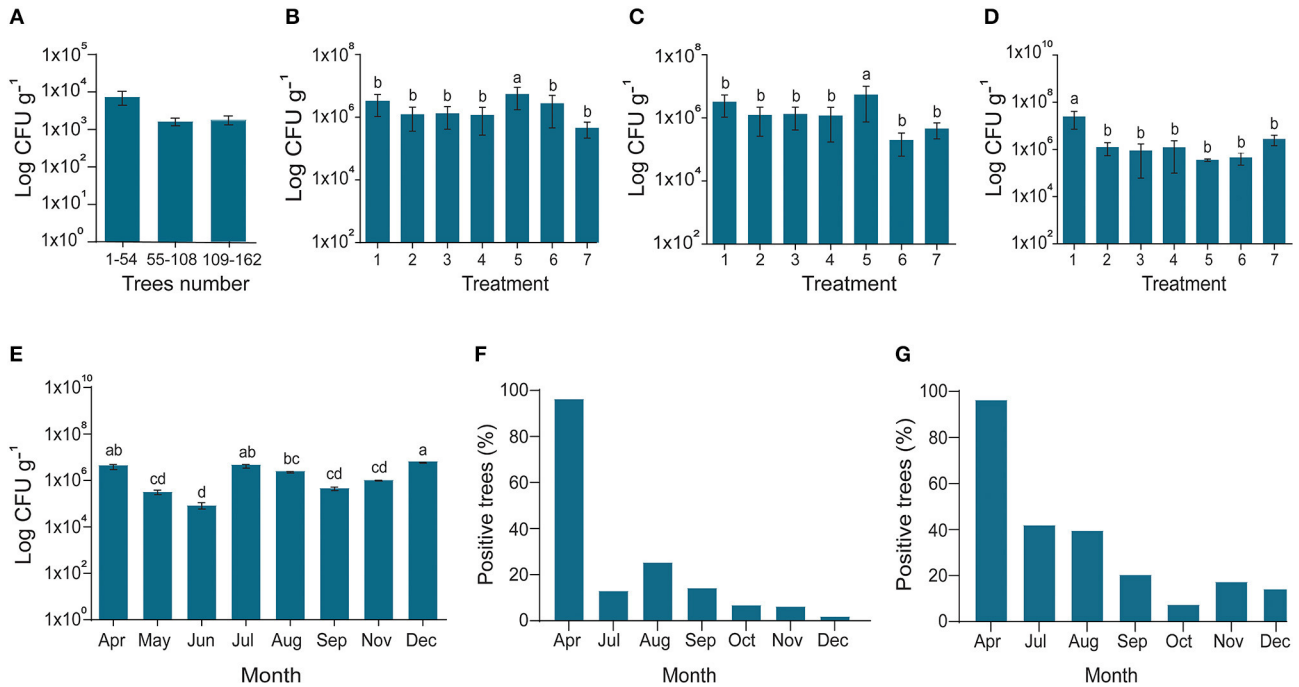

FIGURE 4 | Endophytes density and reduction of *C*Las pathogen. **(A)** In March 2017, the diseased citrus trees were checked for possible assessment endophyte before starting experiment; **(B)** In April 2017, all the trees treated with fertilizer 1 with *Bacillus amyloliquefaciens* Y2; the X-axis represents endophytes and penicillin injection through the trunk (1); endophyte injection (2); Penicillin spray (3); Endophyte spray (4); Penicillin injection (5); Control CK1 and CK2 (6,7); **(C)** Fertilizer 2 with *B. amyloliquefaciens* Y2; CK2 and CK3 (6,7); **(D)** No organic fertilizer (F_0) was used as control; (6,7) indigenous endophytes showing increase in the number of endophytes due to dispersal of endophytes in all the trees even control samples; **(E)** Endophytes population during different sampling times along with; and **(F)** Successive reduction of *C*Las pathogen inside disease trees using conventional PCR and **(G)** Nested PCR. The populations of endophytic bacteria were calculated based on the average logarithm (base 10) of bacteria recovered from the plant leaves. The log cfu values were analyzed with the GraphPad Prism version 8 (San Diego, California, USA). The values are means ± SD with statistically significant difference among different treatments with different letters ($p \leq 0.05$).

and leaves. The leaf density of citrus endophytes was initially reduced by the application of antibiotic but later recovered to more than 10^7 cfu g^{-1}. By the second quarter of the experiment, the number of diseased trees reduced to 69, in which the endophyte density was similarly increased; by the final quarter, the number of diseased citrus trees reduced to three, and trees in the grove finally yielded fruit suitable for the commercial market. Thus, we confirmed that the endophyte *B. subtilis* L1-21 was the most effective control agent for the management of HLB and a reliable yet cheap option for citrus growers worldwide. Strengthening citrus microbe density using an endophyte with diverse antagonistic activities through trunk injection and foliar spray application may elevate microbe density in diseased citrus trees to levels where *C*Las may more easily be controlled (**Figure 4E**). Previously, we found the number of the endophyte types was lower in diseased citrus trees, indicating that greater density of indigenous citrus endophytes may contribute to the control of *C*Las in diseased trees (Munir et al., 2019).

The copies of *C*Las in leaf samples were reduced following 6 months of monthly treatments with *B. subtilis* L1-21 (**Figures 4F,G**), confirming our hypothesis that indigenous citrus endophytes may reduce disease incidence in the field by >95%. After a year of monthly applications of the endophyte, population of *B. subtilis* L1-21 in citrus leaf midribs increased from 10^3 to around 10^9 cfu g^{-1} in most trees, showing that indigenous endophytes may represent a novel management strategy for the control of *C*Las in citrus plants (**Figure 5**). We have found similar effects of this endophyte on *C*Las in 2 other citrus groves in the study region and one each in the Genma county of Yunnan Province (**Figure 6**).

Effects of Long-Term Field Applications of *Bacillus subtilis* L1-21 on *C*Las

To confirm the results from the 1-year field experiments, we regularly treated diseased citrus groves, comprising 525 trees, with *B. subtilis* L1-21 in 2018–2019; these regular applications reduced copies of *C*Las in the citrus trees so that the number of diseased trees was reduced. Finally, we selected 93 among the 525 trees, which represented three replicates of 31 trees and found that after 1 year, regular applications of the endophyte had reduced the *C*Las from 10^9 to 10^4 g^{-1} of leaf midrib (**Figure 7A**) (99% control; **Table 1**). *C*Las copies were very high in April 2018 but decreased within 3 months (**Figure 7B**). In April 2019, we found 16 of the 93 diseased trees contained 100 copies of the pathogen g^{-1} of leaf material, while 39 diseased trees contained < 100 copies, representing a 91.39% reduction in *C*Las (**Figure 7C**) and there was a decrease in the number of yellow leaves and shoots and an increase in the growth

FIGURE 5 | Huanglongbing affected diseased citrus groves before and after application of endophyte L1-21 in Binchuan county, Yunnan Province, China. The citrus groves were checked for *C*Las pathogen detection before the start of the experiment to make sure that all the citrus trees are affected with HLB. **(A,C,E)** In March 2017, the citrus grove was affected with HLB and **(B,D,F)** In June 2017, trees displayed more robust growth with green leaves after *Bacillus subtilis* L1-21 application.

of new shoots (**Figure 7D**). Our findings indicated that *C*Las density remained constant in around 25% of the citrus trees during the endophyte treatment and was undetected in 16 of the diseased citrus trees in April 2019. Thus, we suggested that *C*Las was successfully eliminated from diseased citrus trees by *B. subtilis* L1-21.

DISCUSSION

In order to demonstrate the specific role of endophytes to manage the alarmingly devastating disease of citrus, we isolated and identified indigenous endophytes from symptomatic, asymptomatic, and healthy leaves from different locations of citrus growing regions in China (Munir et al., 2020a). We tested the efficacy of *B. subtilis* L1-21, which showed marked pink colonies differed among all the isolated endophytes, used as a potential endophyte isolated from citrus trees in the experimental fields. The novel half-leaf method advanced the understanding of pathogen exclusion within the midrib. The endophyte application positively affected other microbes in citrus leaves, indicating native bacterial density was unaffected. It is likely that healthy and asymptomatic citrus trees harbor disease suppressive endophytic bacteria that may secrete antimicrobial compounds in the host phloem and, perhaps, contribute to protection against *C*Las. We found that there was no effect on *C*Las copies in diseased citrus leaves of treatment with LB and water as controls, indicating that reduction of the pathogen within the leaf was due to application of *B. subtilis* L1-21. Previously, citrus endophytes, such as *Methylobacterium* and *Sphingomonas*, were also found to be involved in the protection of plants against various devastating pathogens (Innerebner et al., 2011; Ardanov et al., 2012).

The pathogen reduction is associated with competition for the nutrients among pathogen and endophytes and substances

FIGURE 6 | Management of HLB in diseased citrus fields using endophyte *Bacillus subtilis* L1-21: In 2018, an experiment was performed to check the reduction of diseased severity in a citrus grove in Genma county, Yunnan Province, China. Trees showed diseased symptoms to a healthy state after 7–8 months regular treatment with indigenous citrus endophyte L1-21. **(A)** April 2018; **(B)** June 2018; and **(C)** November 2018 (Genma county, Yunnan Province, China).

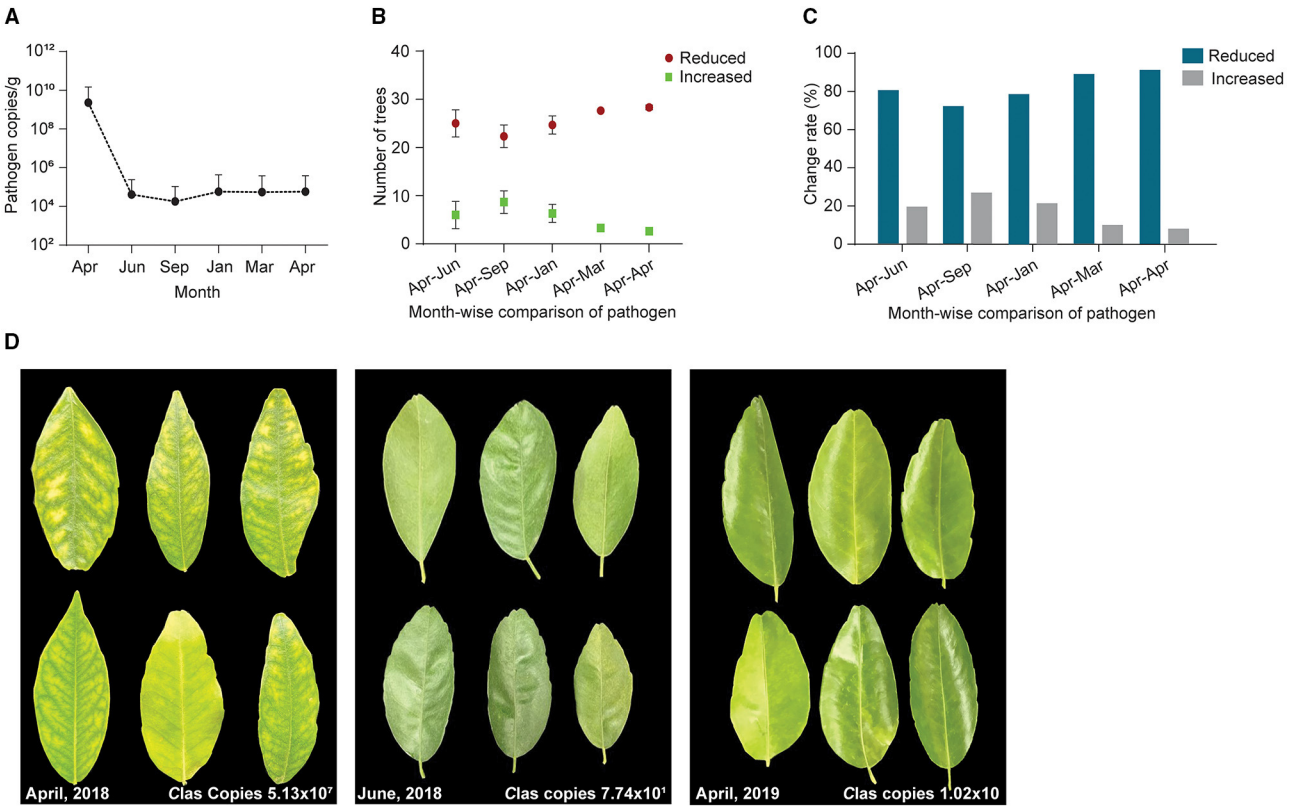

FIGURE 7 | A total of 93 citrus diseased sampled trees were treated with endophytes and *C*Las pathogen was monitored throughout the study period from April 2018 to April 2019. **(A)** CT values obtained through qPCR were changed to pathogen copies per gram of citrus leaf using a standard curve generated from pUC18-382 recombinant plasmid; **(B)** Number of citrus trees displayed a reduction of *C*Las pathogen during different time intervals, April to September 2018 and January, March, and April 2019. Each repeat contains 31 citrus trees; **(C)** Percentage reduction of pathogen across month-wise indicating reduction of the pathogen from time to time; and **(D)** Citrus leaves showing yellow symptoms before application of endophytes (April 2018) to more greenish leaves with the different time intervals decreasing pathogen copies 10^7 to 10 *C*Las/gram citrus leaf midribs.

released by endophytes that could have possible interference with the pathogen quorum sensing signaling (Miller and Bassler, 2001; Piewngam et al., 2018). Endophytic strain *B. subtilis* L1-21 was labeled with RFP that can express *mKate2* gene. In light of our finding, CLSM visualization clearly showed successful colonization in the phloem of citrus leaves, which may open more

TABLE 1 | Reduction of *Candidatus* Liberibacter asiaticus (CLas) pathogen copies during different time intervals 2018–2019.

	*C*Las copies in diseased citrus grove				
Month	**I**	**II**	**III**	**Mean**	**Reduce from April**
April, 2018	8.34×10^8	4.98×10^9	3.68×10^5	1.94×10^9	-
Jun, 2018	4.26×10^3	2.24×10^4	3.86×10^4	2.18×10^4	99.9989%
Sept, 2018	8.91×10^3	3.31×10^4	4.59×10^3	1.55×10^4	99.9992%
Jan, 2019	2.62×10^4	1.44×10^5	1.57×10^3	5.74×10^4	99.9970%
March, 2019	2.64×10^4	1.29×10^5	9.76×10^3	5.51×10^4	99.9971%
April, 2019	2.09×10^4	1.52×10^5	1.72×10^3	5.81×10^4	99.9970%

Repeat I, II, and III: Each consists of 31 citrus trees. The CLas reduction (pathogen copies g^{-1}) was tested during 1-year period. Each of these values displayed in Table 1 represents the average pathogen copies of 31 trees. Each tree was randomly sampled for 12–15 leaves.

doors for future research to study the mechanisms involved in the interaction of the endophyte with non-culturable *C*Las. The endophyte colonization in a similar niche as *C*Las might have employed direct inhibition of pathogen by producing antibiotics or lipopeptides, bacteriocin proteins (Kamada et al., 2013). On the other hand, core microbiomes present in the citrus host may be regulated by these endophytes, creating the possibility of pathogen elimination, most probably through induced systemic resistance (ISR) (Pertot et al., 2013; Zhao et al., 2018).

Currently, *C*Las is being treated with antibiotics (oxytetracycline hydrochloride, penicillin G potassium, ampicillin, and others) in the greenhouse and field experiments, but these are phytotoxic and persist as residues inside fruits (Zhang et al., 2014; Munir et al., 2018a). Proper use of antibiotics at the right time, adequate dosage, and application techniques can avoid problems related to the residue and phytotoxicity (McVay et al., 2019). Dissemination of antibiotics in the agriculture system is a potential threat to human health, agricultural productivity, natural ecosystem functioning, development of resistance in the pathogen, and negative effects on native microbiota (Williams-Nguyen et al., 2016), which urge the implementation of environmental-friendly alternatives for disease control. The use of antibiotics in citrus groves is common practice, which has been shown to drastically reduce the bacterial community richness and diversity (Zhang et al., 2013b); however, studies focusing on antibiotic effect solely on an endophytic community are scarce. Other common management practices such as glyphosate applications between the trees likely affect the endophytic microbial community over time. Our field experiment demonstrated successful management of *C*Las in HLB-diseased citrus with regular applications of *B. subtilis* L1-21. Pathogen copies in the diseased trees were reduced to $<10\ g^{-1}$ of leaf midrib and growth of green leaves and shoots was increased, including in previously moribund trees; similarly, marketable fruit yields also improved. However, the underlying mechanisms of endophytes on reductions in *C*Las copies and disease development remain unclear. These results indicated disease suppressive activities, such as quorum sensing attenuation by pathogen diffusible antibacterial compounds, niche and nutrient competition, synthesis of plant growth promoting hormones, siderophores or other bioactive compounds, and induction of plant defense, may have occurred with field applications of the endophyte, which limited in planta transmission of the pathogen, but warrants detailed study (Munir et al., 2021). Further, we assume that reduction of the pathogen may be related to increasing within plant endophyte density because the previous study has documented that *C*Las present inside citrus leaves regulated HLB symptoms positively and the leaf microbiome negatively (Blaustein et al., 2017; Munir et al., 2019). Pathogen exclusion effects were related to the increased density of *B. subtilis* L1-21 within the diseased trees. To determine *in planta* endophyte interactions with *C*Las, we first quantified their pretreatment densities in the diseased trees. Following treatment with *B. subtilis* L1-21, the endophyte densities in the citrus trees increased and the density of pathogen reduced. Notably, high copies of *C*Las remained present in untreated diseased trees. Thus, this study confirmed that the population dynamics of native citrus bacteria affect *C*Las titers (Sagaram et al., 2009; Zhang et al., 2016). It has been suggested that the endophyte application may also inhibit pathogen indirectly through activation of plant defense or recruitment of other endophytes in the citrus that causes the *C*Las reduction. We found significant genes and pathways associated with the elimination of *C*Las. Some pathways were effectively regulated in the diseased host, but not in healthy trees after endophytes treatment confirming different roles of endophytes in the host (data not shown).

Timing and effective dosage of application are much important to use biocontrol in field conditions (Brilli et al., 2019). Desired results can be achieved using this endophyte in the field during any time of the year in citrus fields. Later, we found that this endophyte can colonize for a longer duration inside citrus plants and maximum abundance was present even after 3 months, which makes it economically feasible for farmers. Using antibiotics to manage HLB disease could raise major concerns with respect to the uptake and distribution of antibiotics in citrus plants (Hijaz et al., 2020). Antibiotics such as penicillin and oxytetracycline showed uneven distribution in different parts of citrus plants and activity against the disease was also not stable (Al-Rimawi et al., 2019). In addition, endophyte-based biocontrol products will cost 100 dollars/hectares during 3 months of application and citrus growers and overall industry

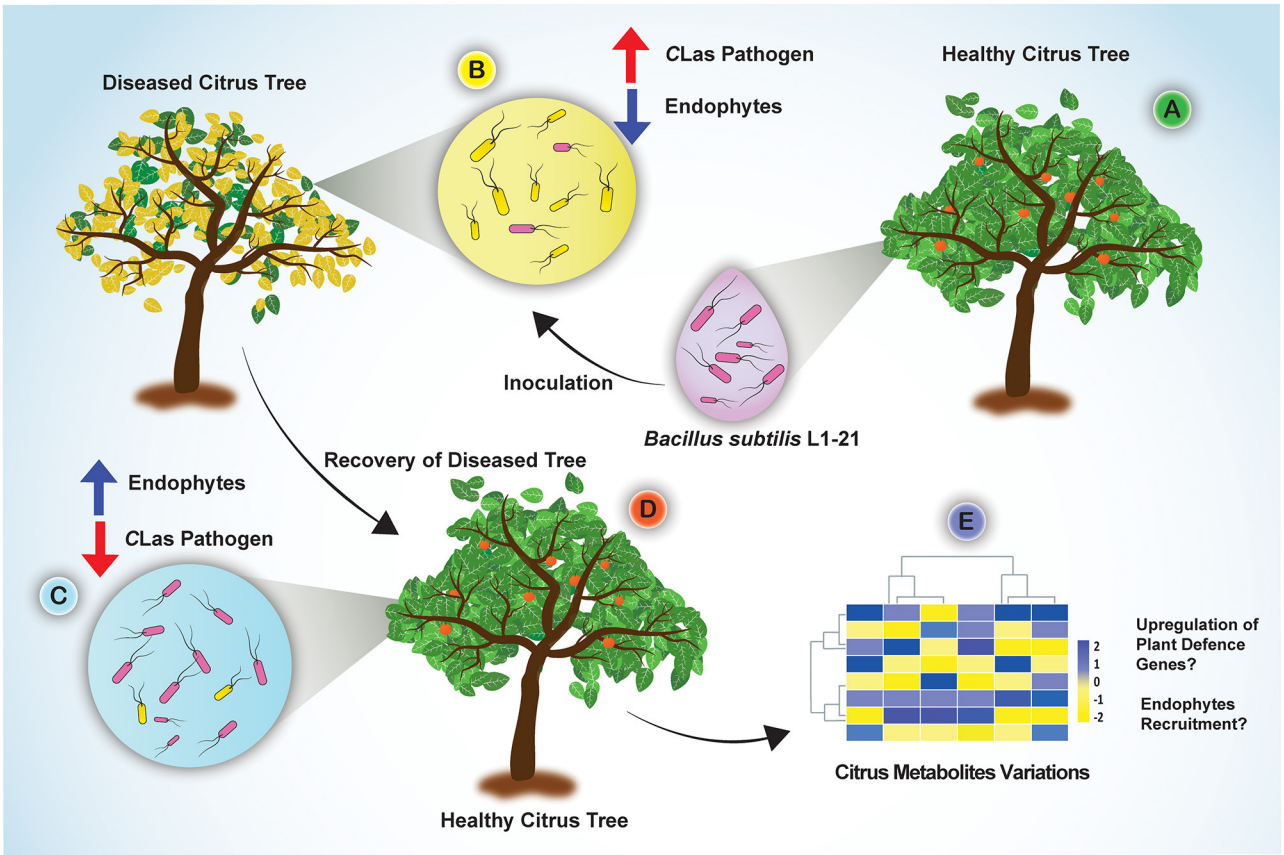

FIGURE 8 | Concluding sketch illustrating the different events taking place in the diseased citrus fields through restructuring bacterial endophytes for management of HLB disease. **(A)** Indigenous endophyte *B. subtilis* L1-21, displaying marked pinkish colonies, was isolated from healthy citrus plants; **(B)** Diseased citrus trees with more pathogen and less endophytes were inoculated with the endophyte *B. subtilis* L1-21; **(C)** Endophyte L1-21 shifted the bacterial endophytic community and excluded/restricted the pathogen colonization in diseased citrus trees from two different regions by regular application for 1 year; **(D)** The recovered tree showing more robust leaves and fruits; **(E)** Significant citrus metabolites were upregulated in the diseased citrus host after application of potential endophyte *B. subtilis* L1-21 (Munir et al., 2020b).

could minimize the use of high costing antibiotic applications (Sundin and Wang, 2018).

Candidatus Liberibacter asiaticus density in citrus trees is driven by an inversely proportional density of endophytes. Potential endophyte *B. subtilis* L1-21 for the management of citrus HLB using novel half-leaf method *in vitro* demonstrated that treatment of diseased citrus leaves reduced pathogen density in leaf midribs by 1,000-fold. In addition, phloem localization of endophyte L1-21 has also been confirmed in citrus revealed the possible role of pathogen exclusion. In light of our finding, this endophyte can efficiently colonize the phloem of citrus leaves, which may open more doors for future research to study the mechanisms involved in the interaction of endophytes with vascular pathogens. Based on the results, how the potential endophyte leads to a restructuring of the bacterial endophytic microbiome in diseased citrus groves is given in **Figure 8**. We suggested that indigenous endophyte application reduces the pathogen inside diseased citrus canopy from >90 to <3% within a year to a level that will bring benefit to the farmer in the future. Although the suppressive mechanism of endophyte against pathogen requires further exploration, however, we gained valuable insights about multiple metabolites variations in HLB-affected citrus trees, which mounted a defense against pathogen posttreatment with endophyte (Munir et al., 2020b). This study is the first initiative to highlight the restructuring of the citrus bacterial endophytic microbiomes by indigenous endophytes to mitigate the devastating disease HLB in economically important citrus crops.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SM, YL, PengfH, PengbH, PengjH, WC, YiW, YuW, JG, SK, and XL performed the laboratory experiments and data analyses. SM, SZ, ZL, YX, WW, KZ, ZL, YY, QL, SY, CM, HW, and

YH performed field experiments and collected samples, and supervised the study. SM and YH drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.789065/full#supplementary-material

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Increased Yield and High Resilience of Microbiota Representatives With Organic Soil Amendments in Smallholder Farms of Uganda

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Organic matter inputs positively affect soil fertility and quality but management effects on the soil and plant microbiome are less understood. Therefore, we studied the response of microbial colonization of the East African highland banana cultivar "Mpologoma" (AAA genome) under different mulch and manure treatments on three representative smallholder farms in Uganda. In general, the gammaproteobacterial community appeared stable with no significant response to organic matter inputs after 24 months of treatment. Significant differences ($p < 0.05$) in the plant-associated carpo-, phyllo-, and rhizosphere microbial community composition and diversity were found among individual sampled farms, independent of added soil inputs. Across farms, banana fruit harbored a richer and more balanced gammaproteobacterial community than the rhizo- and endospheres. Gammaproteobacterial beta diversity was shaped by the microenvironment (44%) as well as the sampling site (4%). Global effects of treatments in the rhizosphere analyzed using linear discriminant analysis effect size showed significantly enriched genera, such as *Enterobacter*, under manure and mulch treatments. As shown in previous works, bunch size and total yield were highly increased with manure and mulch, however, our results highlight general short-term microbial stability of Ugandan banana cropping systems with increases in the gammaproteobacterial community.

Keywords: ***Gammaproteobacteria*, banana fruit microbiome, organic soil amendments, manure, mulch, smallholder farms, plant-associated microbiota**

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INTRODUCTION

The plant and its highly diverse plant-associated microbial community, including bacteria, archaea, fungi, and protists, are nowadays recognized as holobiont (Vandenkoornhuyse et al., 2015). Essential functions are outsourced to symbiotic microbiota partners, and therefore plant-associated microbiomes play a key role in plant survival and health (Gilbert et al., 2012; Guttman et al., 2014). The species- and habitat-specific plant microbiota contribute to multiple aspects in functioning of the plant holobiont, such as (i) seed germination and growth, (ii) nutrient supply, (iii) resistance against biotic and abiotic stress factors, and (iv) production of bioactive metabolites (Berg et al., 2016, 2020). The plant microbiome and its interaction with the host were identified as an important

research focus for sustainable crop production and has been identified as key to the next, and sustainable, agricultural revolution (Schlaeppi and Bulgarelli, 2015; Bender et al., 2016). Despite ever-increasing interest in microbial strains as biopesticides, they are, however, far from overtaking chemical pesticides used in agriculture (Weller, 2007; Berg et al., 2013). In addition to microbial inoculants, an extensive list of microbiome management strategies and products were developed in agriculture including (i) microbiome transplants (straw dung, manure, mulch, and biodynamic additives), (ii) microbial and plant extracts as well as (iii) methods to change environmental conditions (Berg et al., 2020, 2021; French et al., 2021). Nevertheless, many parts of the underlying mechanisms are yet not fully understood.

African farming systems largely comprise a tapestry of crops and livestock dominated by smallholders with low crop yields but high biodiversity. All traditional farming systems in East Africa produce a variety of bananas. Bananas are also fruits of global importance with Latin America and the Caribbean (LAC) as the main producers worldwide (Lescot, 2011; FAOSTAT, 2018). Threatened by major *Fusarium wilt* disease outbreaks in the past and a new even more devastating strain currently menacing new areas, the banana industry is always on the look out for ways to control pathogens efficiently with promising advances with microbial-based suppressive soils (Butler, 2013; Dita et al., 2018; Bubici et al., 2019; García-Bastidas et al., 2020). While less is known about the banana fruit microbiome, a study on the rhizosphere and endosphere revealed *Gammaproteobacteria* as an important and dominant group in banana farms in Uganda contributing a third of the total bacterial population (Rossmann et al., 2012). Agroforestry was found to have especially an effect on plant-associated *Gammaproteobacteria* (Köberl et al., 2015). Moreover, *Gammaproteobacteria* are generally an important and dominant group within the plant microbiome comprising a variety of PGPR and biological control agents (e.g., *Pantoea sp., Pseudomonas sp., Serratia sp.*) but also potential plant pathogenic members (e.g., *Xanthomonas sp.*, *Xylella sp.*, *Pseudomonas sp.*) (Eastgate, 2000; Buchholz et al., 2011; Fürnkranz et al., 2012; Rastogi et al., 2012). Hence, this class was identified as a potential health indicator of banana plants (Köberl et al., 2017). Some members are also considered to be opportunistic human pathogens and have been implicated in foodborne diseases and outbreaks (Bloch et al., 2012; Berg et al., 2015); which indicates them, together with their dominant abundance in banana, as a model group of interest.

In this study, the microbial colonization of the East African highland banana cultivar "Mpologoma" (AAA genome) was investigated on three smallholder farms in Uganda from a larger study of plots on 54 farms, in order to reveal differences according to applied organic soil amendments. Different combinations of mulch and manure (mulch, manure, mulch + manure, non-treated control) were investigated. Previous results from the 54 farms have already shown, that shrubs and trees grown on the farms (e.g., *Calliandra*, *Magnifera*, *Sesbania,* or *Tithonia*) provide a rich source of nutrients such as nitrogen, potassium, and phosphorus, changing the physiochemical properties (Mpiira et al., 2013; Kongkijthavorn, 2017) and yield (50–100%) (Staver et al., 2015). Moreover, organic matter input to the soil affects biotic factors related to the soil microbiota, such as microbial biomass, microbial diversity, and community structure (Saison et al., 2006; Wallis et al., 2010; Bonilla et al., 2012, 2015). Here, we investigated if (I) specific soil amendments induce significant shifts in the banana gammaproteobacterial community and if effects can be found within (II) specific compartments (carpo-, phyllo-, and rhizosphere) or locations (III). Moreover, we investigate the currently unknown (IV) banana fruit microbiome and whether it reflects soil management practices. This study increases our knowledge of the impact of soil amendments on the plant-associated microbial community. Further studies based on more holistic population microbiology and over longer time periods are, however, necessary to potentially extend the gammaproteobacterial shifts/resilience to the entire microbial community.

MATERIALS AND METHODS

Experimental Design and Sampling Procedure

The rhizosphere, pseudostem endosphere, and carposphere samples were collected from the East African highland banana cultivar "Mpologoma" (AAA genotype) in July 2013. The same set of samples was collected at three smallholder farms in Uganda: RB farm in Matete in the district Sembabule, NSK farm in Semuto in the district Nakaseke, and KBG farm in Kisweeka in the district Kiboga (**Figure 1**). Soils in the areas are classified as Ferric Acrisols (FAO, 1977) comprising approximately 30% clay (FAO, 2004). Soil pH was 6.1 (NSK), 6.4 (KBG), and 6.6 (RB). Additionally, chemical soil properties were analyzed by (Kongkijthavorn, 2017). The whole region is characterized by a bimodal rainfall pattern, with the first peak from March to June and a second peak from August to December. The annual mean temperature is similar for all the sites around 23°C with 15°C minimum to near 30°C maximum. Plots did not have previous banana cultivation. Four treatments were planted on each farm with 10 plants/treatment—a control and three combinations of mulch and manure (mulch, manure, mulch + manure). In the manure and mulch + manure plots, 10 kg of air-dried cow manure was applied in each banana mat at the time of planting. Farmers followed up with yearly applications of 8–10 kg/plant of goat manure produced on the farm. Farmers used their own sources of mulch to maintain the soil surface covered—maize stover, cut grass, shrubs and tree trimmings, and unconsumed fodder materials from goat feeding. Sampling for microbial communities was completed 2 years after plot establishment. All the plants sampled were at the same physiological stage with a bunch approaching harvest. For each microenvironment and treatment, two replicate composite samples consisting of subsamples from five plants were taken. Additional information about the region, the banana cropping system, and the use of trees and shrubs to improve banana productivity are given by (Mpiira et al., 2013; Kongkijthavorn, 2017). Banana yield data were previously reported by (Staver et al., 2015).

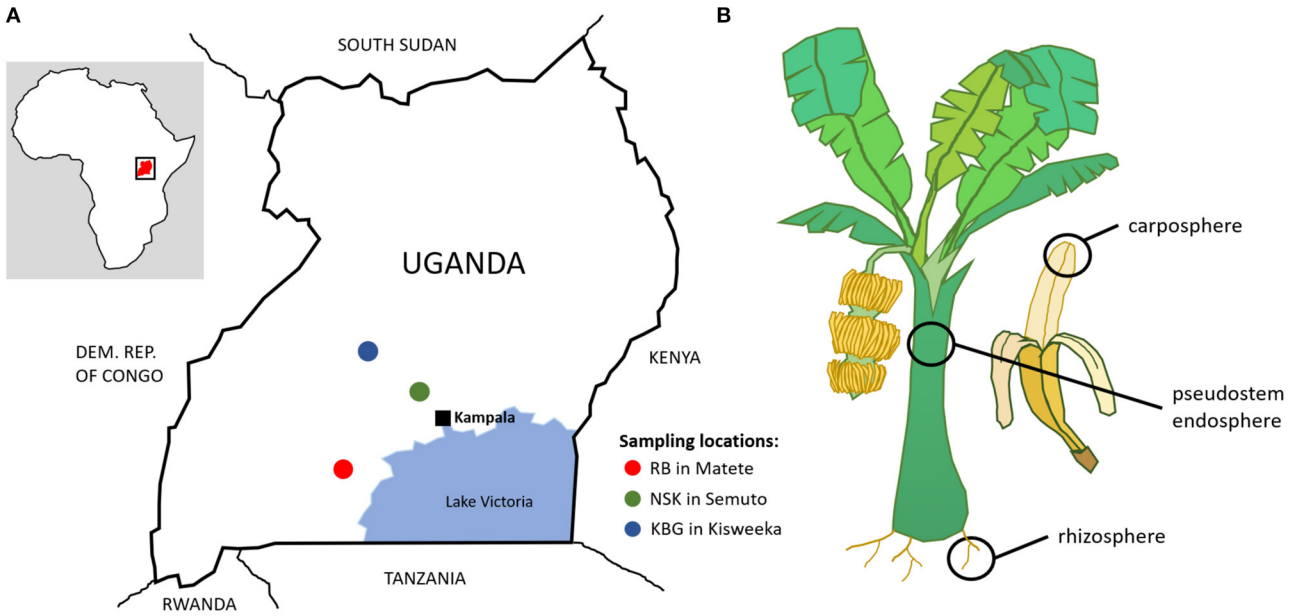

FIGURE 1 | Geographic location of sampling spots in Uganda **(A)** and schematic representation of samples **(B)**.

Total Community DNA Isolation

For the extraction of metagenomic DNA from the rhizosphere, 5 g of roots with adhering soil were added to 40 ml of sterile 0.85% NaCl solution and mixed for 5 min. Pseudostem samples (5 g) were washed with sterile distilled water, transferred to Whirl-Pak bags (Nasco, Fort Atkinson, Wisconsin, USA), and after 10 ml of 0.85% NaCl were added, homogenized with mortar and pestle. From the banana fruits, 5 g of pulp was washed with sterile distilled water, before homogenization with 10 ml of 0.85% NaCl. From the liquid parts, 4 ml were centrifuged (10 min, 16,000×*g*, 4°C) and the resulting pellets were stored at −70°C until further processing. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio, USA) and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Metagenomic DNA samples were encoded using abbreviations indicating: (1) microenvironment [R = rhizosphere, PS = pseudostem, C = carposphere (fruit)], (2) farm (RB, NSK, KBG) and (3) organic matter input (mulch, mulch + manure, manure, control = non-treated).

Gammaproteobacterial 16S RRNA Gene Profiling by Illumina MiSeq Sequencing

For a deep-sequencing analysis of the banana-associated *Gammaproteobacteria* community, the hypervariable V4 region of the 16S rRNA gene was amplified in a nested PCR approach with first a *Gammaproteobacteria*-specific primer pair Gamma395f/Gamma871r (Mühling et al., 2008) and then the universal primer pair 515F/806R (Caporaso et al., 2011), which carried sample-specific barcodes for demultiplexing. The reaction mixture for the first PCR (20 μl) contained 1× Taq&Go (MP Biomedicals, Eschwege, Germany), 2 mM $MgCl_2$, 0.1 μM of each primer, and 1 μl (∼10 ng) of template DNA dilution (96°C, 4 min; 30 cycles of 96°C, 1 min; 54°C, 1 min; 74°C, 1 min; and elongation at 74°C, 10 min). The second PCR (30 μl) was performed by using 1× Taq&Go, 0.2 μM of each primer, and 1.2 μl from dilutions (1:10^3) of the first PCR mixtures (94°C, 3 min; 32 cycles of 94°C, 45 s; 60°C, 1 min; 72°C, 18 s; and elongation at 72°C, 10 min). PCR products of three technical replicates per sample were purified by employing the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Samples were pooled in equimolar concentrations and amplicon libraries were sequenced with a paired-end approach (2 × 300 bp, chemistry v3) using an Illumina MiSeq platform (Eurofins Genomics, Ebersberg, Germany).

Data Processing

Data analysis was performed by employing QIIME version 1.9.1 (Caporaso et al., 2010b). Joined paired-end reads with more than three consecutive low-quality base calls (Phred quality score ≤ 25) were truncated at the position where their quality began to drop, and only reads with > 75% consecutive high-quality base calls, without any ambiguous characters, and longer than 200 nucleotides in length were retained for further analyses. Demultiplexed high-quality reads were *de novo* clustered into operational taxonomic units (OTUs) with uclust (Edgar, 2010), using a 97% similarity threshold. From each OTU, the most abundant sequence was selected as representative, and the taxonomy was assigned with the uclust-based consensus taxonomy assigner against the Greengenes database (version 13.8). The representative sequence set was aligned with PyNAST (Caporaso et al., 2010a), and potential chimeric sequences were discarded based on a check with ChimeraSlayer. OTU tables were constructed, and OTUs not assigned to the class

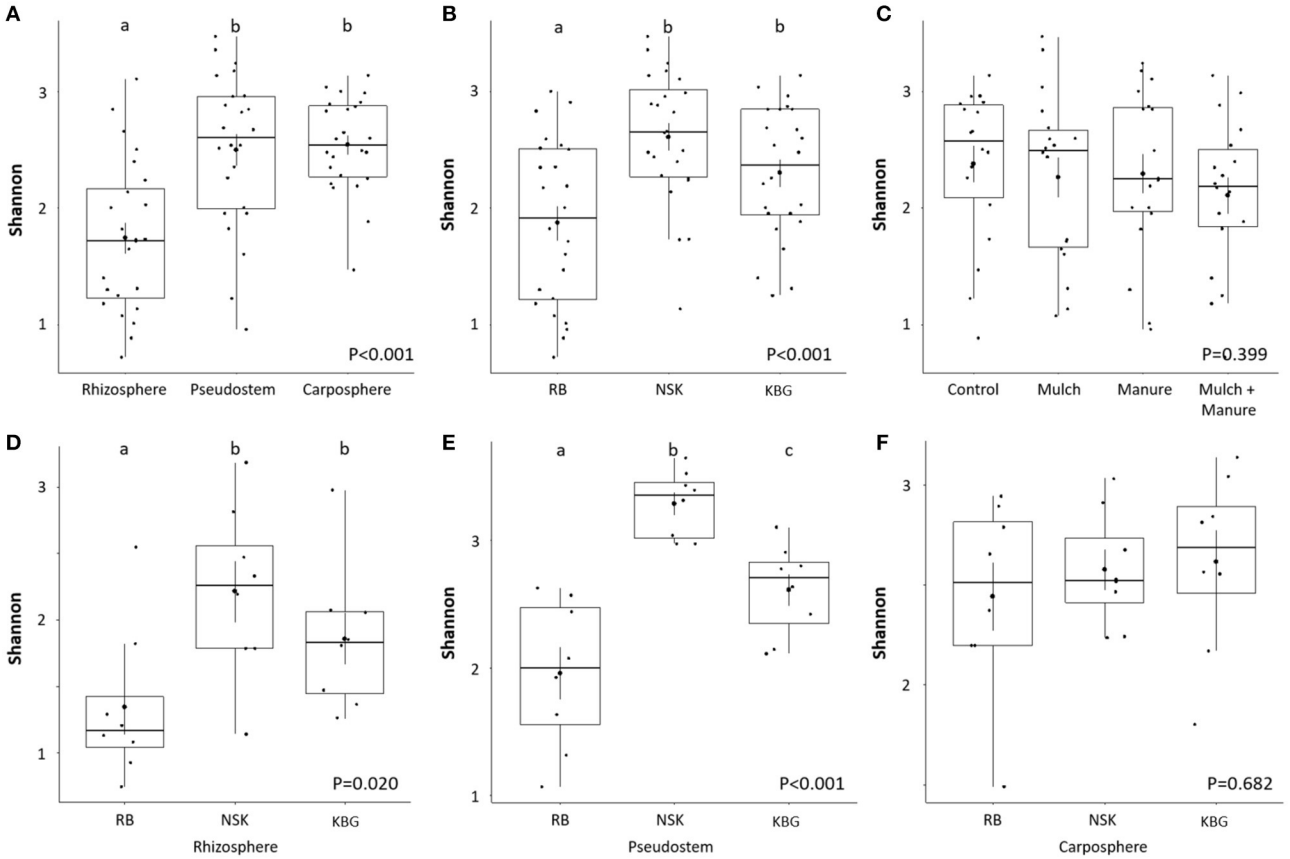

FIGURE 2 | Shannon indices of gammaproteobacterial diversity in different microhabitats **(A)**, smallholder farms **(B)**, organic soil amendment treatments **(C)**, and for different microenvironments in different smallholder farms **(D–F)** at a genetic similarity level of 97%. Significant differences ($p \leq 0.05$, ANOVA with Tukey-HSD *post hoc* test) within tested factors are indicated by different letters above the boxplot. Smallholder farms are abbreviated with RB, NSK, and KBG.

of *Gammaproteobacteria* and singletons were removed from the dataset.

Statistical Analysis

The datasets were normalized by rarefying to the lowest number of reads (3,351 high-quality reads) and MetagenomeSeq's cumulative sum scaling (Paulson et al., 2013) was used for alpha and beta diversity analysis, respectively. Phyloseq, MicrobiomeAnalyst, and vegan R packages implemented in RStudio v1.3.1093 were used to analyze bacterial community diversity and composition (Oksanen et al., 2007; Allaire, 2012; Core Team, 2013; McMurdie and Holmes, 2013; Dhariwal et al., 2017; Chong et al., 2020). Significant differences in alpha diversity based on Shannon index (H') were determined using ANOVA and Tukey-HSD *post hoc* test. The normalized weighted Unifrac dissimilarity matrix (Lozupone et al., 2011) was subjected to permutational ANOVA (PERMANOVA) to test for significant effects of factors on microbial community structure. The Adonis test with 999 permutations was used for pairwise comparisons. Biomarkers at the bacterial genus level between the tested factors were identified using a linear discriminant analysis effect size (LefSe) (Segata et al., 2011). The threshold of linear discriminant analysis was set as 1 with cutoff p values of 0.1.

RESULTS

After sequencing, a total of 6,550,680 quality reads and 72,861 OTUs were retrieved. Filtering singletons, chimeric and non-gammaproteobacterial reads left 5,011,782 high-quality reads that were retained and clustered into 17,824 gammaproteobacterial OTUs. Rarefaction curves indicate that the sequencing depth was sufficient to capture gammaproteobacterial diversity (**Supplementary Figure S1**).

Microbial Alpha Diversity Is Shaped by Location, Microenvironment, and Treatment

Analysis of variance indicated that microhabitat and sampling location influenced gammaproteobacterial diversity ($p < 0.001$), whereas different soil amendments showed unexpectedly no significant effect overall ($p = 0.399$). Microbial alpha diversity calculated based on the Shannon index showed a generally lower gammaproteobacterial diversity in rhizosphere samples compared to endo- and carposphere ($p < 0.001$; **Figure 2A**). Within smallholder farms, we identified a highly site-specific microbiome fingerprint: the lowest alpha diversity was observed in RB sampling sites ($p < 0.001$; **Figure 2B**). Systematical

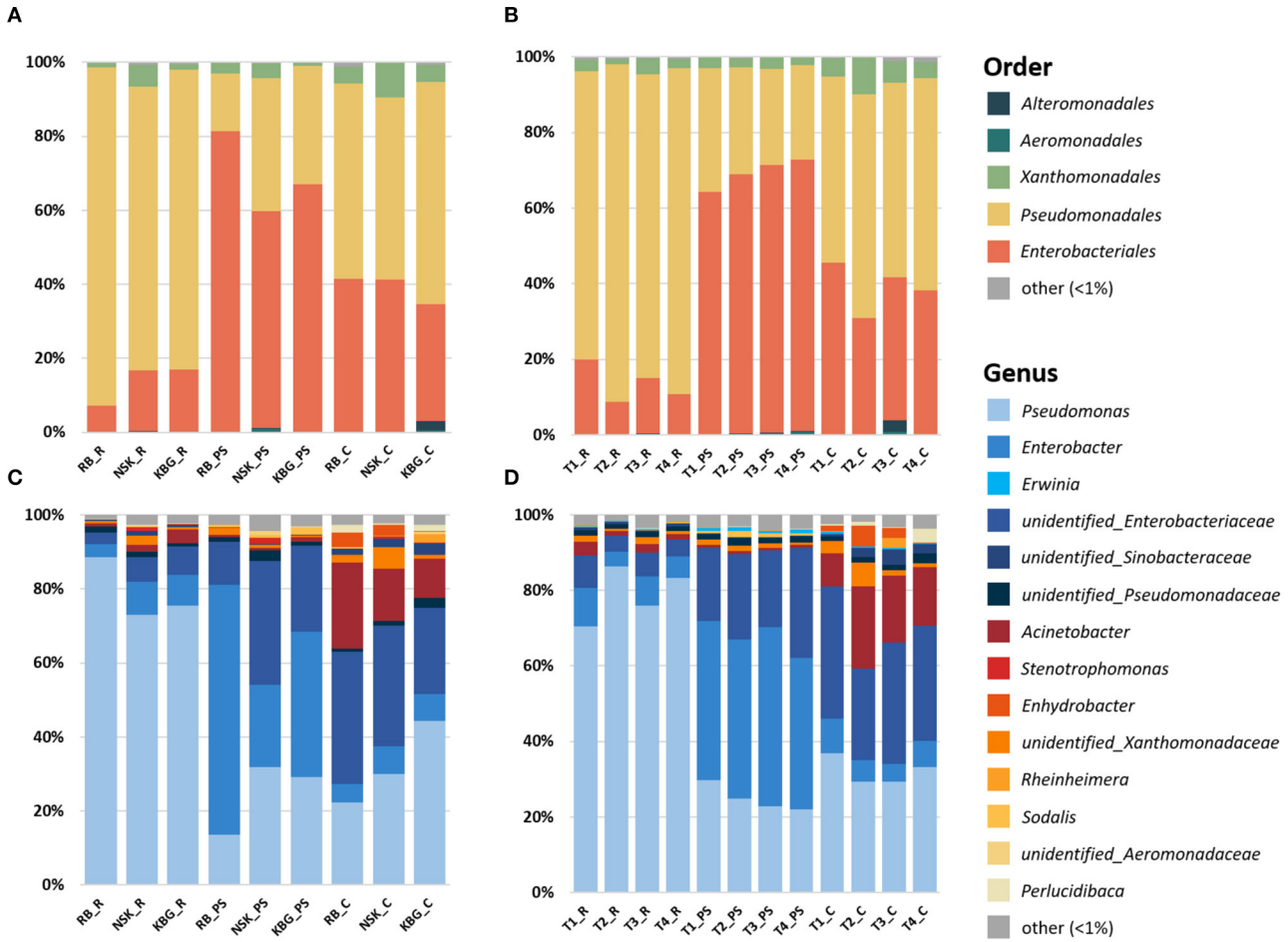

FIGURE 3 | Taxonomic composition of *Gammaproteobacteria* communities inhabiting rhizosphere, pseudostem endosphere, and carposphere of banana plants grown at three smallholder farms in Uganda under varying organic management regimes. Sequences were classified at order **(A,B)** and genus **(C,D)** levels. Groups were encoded using abbreviations indicating: (1) farm (RB, NSK, KBG), (2) microenvironment (R, rhizosphere, PS, pseudostem endosphere, C, carposphere), and (3) organic matter input (T1 = mulch, T2 = mulch + manure, T3 = manure, T4 = non-treated).

analysis was performed to determine significant differences among treatments. While there was no significant treatment effect on the alpha diversity overall ($p = 0.399$; **Figure 2C**), some significant treatment-based changes were observed when farms were analyzed separately. Soil amendment changed the gammaproteobacterial diversity in the endosphere of the farm NSK ($P = 0.035$, **Supplementary Figure S2**). The combination of mulch and manure increases the gammaproteobacterial diversity in comparison to the control (**Supplementary Figure S2**). Soil amendment also changed the gammaproteobacterial diversity in the rhizosphere and endosphere of KBG farm. However, these findings were only significant at the 90% confidence level (rhizosphere—$p = 0.076$ and carposphere—$p = 0.077$). Mulch treatment increased gammaproteobacterial diversity in the rhizosphere in comparison to control. Mulch, manure, and their combination showed a higher diversity in the carposphere of the KBG farm compared to the control (**Supplementary Figure S2**). Looking closer into differences within smallholder farms, the highest alpha diversities were found in the rhizo- and endosphere of NSK, while RB showed generally lower diversities ($p < 0.001$; **Figures 2D–F**).

The Microbial Composition of Banana Plants Is Microhabitat Specific

Gammaproteobacterial community composition was analyzed on different phylogenetic levels. Already at the order level, differences between the three microhabitats (rhizosphere, endosphere, and carposphere) were visible (**Figure 3A**). The rhizosphere and carposphere were dominated by a major fraction of *Pseudomonadales* [76–92% (rhizosphere); 49–60% (carposphere), and 16–36% (pseudostem endosphere)]. In contrast, the pseudostem endosphere was dominated by a high fraction of *Enterobacteriales* (59–81%), which was also present in the rhizosphere (7–17%) and carposphere (32–41%). Moreover, minor fractions of *Xanthomonadales* were found in all three microenvironments (1–9%). At the genus level, *Pseudomonas*

was dominating the rhizosphere (73–89%) with minor fractions of *Enterobacter* (3–9%) and an unidentified *Enterobacteriaceae* (2–8%). In the pseudostem endosphere, *Enterobacter* (22–67%), *Pseudomonas* (14–32%), and unidentified *Enterobacteriaceae* (12–34%) were present. In contrast, the carposphere showed, additionally to *Pseudomonas* (22–44%), *Enterobacter* (5–8%), and unidentified *Enterobacteriaceae* (23–36%), a high fraction of *Acinetobacter* (10–23%) (**Figure 3C**). If samples were grouped by organic matter treatment (non-treated, mulch, manure, mulch + manure), similar patterns between the three microenvironments were visible, however, major effects induced by treatments were not observed (**Figures 3B,D**). Mulch and manure treatment increased the abundance of *Enterobacteriales* from 11% (control) to 20% (mulch) and 15% (manure) in the rhizosphere. Moreover, mulch also increased the *Enterobacteriales* fraction in the carposphere [from 38% (control) to 45% (mulch)]. The abundance of *Pseudomonadales*, on the other hand, was decreased by manure and mulch treatment in parallel to the increase of *Enterobacteriales*. Contrastingly, in the pseudostem endosphere, all treatments decreased the abundance of *Enterobacteriales* and increased the abundance of *Pseudomonadales*. To investigate detailed changes induced by treatments on genus level LEfSe analysis was performed below.

Microbial Community Structure and Taxonomic Changes Induced by Treatments

Beta diversity analysis indicated that microhabitat was the main factor that influenced the gammaproteobacterial community structure ($p = 0.001$; **Table 1**). This factor explained 43.9% (as shown by PERMANOVA) of total gammaproteobacterial community variation as indicated by separated clustering according to the microhabitat (**Figure 4A**; **Table 1**). Sampling sites also influenced the gammaproteobacterial community structure ($p = 0.003$) but only explained a small variation of the community (3.7%). To test if the variation of gammaproteobacterial communities in each microhabitat is influenced by soil amendment treatment and/or farm, deepening analysis by separating datasets from each microhabitat was performed. Principal coordinate analysis (PCoA) plots indicated that the gammaproteobacterial communities of banana plants showed significant grouping in rhizo- and endosphere by the different farms (**Figures 4B,C**; **Table 1**). However, there was no grouping for the carposphere (**Figure 4D**; **Table 1**).

Soil amendment treatment only influenced the gammaproteobacterial community structure in the rhizosphere ($p = 0.050$, **Supplementary Table S1**). When pairwise comparisons using Adonis between control and each treatment were performed, a significant shift in gammaproteobacterial community structure was observed after amendments of mulch ($p = 0.034$) and manure ($p = 0.034$). Interestingly, a combinatory treatment (mulch and manure) did not affect the gammaproteobacterial community structure ($p = 0.612$). Subsequently, LEfSe analysis was conducted to identify taxa that were significantly affected by soil amendment treatments. Using a stringent p value, there was no bacterial genera significantly affected by treatment. However, when the stringency was reduced (from $p > 0.01$ to $p < 0.1$), LEfSe analysis indicated that four bacterial genera and two taxa that could not be classified into genus levels were affected by the treatments. These taxa belonged to *Enterobacter*, *Citrobacter*, *Perlucidibaca*, unclassified *Pisciricettsiaceae*, unidentified *Coxiellaceae,* and *Cellvibrio* (**Figure 4E**). The first two taxa were consistently enriched in the mulch as well as manure-treated samples. *Cellvibrio* was enriched in manure and in the combinatory manure and mulch treatment. Moreover, the abundances of *Perlucidibaca* and an unclassified *Pisciricettsiaceae* were higher in the control.

TABLE 1 | Permutational ANOVA (PERMANOVA) performed on the weighted Unifrac dissimilarity matrix showing influencing factors for the whole dataset and each microenvironment.

Factor	*P* value	R^2 value
All datasets		
Microenvironment	0.001	0.439
Farm	0.003	0.037
Treatment	0.538	0.018
Rhizosphere datasets		
Farm	0.004	0.282
Treatment	0.050	0.182
Endosphere datasets		
Farm	0.001	0.304
Treatment	0.712	0.077
Carposphere datasets		
Farm	0.651	0.076
Treatment	0.817	0.105

DISCUSSION

In this study, we investigated the effect of organic management regimes on the gammaproteobacterial community in the rhizo-, endo-, and carposphere of banana in three smallholder farms in Uganda. Previous results showed that nutrient input by organic matter, often produced from local sources, increases nutrient availability and has a tremendous effect on the achieved bunch sizes and yields (Mpiira et al., 2013; Staver et al., 2015). Nevertheless, our results show that treatments overall did not have a significant effect on gammaproteobacterial alpha diversity. This was in contrast to our expectations but indicates a generally stable gammaproteobacterial community. When farms were separated, significant differences induced by organic soil amendments were found. For instance, manure increased gammaproteobacterial diversity in the rhizosphere and carposphere in the farm KBG. Despite the fact, that the effect was specific for each of the three selected farms, still general microbiota patterns were identified.

Banana habitats (rhizosphere, pseudostem, and fruit) and their specific biotic and abiotic parameters were identified as the main drivers of the microbiota. Overall, gammaproteobacterial

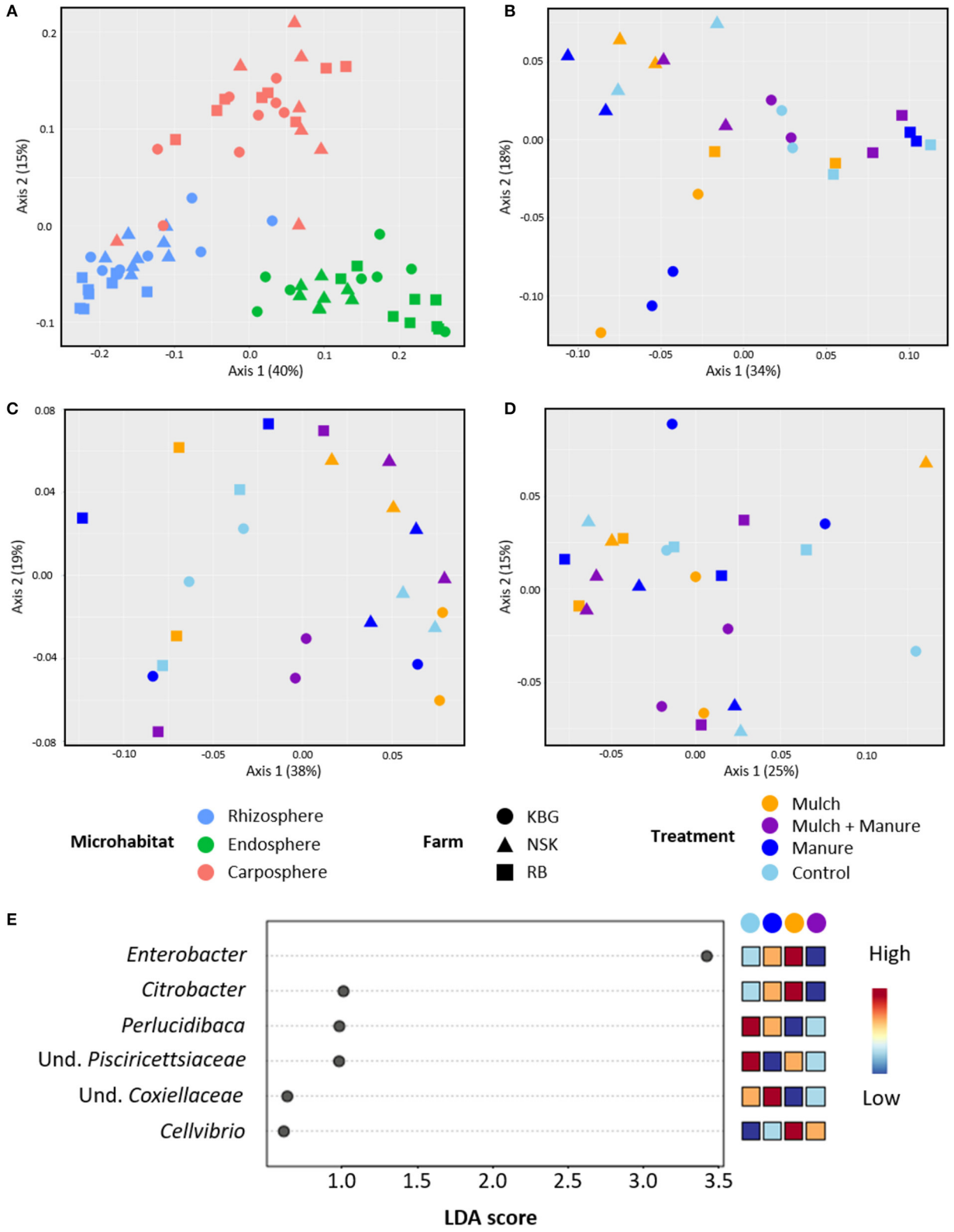

FIGURE 4 | Principal coordinate analysis (PCoA) plots of the gammaproteobacterial communities inhabiting all microenvironments **(A)**, rhizosphere **(B)**, pseudostem endosphere **(C)**, and carposphere **(D)** of banana plants grown at three different farms (RB, NSK, and KBG) in Uganda under varying organic management regimes. Linear discriminant analysis effect size (LEfSe) analysis identifying considerably different taxa between treatments in the rhizosphere across all locations **(E)**. PCoA plots are based on weighted UniFrac distances. The plots indicate the grouping of samples by the farm (different shapes) and individual treatments (different colored symbols).

diversity was lower in the rhizosphere compared to endophytic aboveground parts (pseudostem and banana fruit). This is in contrast to most published studies about the plant microbiota, but confirms our previous study in banana, when we reported this unexpected finding for the first time (Rossmann et al., 2012). In addition, the diversity values observed for aboveground plant parts were comparable to our previous findings in other banana plantations in Central America (Köberl et al., 2015, 2017). Beta diversity grouping was mainly due to microhabitat, while effects of sampling farm and treatment were only visible when samples were separated by habitat first. Similarly to alpha diversity measures, the three farms RB, NSK, and KBG significantly differed in microbial composition in the rhizo- and endosphere, while no differences were found for the carposphere.

Soil amendments were found to influence the gammaproteobacterial community structure only in the rhizosphere. Further taxonomic composition analysis was in line with our previous observations. The microenvironment effect was more visible than the treatment effect. Confirming our expectations, an increase in *Enterobacteriales* abundance due to manure amendment was observed in the rhizosphere. Moreover, the mulch amendment did have this effect on the rhizo- and carposphere. Interestingly, combinatory treatments did not have such an effect. On the contrary, treatments had the opposite effect in the pseudostem endosphere, which was generally dominated by *Enterobacteriales*. Here, all treatments increased the abundance of *Pseudomonadales* and decreased *Enterobacteriales* fractions. Both *Pseudomonadales* and *Enterobacteriales* species were previously discovered to play a key role in plant-associated communities (Berg et al., 2014; Wassermann et al., 2017; Cernava et al., 2019) and are especially high in the pseudostem of banana (Rossmann et al., 2012; Nimusiima et al., 2015).

We highlight specifically that manure and mulch treatments in the soil affected both above- and belowground communities. The strong connectivity of soil microbial communities with the aboveground and especially fruit community was just recently discovered (Bergna et al., 2018; Kusstatscher et al., 2020). Microbial diversity in the soil, as well as management regime, has significant effects on carposphere communities (Abdelfattah et al., 2016; Wassermann et al., 2019). Moreover, *Gammaproteobacteria*, which can be usually found in high abundances within plant-associated communities, were previously discovered to be affected above- and belowground by agroforestry treatments (Fürnkranz et al., 2012; Köberl et al., 2015). In contrast to previous studies, we here analyzed also the effect of different organic soil inputs. While no major community shifts were induced by the applied treatments, LEfSe analysis elucidated considerable taxonomic shifts at the genus level. Interestingly, both *Enterobacter* and *Citrobacter* were significantly increased in the rhizosphere by manure and mulch treatments. Moreover, *Cellvibrio* was significantly increased by both manure and combinatory treatment (manure and mulch). All those taxa were previously discussed as members of the core microbiome of leafy greens, citrus fruits, grapevine, and banana (Zarraonaindia et al., 2015; Köberl et al., 2017; Xu et al., 2018; Cernava et al., 2019). *Enterobacter* was moreover associated with diseases, such as *F.* wilt, in banana but is at the same time a well-known antagonist and important keystone taxa against fungal diseases (Köberl et al., 2017; Liu et al., 2019).

To our knowledge, we were the first to specifically investigate the banana fruit microbiome. Interestingly, banana fruit harbored a richer and more balanced gammaproteobacterial community than the rhizo- and endosphere. Compositional analysis showed a higher relative abundance of *Pseudomonas* (22–44%), unidentified *Enterobacteriaceae* (23–36%), *Acinetobacter* (10–23%), and *Enterobacter* (5–8%). Studying the edible microbiome, e.g., microbiome of fruits, became recently a trend and is important to assess risks for human consumption as well as storability (Berg et al., 2015; Droby and Wisniewski, 2018; Kusstatscher et al., 2020). The group of *Enterobacteriaceae,* even though comprising a number of human pathogens, were previously shown to be part of the native fruit microbiome in mango and strawberry and harbor specific functions (Diskin et al., 2017; Zhang et al., 2020). Therefore, a higher abundance in banana fruit is not surprising.

Plant and soil management practices to change soil characteristics have been discussed as a potential strategy to prevent panama disease in banana (Mena et al., 2015; Omondi et al., 2020). Organic matter sourced from local shrubs and trees harbors a rich source of nitrogen, phosphorous and potassium, needed for banana production (Kongkijthavorn, 2017). While bunch size and yield could be improved (Staver et al., 2015), this study shows the high resilience of gammaproteobacterial communities in African smallholder cropping systems. Highly diverse soils were previously discussed to be more resistant to changes and can harbor suppressive characteristics (Raaijmakers and Mazzola, 2016). In this study even though organic amendments were applied over 2 years compared to a control without amendments, no major shifts but only changes in a low number of taxonomic groups were observed. Our previous studies showed that major shifts are associated with plant diseases, however healthy banana plants were associated with similar communities independently from their cropping system or planting location (Köberl et al., 2015, 2017). Similarly, Wemheuer et al. (2020) showed the stability of bacterial communities in cocoa agroforests, while fungal communities changed (Wemheuer et al., 2020). Taken together, we here, confirmed former observations and moreover, increased our knowledge of the complex banana agroecosystem. Microbiome management in the soil will be of significant importance to maintain healthy agricultural soils, not only in banana farming. Even though gammaproteobacterial communities were shown to be stable under multiple conditions, future investigations including the whole bacterial as well as multi-kingdom communities should give a better look into soil amendments impact on the banana-associated microbial community. Organic amendments applied over a longer period also deserve attention.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB48209.

AUTHOR CONTRIBUTIONS

MK, CS, and GB designed the study. SM and FK conducted the fields experiment and collected the samples. MK, PK, and WW analyzed the data and wrote the manuscript. CS and GB critically reviewed the manuscript. All authors approved the final paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.815377/full#supplementary-material

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Microbiome Variation Across Populations of Desert Halophyte *Zygophyllum qatarensis*

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Microbial symbionts play a significant role in plant health and stress tolerance. However, few studies exist that address rare species of core-microbiome function during abiotic stress. In the current study, we compared the microbiome composition of succulent dwarf shrub halophyte *Zygophyllum qatarensis* Hadidi across desert populations. The results showed that rhizospheric and endosphere microbiome greatly varied due to soil texture (sandy and gravel). No specific bacterial amplicon sequence variants were observed in the core-microbiome of bulk soil and rhizosphere, however, bacterial genus *Alcaligenes* and fungal genus *Acidea* were abundantly distributed across root and shoot endospheres. We also analyzed major nutrients such as silicon (Si), magnesium, and calcium across different soil textures and *Z. qatarensis* populations. The results showed that the rhizosphere and root parts had significantly higher Si content than the bulk soil and shoot parts. The microbiome variation can be attributed to markedly higher Si – suggesting that selective microbes are contributing to the translocation of soluble Si to root. In conclusion, low core-microbiome species abundance might be due to the harsh growing conditions in the desert – making *Z. qatarensis* highly selective to associate with microbial communities. Utilizing rare microbial players from plant microbiomes may be vital for increasing crop stress tolerance and productivity during stresses.

Keywords: microbiome, desert succulents, *Zygophyllum qatarensis*, microbial communities, microbial diversity, core-microbiome

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INTRODUCTION

Arid land ecosystems cover over 30% of earth and are inhabited by nearly a billion people. Both plant and microbial life are confronted with extreme living conditions that greatly depend on a scarcity of water and nutrients from soil. In plants, xerophytic succulents and annuals are the key species well-tailored to continuous episodes of abiotic stresses (drought, heat, and salinity) (Ndour et al., 2020; Peguero-Pina et al., 2020; Zeng et al., 2021). Succulent plants are common in arid land ecosystems due to their ability to store significant amounts of water in cells, their potential to withstand or avoid extreme drought periods, and growth patterns (Griffiths and Males, 2017; Heyduk, 2021). In addition, the survival of succulent plants in arid

environments is often attributed to their (i) genetic makeup, (ii) physio-photosynthetic responses, (iii) essential metabolite production, and (iv) associated microbial symbionts (Heyduk, 2021). In the latter case, the mutualistic microbiota (mainly bacteria and fungi) inhabiting the soil around roots (rhizosphere) and the shoot surface (phyllosphere) play a considerable role in improving physiological responses, beneficial metabolite production, and nutrient uptake (Khan et al., 2015, 2020; Trivedi et al., 2020). Though the phytobiome consists of a great diversity of micro and macroorganisms in and around plants, disentangling the effects of the phytomicrobiome on plant performance has emerged as a potential solution for economically important plants to deal with changing global climate and improve productivity and disease resistance (D'Hondt et al., 2021).

The plant-associated microbiome has been coined as the plant's "second genome" that is highly variable in diversity, abundance, and composition (Berendsen et al., 2012). This microbial variability is due to (i) abiotic factors like temperature, water (wet or dry), soil chemistry, and nutrient cycling, (ii) the plant species, developmental stage, its ability to establish successful associations with the microbiome, the interaction of the microbiome with hub microbiota and keystone species, and (iv) how root exudates influence microbial growth and reproduction (Trivedi et al., 2020; D'Hondt et al., 2021). There has been a considerable effort to elucidate the plant-microbiome interactions and the microbial niches in arid land ecosystems. Indeed, extreme environments may contain highly beneficial culturable microbes that can, in turn, withstand the adverse impacts of stressful conditions. However, interactions between the microbial communities and succulent plants have been minimally investigated, particularly in arid ecosystems (Pfeiffer et al., 2017). Previous studies (Jorquera et al., 2016; Citlali et al., 2018; Delgado-Baquerizo et al., 2018; Mandakovic et al., 2018; Araya et al., 2020; Astorga-Eló et al., 2020; Khan et al., 2020) have evaluated the microbiome, especially bacterial communities from arid soil. However, little work has explored the phyllosphere and rhizosphere microbiome across different populations of the same host plant species. Notably, more diversity in sampling may provide additional significant insights into the plant microbiome and help discover new beneficial microbes.

The Earth Microbiome Project has estimated nearly 10 million microbial species globally, whereas other estimates suggest a trillion species (Locey and Lennon, 2016; Thompson et al., 2017). However, only a small fraction of the Earth microbial species has been sequenced or are available in culture stocks. Hence, there is a great need to explore the unique phytomicrobiome and the keystone species of extreme arid environments. Some of the succulents recently analyzed for their microbiome are *Agave* species (Flores-Núñez et al., 2020), *Aloe vera* (Akinsanya et al., 2015), cacti (Fonseca-García et al., 2016), CAM plants (Citlali et al., 2018), pineapple (Putrie et al., 2020), and Aizoaceae (Pieterse et al., 2018). These microbiome studies showed remarkably high and diverse rhizosphere colonization by the bacterial phyla *Actinobacteria, Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria,* and *Bacteroidetes* (Citlali et al., 2018; Flores-Núñez et al., 2020). However, the importance of understanding the microbiome composition of wild plants growing in arid environments has been underappreciated until recently.

In the present study, we investigated the phytomicrobiome of salt-tolerant dwarf shrub *Zygophyllum qatarensis* Hadidi (Basionym of *Z. hamiense* var *qatarensis*, *Tetraena qatarensis*) and its four major populations. *Z. qatarensis* is a drought and salinity resistant plant endemic to the Arabian Peninsula (Beier et al., 2003; Alzahrani and Albokhari, 2017), where it grows on coarse, stony, or calcareous sandy soils (Sayed, 1996; Abbas, 2005). The plant grows well in arid desert ecosystems despite exposure to high drought, heat, and intense UV conditions. The leaves are fleshy and succulent and can store enough water to sustain plants through arid periods. However, the unifoliate xeromorphic leaf morphology changes depending on water availability (Sayed, 1996; Abbas, 2005). The leaves gradually shed depending upon the intensity of drought and heat in desert conditions. The seed has a tough outer coat and only germinates upon a considerable amount of rainfall. The immature leaves are used by humans as a vegetable and possess several medicinal properties used to treat diabetes and dysmenorrhea. The plant sources biologically active phytochemicals such as terpenoids, phenolics, and essential oils (Zaman and Padmesh, 2009; Shawky et al., 2019). In addition, due to their outstanding resistance to arid conditions, the unique *Z. qatarensis* microbiome has a high potential for identifying beneficial microbial strains that aid crop drought-stress tolerance. Herein, we investigated for the first time the rhizosphere and root/shoot endosphere microbiome of *Z. qatarensis* to describe its microbiome across different populations growing in two different soil conditions (gravel and sandy).

MATERIALS AND METHODS

Soil, Rhizosphere, and Plant Endosphere Sampling

The *Z. qatarensis* plants (shoot and root), rhizosphere and bulk soil were collected from four major population locations (T1, T2, T3, and T5) in the extreme desert of Empty Quarter in Oman (**Supplementary Table 1** and **Figure 1**). The *Z. qatarensis* plant populations are distributed in a conserved and consistent ecological pattern across the four distinctive locations in desert areas. Each population was approximately 100 km apart and samples within a population were collected in triplicate. Each replica from the individual population was representative of ten plants/soils that were pooled for DNA extractions and chemical analysis. The rhizosphere soils adjacent to the root surface (10 to 15cm deep) were collected (**Supplementary Figure 1**). These were mature plants with a reasonably defined rooting system and the sandy soils were removed with the help of a sterile blade. There were no specific signs of rhizosheath with the root, so the soil attached to root parts was categorized as rhizosphere – following the classification of Pang et al. (2021). The bulk soil samples were collected from a depth of 10 to 15cm with no signs of *Z. qatarensis* presence. The root parts were carefully collected by removing the sand and particulate matters. For endosphere, the root and shoot

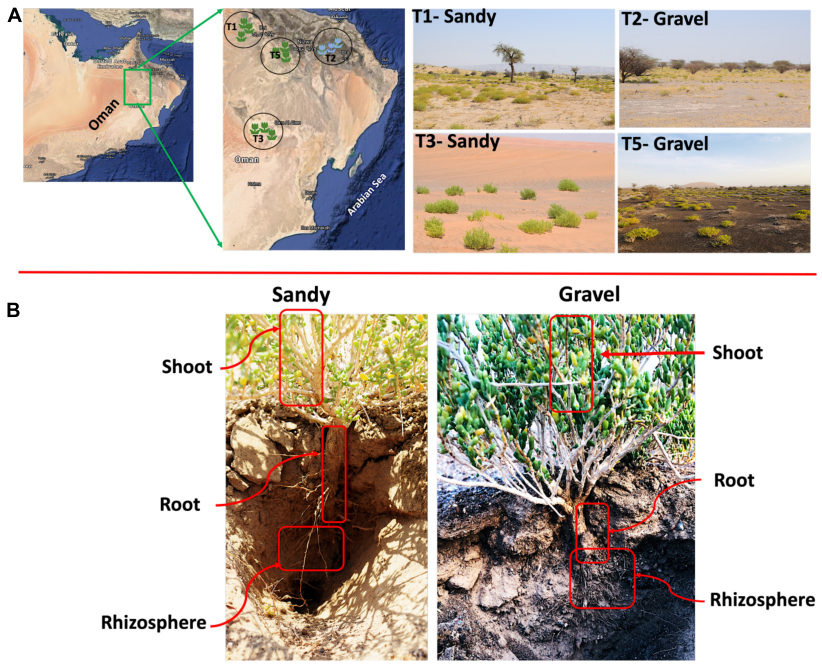

FIGURE 1 | Habitat of *Z. qatarensis*. **(A)** Location map of samples across different parts of desert ecosystem where *Z. qatarensis* grows. Mainly two populations (growing in gravel and sandy soils) were scattered from north to southern regions. **(B)** Samples were collected from bulk soil, rhizosphere, and root/shoot endosphere of sandy and gravel populations.

samples were washed with sterile distilled water and sodium hypochlorite to remove epiphytic microbes following the method of McPherson et al. (2018). Briefly, the pre-sterilized scalpel (with 70% ethanol) was used to prepare individual tissues from about 10 to 15 roots and shoot parts ranging from 4 to 6 cm in length. The excised tissues were placed in autoclaved phosphate buffer (NaH_2PO_4 – 6.33 g/L, Na_2HPO_4 – 8.5 g/L with pH = 6.5: McPherson et al. (2018). The sterilized samples were stored at – 20°C for DNA extractions. All the samples were stored at 4°C for soil chemical analysis. The sampling area's climate is dry with annual rainfall lower than 30 mm and summer temperature can reach up to ±48°C with a relative humidity of 20–30% (**Supplementary Table 2**). The samples were collected during the dry summer season (May–June 2020).

DNA Extraction, Library Preparation, and MiSeq Sequencing

After pooling the soil/plant samples within each replicate, 10.0 g mixtures were subjected to total DNA extraction using the MoBio Power Soil DNA Extraction Kit. The DNA was quantified using Qubit 4.0 and high sensitivity kit (Invitrogen, United States). Libraries of each DNA sample were generated by amplifying the internal transcribed spacer (ITS region) and 16S rRNA gene (V4 region) using Nextflex PCR I Primer Mix (Perkin Elmer, United States) for fungal and bacterial communities, respectively (**Supplementary Figure 2**). Illumina amplicon primers for 16S (Forward 5′–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG–3′; Reverse 5′– GTCTCGTGGGCTCG GAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAA TCC –3′) and ITS (Forward 5′–AATGATACGGCGACCAC CGAGATCTACACGG CTTGGTCATTTAGAGGAAGTAA–3′; Reverse 5′–CAAGCAGAAGACGGCATACGAGA TCGGCTG CGTTCTTCATCGATGC–3′) were used. For the 16S rRNA gene, peptide nucleic acid (PNA) clamps were used to reduce mitochondrial and chloroplast contamination.

A paired-end sequencing approach with read lengths of 250 bp was conducted on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, United States) operating with v2 chemistry (User Guide Part # 15027617 Rev. L). All quality reads related to the study are available at NCBI under BioProject PRJNA771947 (SRP341951) and PRJNA767523 (SRP339516) for bacteria and fungi, respectively.

Bioinformatics Analysis of Sequencing Reads

The sequencing reads were analyzed with QIIME 2.0 (Bolyen et al., 2019). First, reads from ITS and 16S rRNA amplicons were separated into different files. Then, the average quality of forward and reverse reads was observed in each dataset. Only the forward reads were used for the following analyses due to the low quality of the reverse reads in both datasets. We used the DADA2 algorithm for denoising and generating the amplicon sequence variants (ASV) table for each dataset (Callahan et al., 2016). The reads clustered in the same ASV have nucleotide sequences that are 100% identical (Callahan et al., 2017). In the denoising, sequences were filtered

by overall quality, trimmed in low-quality regions, and chimeric sequences were removed. The 16S rRNA gene reads were trained on the SILVA database for taxonomic classification (Quast et al., 2012), while the UNITE database was used to classify the ITS sequences (Nilsson et al., 2019). Sequences classified as mitochondria and chloroplast were removed from the 16S rRNA gene ASV table. The 16S rRNA gene and ITS ASV tables were rarefied to 6000 and 100 reads for diversity analyses containing all samples from each dataset, respectively. The ASV tables of each dataset were then split for each sampling compartment for a more detailed analysis. For the bacterial diversity analyses, the bulk soil, rhizosphere, root endosphere, and shoot endosphere ASV tables were rarefied to 30,000, 37,000, 7,500, and 6,200 reads, respectively. For the fungal diversity analyses, the bulk soil, rhizosphere, root endosphere, and shoot endosphere ASV tables were rarefied to 120, 100, 1,300, and 1,200 reads, respectively. For beta-diversity analyses, the Bray-Curtis distance matrix was generated for each dataset and exported to statistical software. For alpha-diversity analyses, the Shannon index and Observed ASVs were calculated in each dataset to infer species diversity and richness, respectively.

Microbiome Analyses

The Bray-Curtis distance matrices were exported to R for multivariate statistical analyses. Non-metric multidimensional scaling (NMDS) was performed with the metaMDS function to show the ordination of samples according to the major factors (plant compartment) affecting the microbial communities, while constrained analysis of principal coordinates (CAP) was performed with the capscale function to show the ordination of samples according to more specific factors (sampling locations and soil type) using the "vegan" package v. 2.5-6 (Dixon, 2003). Both NMDS and CAP results were visualized with the ggplot function in the "ggplot" package v. 3.3.0 (Wickham, 2011). Permutative multivariate analysis of variance (PERMANOVA, 999 permutations) was used to test for significant effects of the factors studied (plant compartment, sampling location and soil type) and their interaction on bacterial and fungal beta-diversity using the "adonis function." Analysis of similarity (ANOSIM) was also used as a second method to test the effects of the factors studied on the fungal and bacterial communities using in R. Differences in species diversity (Shannon index) and richness (Observed ASVs) for the same factors were assessed using the Kruskal-Wallis test in QIIME 2.0 (Bolyen et al., 2019). Changes in the relative abundance of microbial genera between soil types were evaluated with the Kruskal-Wallis test and Bonferroni *p*-value correction using STAMP software (Parks et al., 2014).

Chemical Properties and Nutrient Composition

The four populations were sampled from soils characterized as sandy (T1/T3) and gravel (T2/T5), where the pH ranges from 6.7 to 7.2 and electrical conductivity from 27 to 39 dS m^{-1}. The soils with more gravel structure have higher nitrates than sandy ones (**Supplementary Table 2**). A detailed soil chemical analysis was performed (Khan et al., 2020). Furthermore, essential nutrients such as silicon (Si), magnesium (Mg) and calcium (Ca) were quantified from plants and soil from the four populations as described previously (Bilal et al., 2018) using inductively coupled plasma mass spectrometry (ICP-MS; Optima 7900DV, Perkin-Elmer, United States). All the measurements were carried out in triplicate.

Statistical Analysis

At least three replicate samples were analyzed during this study. The data for the enzyme study is presented as the mean ± standard error (SEM). The significant differences were determined using one-way analysis of variance (ANOVA). The differences were considered significant at $P < 0.05$ and were calculated by GraphPad Prism Version 6.01 (GraphPad Software, San Diego, CA, United States). Duncan's multiple range test at $P < 0.05$ (SAS 9.1, Cary, NC, United States) was used to compare the mean values.

RESULTS

Shifts in Microbial Community Structure and Diversity Between Plant Compartments, Sampling Locations, and Soil Types

A total of 58,648 ITS and 2,715,959 16S rRNA sequence reads passed all quality filters and were used for the following analyses. We analyzed the differences in bacterial/archaeal and fungal community structure between four locations where *Z. qatarensis* was naturally found (**Figure 1A**). Location T1 and T3 have sandy soils, while locations T2 and T5 have gravel soils (**Figure 1A**). The microbiomes of two soil (bulk soil and rhizosphere) and two plant endosphere (roots and shoots) compartments were investigated (**Figure 1B** and **Supplementary Figure 3**). The bacterial/archaeal community was dominated by the phylum Proteobacteria in the root and shoot endosphere compartments and Acidobacteria in the rhizosphere and bulk soil compartments (**Figure 2A**). Proteobacteria had a higher relative abundance in sandy than gravel soils in the bulk soil and shoot endosphere. In the rhizosphere and root endosphere compartments, the phylum Firmicutes was relatively more abundant in sandy than in gravel soils (**Figure 2A**). On the other hand, in the shoot endosphere *Firmicutes* were proportionally more abundant in gravel than in sandy soils (**Figure 2A**). PERMANOVA results indicated that sampling compartment was the main factor significantly affecting ($p < 0.001$; $R^2 = 0.34$) the bacterial/archaeal communities, followed by location ($p < 0.001$; $R^2 = 0.08$). The interaction effect between compartment and location was also significant ($p = 0.031$; $R^2 = 0.12$) on the bacterial communities. The bulk soil and rhizosphere samples did not show a clear separation in the NMDS, but clustered separately from the root and shoot endosphere samples (**Figure 2B**). ANOSIM results also showed a significant effect of compartments ($p < 0.001$;

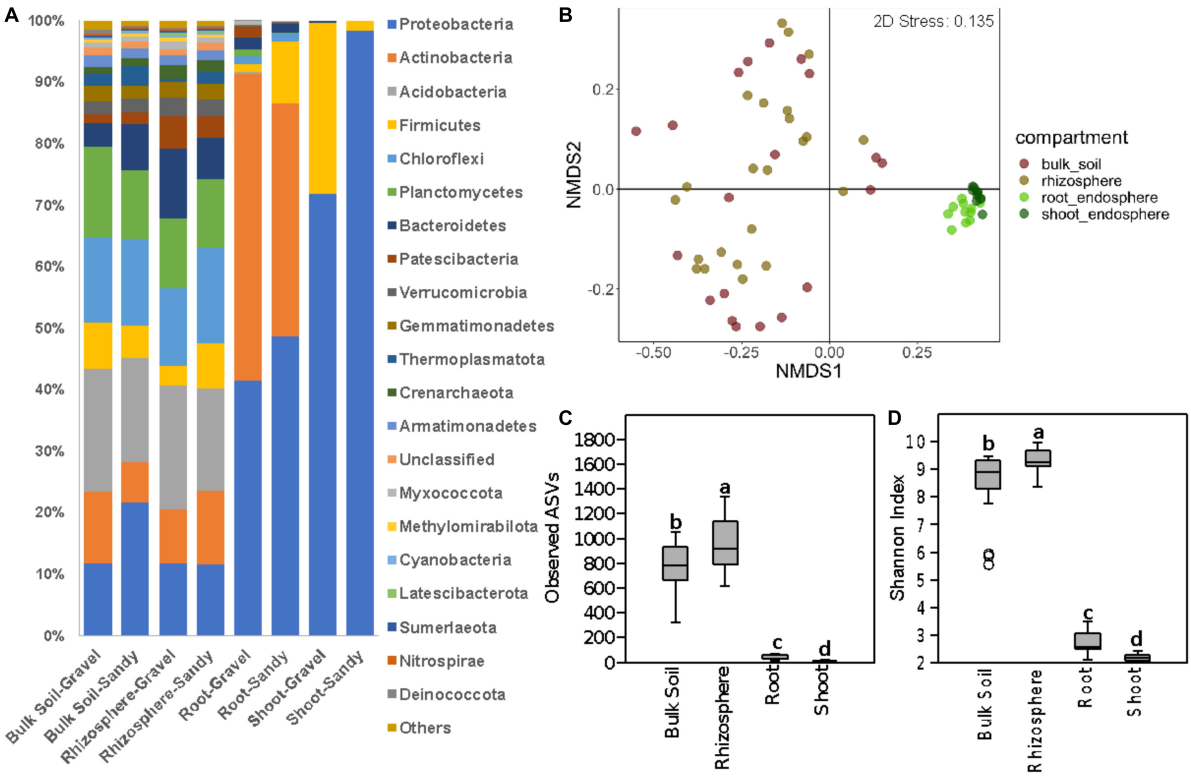

FIGURE 2 | Bacterial community composition and diversity in all sampling compartments. **(A)** Bar charts show the relative abundance of the dominant phyla in bulk soil, rhizosphere, root endosphere, and shoot endosphere samples of plants growing in gravel or sandy soils. "Others" stand for the sum of other phyla with <1.5% of relative abundance. **(B)** Bacterial community structure represented by a non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis distance matrix of the 16S rRNA gene ASV table. Samples are colored according to the sampling compartment. **(C,D)** Differences in bacterial alpha-diversity between the four plant and soil compartments. **(C)** Bacterial species richness based on the number of observed ASVs. **(D)** Bacterial species diversity based on the Shannon index. Different letters indicate significant differences according to the Kruskal-Walli's test ($p < 0.05$).

R = 0.53) and locations (p = 0.022; R = 0.34) on the bacterial communities. When analyzing the samples from all compartments together, soil type was not a significant factor in shaping the bacterial community structure according to PERMANOVA and ANOSIM.

Bacterial alpha-diversity was also significantly different between compartments, with greater species richness and diversity in the rhizosphere, followed by bulk soil, root endosphere, and shoot endosphere (**Figures 2C,D**). The bacterial community distribution and abundances across different soil types and samples have also been shown through heat and networking maps (**Supplementary Figures 4**, **5**). The analysis of each compartment separately indicated that the bacterial community structure was significantly different between the gravel and sandy soils in the rhizosphere (p = 0.025; R^2 = 0.07) and bulk soil (p = 0.013; R^2 = 0.09), but locations had a larger effect than soil type for both rhizosphere ($p < 0.001$; R^2 = 0.29) and bulk soil (p = 0.02; R^2 = 0.17) (**Figure 3**). Location T2 had the most different bacterial community composition among the four locations (**Figure 2**). The root and shoot endosphere bacterial community structure was not affected by soil type and location. The alpha diversity was not affected by soil type and locations in any of the four compartments.

The Ascomycota phylum dominated the fungal community in all samples from the four compartments (**Figure 4A**). This dominance was mainly represented by the ascomycete species *Acidea extrema*. The root and shoot endosphere compartments had proportionally more unclassified sequences than the bulk soil and rhizosphere (**Figure 4A** and **Supplementary Figure 6**). As observed for the bacterial community, the fungal community structure was more affected by plant compartment ($p < 0.001$; R^2 = 0.21), followed by sampling location (p = 0.014; R^2 = 0.08) according to PERMANOVA. The interaction between these two factors also significantly affected the fungal communities (p = 0.032; R^2 = 0.16). However, ANOSIM indicated that only location (p = 0.028; R = 0.05), but not compartment (p = 0.119; R = 0.05) significantly affected the fungal community structure. The separation between samples from the four compartments was unclear in the NMDS ordination (**Figure 4B**). Soil type did not affect the fungal beta-diversity when analyzing the samples from the four compartments together.

As seen for bacteria, the fungal alpha-diversity was also affected by plant compartments, with greater species richness and diversity in the rhizosphere and bulk soil than in the root and shoot endosphere (**Figures 4C,D**). It is noteworthy the low fungal

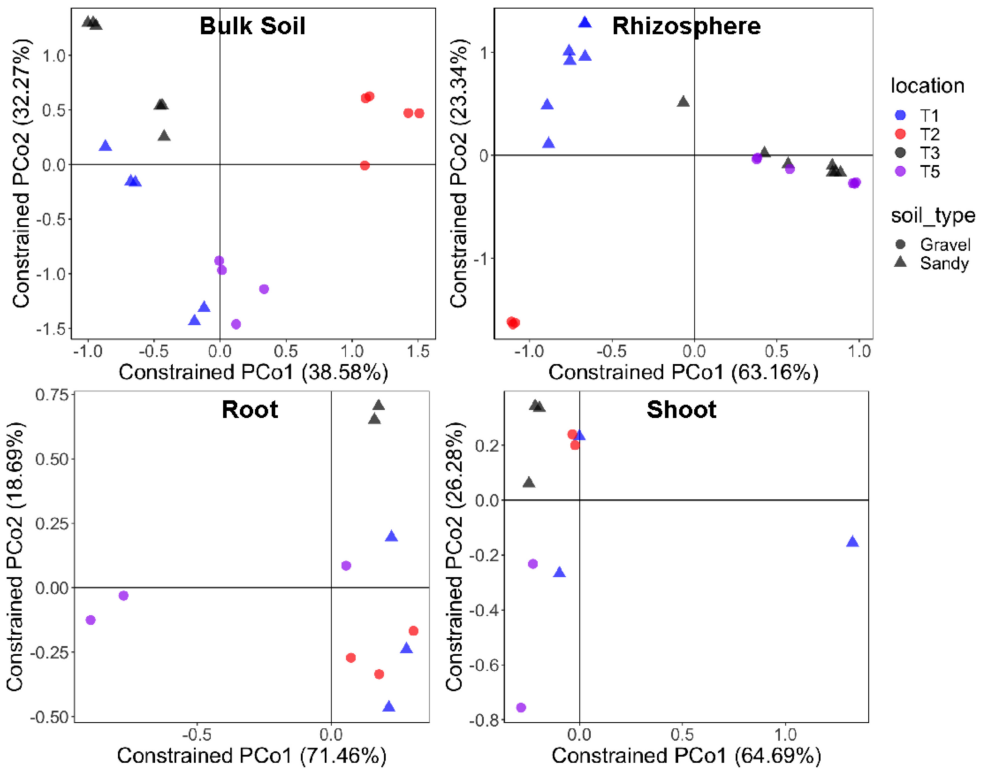

FIGURE 3 | Bacterial community structure in each specific sampling compartment. Constrained analysis of principal coordinates (CAP) based on the Bray-Curtis distance matrix of the 16S rRNA gene ASV table, showing the ordination of samples according to the factors sampling location and soil type in each compartment separately. The percentage of variation explained by the two first axes is provided for each graph. Different colors stand for the four locations, while different symbols stand for the two soil types.

alpha diversity in all compartments. Rarefaction curves showed that the sequencing depth and sample rarefaction (100 reads) used were sufficient to reflect the fungal diversity in these samples (**Supplementary Figure 7**). In addition, the species richness was also affected by different locations, with greater values in T3 and T5 than in T1 and T2 (**Supplementary Figure 5**). When separating the analysis by each compartment, soil type still did not affect the fungal beta-diversity in any compartment. Soil type also did not affect the fungal alpha-diversity in any compartment. However, location significantly affected the fungal community structure of the rhizosphere ($p < 0.001$; $R^2 = 0.37$) and bulk soil ($p = 0.049$; $R^2 = 0.23$) compartments (**Figure 5**). Like what was observed for bacteria, the fungal communities of location T2 were the most different among the four locations, mainly in the rhizosphere (**Figure 5**). Moreover, the fungal species diversity decreased in T2 compared to T3 and T5 in the rhizosphere compartment (**Supplementary Figure 5**).

The Core Microbiome of *Zygophyllum qatarensis* and Differences in the Relative Abundance of Specific Microbial Genera Between Soil Types

After analyzing the microbiome differences between locations and soil types, we aimed to identify the core microbiome of *Z. qatarensis* regardless of soil type and geographical location. We considered the core microbiome to be the microbes shared by >90% of all plant samples along with the four locations. Results indicated that no specific bacterial ASV was in the core microbiome of the bulk soil, rhizosphere, and root endosphere compartments, but one ASV classified in the *Alcaligenes* genus was present in the shoot endosphere of all plant samples. Similarly, no fungal ASV was present in the bulk soil and rhizosphere core microbiomes. However, two ASVs were found in the root endosphere core microbiome and three ASVs in the shoot endosphere core microbiome. One of these ASVs was in the core microbiome of both root and shoot endosphere and was classified in the species *Acidea extrema*, while the others were from unknown fungal genera. Next, we analyzed the core microbiome at the genus level. A total of 27 bacterial genera were in the bulk soil core microbiome, including *Bryobacter*, *Chthoniobacter*, *Gemmata*, *Nitrospira*, *Pirellula*, *Rubrobacter* and other 21 unknown/undescribed genera. On the other hand, no bacterial genus was in the rhizosphere core microbiome, while only one genus was in the root and shoot endosphere core microbiomes: *Alcaligenes*. Only the fungal genus *Acidea* (species *A. extrema*) was in the core microbiomes of all compartments. These results indicate that the different locations and soil types significantly affect the microbial beta-diversity and the core microbiome – shrinking it to none or just a few microbes.

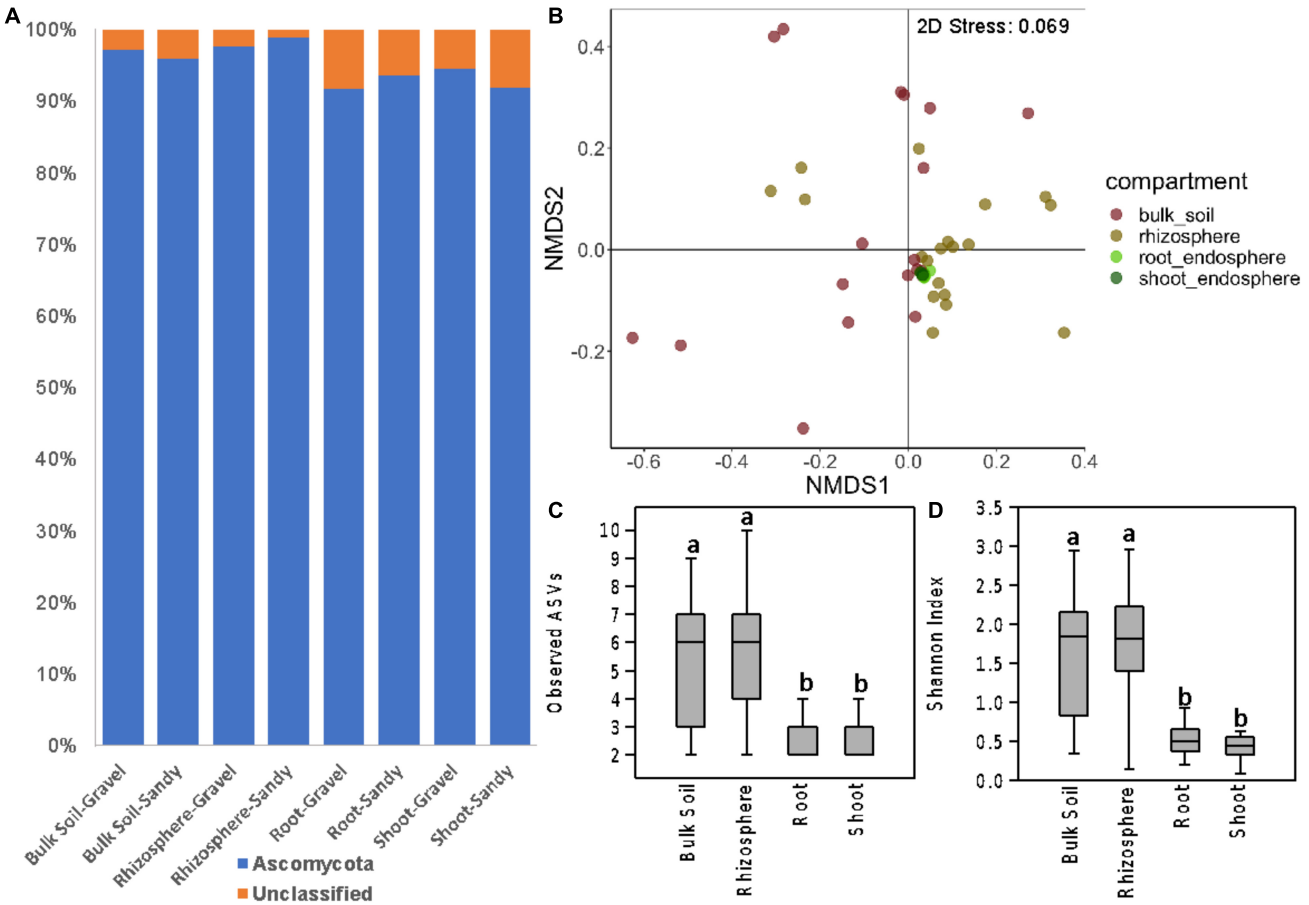

FIGURE 4 | Fungal community composition and diversity in all sampling compartments. **(A)** Bar charts showing the relative abundance of fungal phyla in bulk soil, rhizosphere, root endosphere, and shoot endosphere samples of plants growing in gravel or sandy soils. **(B)** Fungal community structure represented by a non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis distance matrix of the ITS ASV table. Samples are colored according to the sampling compartment. **(C,D)** Differences in fungal alpha-diversity between the four plant and soil compartments. **(C)** Fungal species richness based on the number of observed ASVs. **(D)** Fungal species diversity based on the Shannon index. Distinct letters indicate significant differences according to the Kruskal-Walli's test ($p < 0.05$).

We further investigated the changes in the microbial communities between the soil types by analyzing shifts in the relative abundance of specific bacterial and fungal genera. The bulk soil compartment had the highest number of bacterial genera (30) with significantly different relative abundances between the gravel and sandy soils, from which 18 were enriched in the sandy soils and 12 were enriched in the gravel soils (**Figure 6**). The bacterial genus with the largest relative abundance in sandy soils compared to gravel soils was *Adhaeribacter*. The genus with the largest relative abundance in gravel compared to sandy soils was *Rubrobacter* (**Figure 6**). The rhizosphere was the second compartment with more changes in the relative abundance of specific bacterial genera (17) between the soil types, where 13 genera were enriched in the sandy soils and four genera were enriched in the gravel soils (**Figure 6**). The bacterial genus with the most extensive proportional enrichment in the sandy soils was *Bacillus*. *Blastocatella* was the bacterial genus with the most significant relative abundance in gravel soils compared to sandy soils (**Figure 6**). The root endosphere showed only three changes in the relative abundance of bacterial genera between the soil types, including the enrichment of *Streptomyces* and *Cupriavidus* in the sandy soils and the putative genus WD101_soil_group in the gravel soils (**Figure 6**).

The genera affected by soil types were generally not the same in the rhizosphere and bulk soil, except *Noviherbaspirillum, Marine_Group_II, and Herpetosiphon*, which were enriched in the sandy soils in both compartments (**Figure 6**). Furthermore, *Streptomyces* was enriched in sandy soils in both rhizosphere and root endosphere (**Figure 6**). No bacterial genera had different relative abundances between soil types in the shoot endosphere compartment. No fungal genera changed in relative abundance between soil types in any of the four compartments. Many bacterial genera also had differences in relative abundance between locations within each soil type, but there were more changes in the rhizosphere than in the bulk soil (**Supplementary Figures 4**, **5**). The root and shoot endosphere did not show

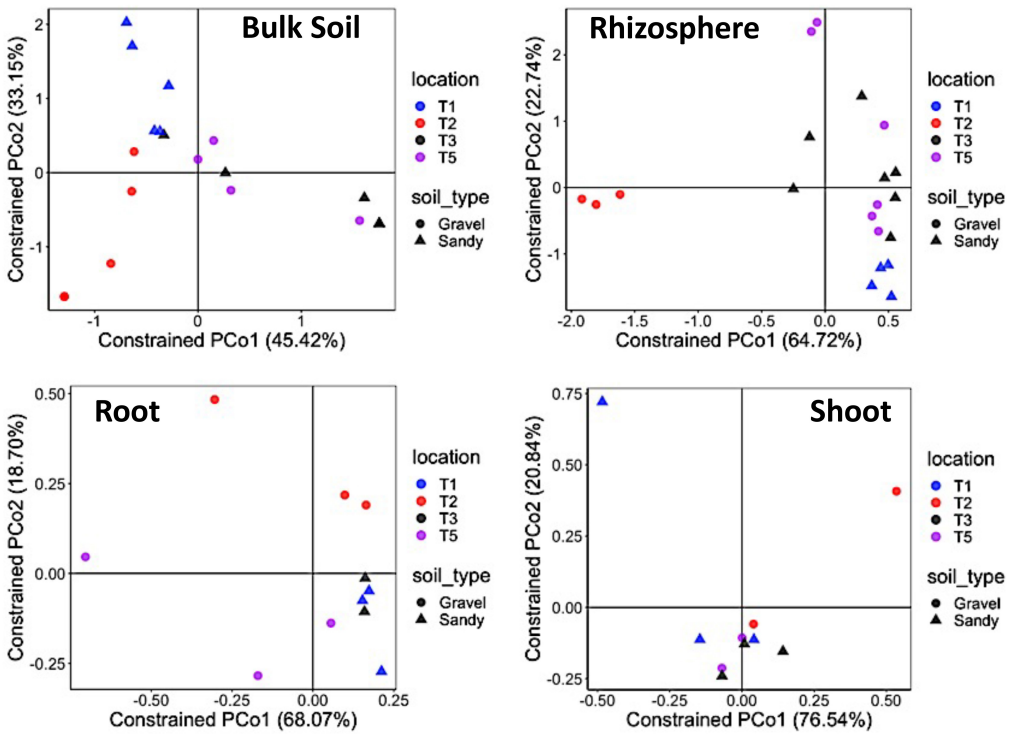

FIGURE 5 | Fungal community structure in each specific sampling compartment. Constrained analysis of principal coordinates (CAP) based on the Bray-Curtis distance matrix of the ITS ASV table shows samples' ordination according to the sampling location and soil type in each compartment separately. The percentage of variation explained by the two first axes is provided for each graph. Different colors stand for the four locations, while different symbols stand for the two soil types.

any differences in genera between locations, and no fungal genera differences in relative abundance between locations in any compartment.

Nutrient Assimilation in Rhizosphere and Phyllosphere of *Zygophyllum qatarensis*

Three major nutrients, *i.e.,* silicon (Si), magnesium (Mg), and calcium (Ca), were quantified in the soil, rhizosphere, and shoots across four populations of *Z. qatarensis*. In addition to major nutrients, we also assessed the soil physical and morphological properties shown in **Supplementary Figure 8**. The results showed that the rhizosphere had significantly ($p < 0.01$) higher Si content than bulk soil. Among the populations growing in each soil type, T3 had a significantly higher Si content than T1 in sandy soils, whereas T2 had a significantly higher Si content than T5 in gravel soils (**Figure 7**). The rhizosphere of T1, T3, and T2 had 23, 21.5, and 15.8% more Si than bulk soil. However, the rhizosphere of T5 had a significantly lower Si content compared to bulk soil. In the case of root to shoot parts, T1 and T3 (sandy soils) had significantly higher ($p < 0.05$; 8.7 and 41.6%, respectively) Si content in roots compared to T2 and T5 (gravel soils). On the other hand, the T2 and T5 had significantly higher ($p < 0.05$; 13.2 and 11.8%, respectively) Si in shoots compared to roots (**Figure 7**).

In the case of Mg, T2 (gravel texture) had a significantly higher ($p < 0.003$; 44.7%) content in bulk soil, while T5 had a higher content in rhizosphere. In contrast, T1 and T3 (sandy) had significantly higher ($p < 0.001$; 4.2 and 21.8% respectively) Mg content in the rhizosphere than in bulk soil. Overall, Mg amounts were more elevated in gravel than in sandy soils across the different populations. In plants, the Mg content was 7- to 8-fold higher ($p < 0.001$) in shoots than in roots. Among populations, shoots of T1 and T5 had a significantly higher Mg content than T3 and T2. Only T5 had a higher Mg content than T1, T2, and T3 (**Figure 7**).

In the case of Ca, it was significantly higher in the rhizosphere of T1 (8.7%) and T3 (7.2%) compared to the sandy bulk soils (T2 and T5) of *Z. qatarensis*. Conversely, in gravel soil, the T2 population of *Z. qatarensis* had a significantly higher (14.2%) Ca content in bulk soil compared to rhizosphere. T5 had lower Ca than the other populations of *Z. qatarensis* growing in different locations. In the case of the plants' organs, shoots of *Z. qatarensis* had in general, a significantly higher Ca content than roots. A considerably higher amount of Ca was observed in T2 > T1 > T3 > T5 in shoots. This increase was four to fivefold higher than in roots (**Figure 7**).

DISCUSSION

There has been an unprecedented emphasis on understanding and exploring unique microbiomes and elucidating their function for greater human benefits in agriculture. This study

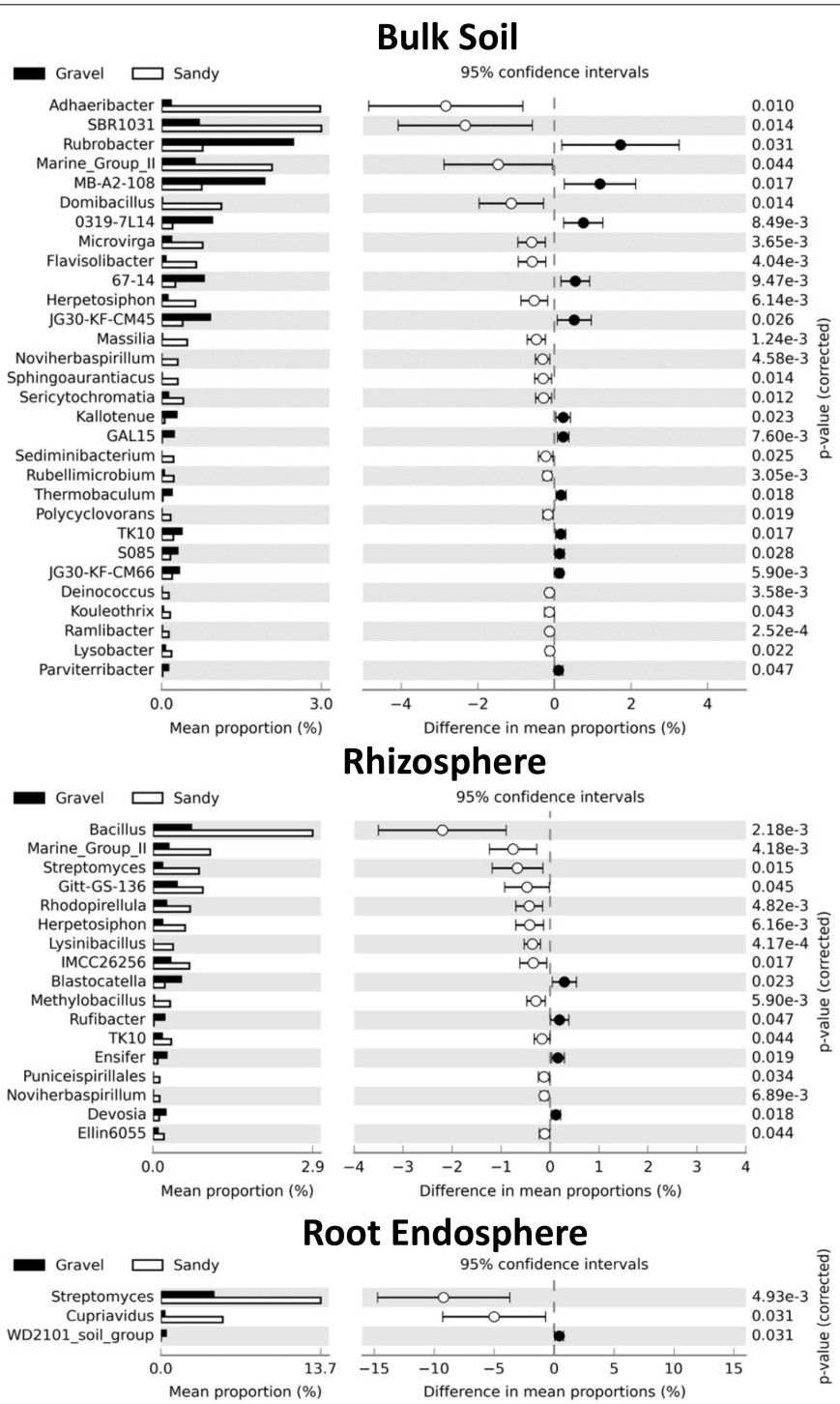

FIGURE 6 | Differences in the relative abundance of specific bacterial genera between the two soil types in each compartment. Set of bacterial genera that significantly shifted in relative abundance according to the Kruskal-Wallis's test with the Bonferroni *p*-value correction ($p < 0.05$) in each sampling compartment. The genera are sorted based on the largest differences in the mean proportion of sequences between samples collected from gravel (black) and sandy (white) soils. The shoot endosphere was not shown in the figure because no significant differences were found in this compartment.

explored the microbiome associated with the desert halophyte *Z. qatarensis* in four populations growing in two soil types in four different locations. We hypothesized that either location or soil type would influence the microbial community structure. Furthermore, we generated the first endosphere datasets for this species, which could help us understand whether and

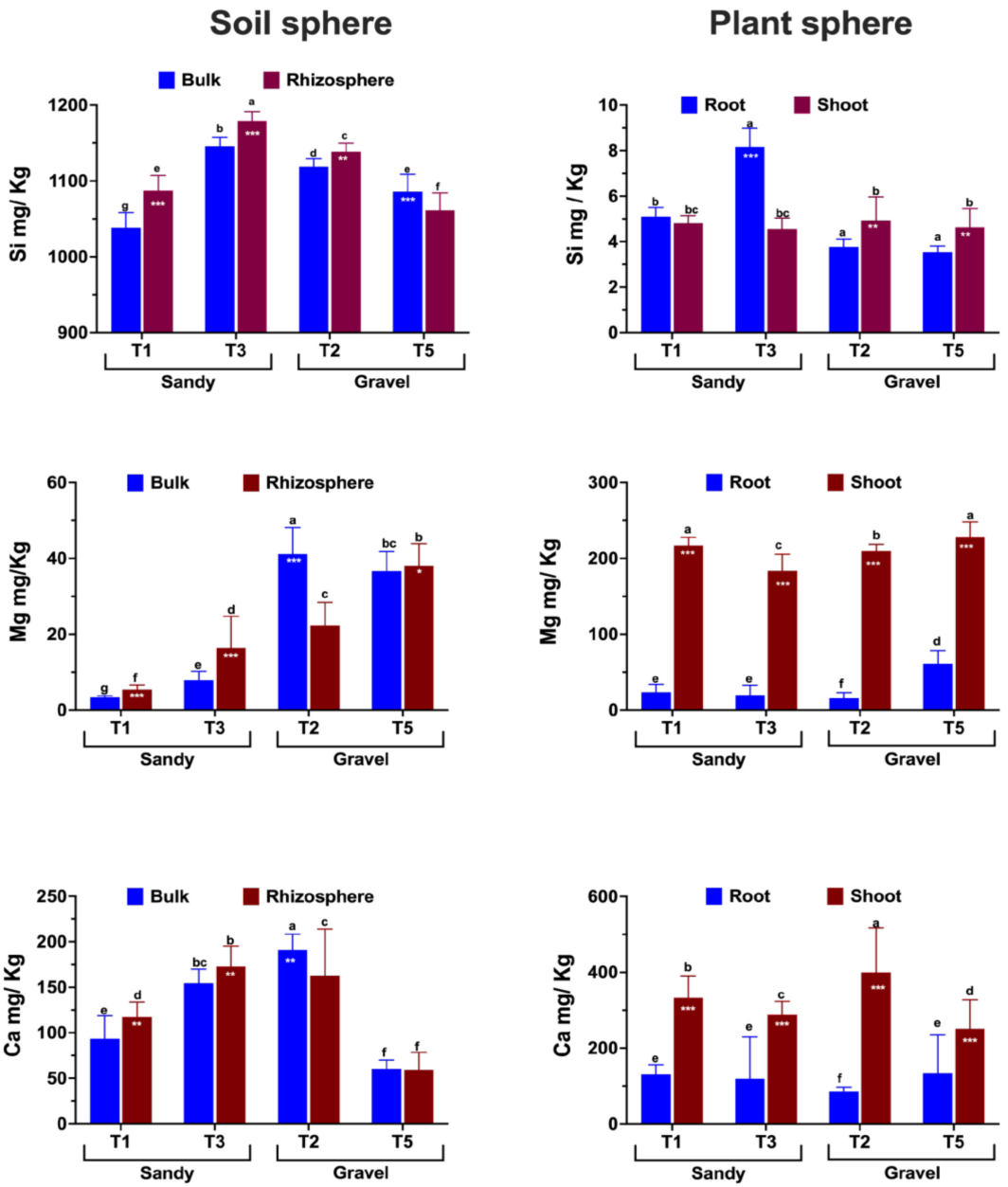

FIGURE 7 | Essential plant growth-related nutrient composition and abundance across soil (bulk and rhizosphere) and plant parts (shoots and roots) of different populations of *Z. qatarensis*. The error bars represent standard deviations. The different letter(s) on the graph indicate significant differences between treatments ($p < 0.05$), as revealed by the DMRT test on SAS 9.0. The $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ on selected bars indicate significant effects of drought treatment.

how the plant's microbiome increases its survival in the harsh desert environment. The results clearly showed that the microbial communities' distribution and diversity were significantly affected by location and soil types (sandy and gravel). This is consistent with previous studies showing that the rhizosphere and root endosphere microbial communities are affected by environmental factors such as soil pH, salinity, moisture content, soil leaching, erosion, and loss of certain nutrients (Mukhtar et al., 2018, 2021; Khan et al., 2020; Bossolani et al., 2021) since different locations and soil types usually show changes in soil physico-chemical factors.

There are several studies on the microbiomes of extreme desert environments such as those found in the Atacama Desert (Araya et al., 2020; Contador et al., 2020; Menéndez-Serra et al., 2020), Lejía Lake (Mandakovic et al., 2018), the Empty Quarters in Oman (Khan et al., 2020), the Sonoran Desert (Andrew et al., 2012; Finkel et al., 2012; Gornish et al., 2020), the Mojave Desert (Pombubpa et al., 2020), the Saline Lakes of Monegros Desert,

Spain (Menéndez-Serra et al., 2020), Eastern Mediterranean (Mazar et al., 2016), and the seed-associated microbiome from Southern Chihuahua Desert (Menéndez-Serra et al., 2020). Some succulent and arid-land plant species were also recently explored for their microbiome structures such as *Agave* spp. (Flores-Núñez et al., 2020), *Aloe vera* (Akinsanya et al., 2015), cacti (Fonseca-García et al., 2016), CAM plants (Citlali et al., 2018), pineapple pp (Putrie et al., 2020), and Aizoaceae (Pieterse et al., 2018). Most of these studies are exploratory and have relied only on bacterial community diversity and structure. Herein, the current study has shown the core-microbiome and culturable microbes' function in plant growth promotion.

In the current study, we assessed both the bacterial and fungal communities of *Z. qatarensis* in the major locations where populations of this plant are naturally present. In bacterial communities, *Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes*, and *Chloroflexi* were the most abundant phyla, whereas, in fungal communities, Ascomycota was the dominant phyla in the rhizosphere of *Z. qatarensis*. Recent studies on the rhizosphere microbiome of desert plants revealed a high portion of extremophilic microbes relative to stress-sensitive plants (Marasco et al., 2012). We also identified several genera of extremophilic microbes such as *Noviherbaspirillum, Marine_Group_II* and *Herpetosiphon*. Species of *Noviherbaspirillum* show considerable potential in denitrification processes in soil (Ishii et al., 2017). Sequences from the archaeal Marine group putative genus have been detected in desert environments (Pombubpa et al., 2020). Previous studies also revealed the presence of halophilic bacterial genera including *Bacillus*, *Halomonas, Halobacillus, Oceanobacillus, Marinobacter, Marinococcus*, and *Nesterenkonia* in the rhizosphere and roots of xerophytes (Etesami and Maheshwari, 2018; Mukhtar et al., 2019). These studies showed remarkably high and diverse rhizosphere colonization of *Actinobacteria, Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria,* and *Bacteroidetes* (Citlali et al., 2018; Flores-Núñez et al., 2020). However, this study helps to understand the microbiome composition of wild plants growing in arid environments has not been demonstrated.

Furthermore, it is worth mentioning that soil nutrient composition was largely variable across populations from different locations, even in similar soil types. The variability was also evidenced in the salinity content and pH range alongside some of the key nutrients. Although some species of *Zygophyllum* prefer a more saline rich soil, however, we noticed that the salt contents were significantly lower in the soil of *Z. qatarensis*. A recent study by Wang et al. (2020) showed (*Z. brachypterum, Z. obliquum* and *Z. fabago*) resistance to 200 mM NaCl and found that the CoA biosynthesis was significantly activated in transcriptome data analysis. In addition, to salts, the Mg was significantly higher in bulk soil than in *Z. qatarensis* rhizosphere. This can be attributed to the overall abundance of Mg across empty quarter desert (Rub'al Khali) desert (McKay et al., 2016). In contrast, the Ca content was higher in the rhizosphere (sandy) than in bulk soil. One of the most abundant nutrients in the earth's crust, Si is higher in the rhizosphere than in the bulk soil. In the case of plant parts, Si was more abundant in roots than in shoots. The Si content in soil increases plant resistance to different biotic and biotic stresses (Epstein, 1999; Ma and Takahashi, 2002; Ma and Yamaji, 2006), including salt and drought stress (Zhu and Gong, 2014; Rizwan et al., 2015), extreme temperature stress (Ma, 2004), nutrient deficiency (Marafon and Endres, 2013), and disease incidence (Marafon and Endres, 2013; Van Bockhaven et al., 2013). The availability, distribution, and concentration of Si have also been associated with a selection of microbial players. For example, Si presence greatly influences the microbial community structure during heavy metal contamination (Zhang et al., 2021), suggesting that extreme environments tend to influence the microbial interactions of the endemic plants, which depends on soil chemical profile.

The soil texture possibly influences the selection of key players and their variability such as *Adhaeribacter,* which was significantly more abundant in sandy than in gravel bulk soils. In contrast, *Rubrobacter* was more abundant in gravel than in sandy bulk soils. Previous studies showed that changes in soil chemistry extend selective effects on bulk soil, rhizosphere and root endosphere microbial community structure (Goss-Souza et al., 2020; Lopes et al., 2021). *Adhaeribacter* has been previously detected in desert soil and is known for its higher cellulolytic activities (Zhou et al., 2018). *Rubrobacter* – a member of the Actinobacteria phylum – was the most abundant soil microbial taxon. In the rhizosphere, many bacterial genera were enriched in sandy compared to gravel soils, mainly *Bacillus*. There is several *Bacillus* spp. —either associated with rhizosphere or endosphere showing remarkable plant growth-promoting and stress tolerance traits. The function of *Bacillus* spp. for *Z. qatarensis* has not been fully explored, which is an important question for microbiome studies in extreme environments.

Extremophilic microbes associated with desert plant species can extend their plant-growth-promoting and stress resistance traits for other plants, such as crops. Previous studies have evaluated the microbiome (especially bacterial communities) from arid soil (Jorquera et al., 2016; Citlali et al., 2018; Delgado-Baquerizo et al., 2018; Mandakovic et al., 2018; Araya et al., 2020; Astorga-Eló et al., 2020; Khan et al., 2020). However, little is known about the function of phytomicrobiome for improving crop growth and resistance to stress. Despite a few extremophile species that have been identified and characterized from desert environments (Yadav et al., 2021), their function in crop-stress tolerance has not been fully explored.

CONCLUSION

The geographic pattern of *Z. qatarensis* microbial communities was established in this study for the first time. The composition and diversity of microbial communities varied between geographical location and soil textures. Each component had different impacts on different microbial groups, and the biogeographic distribution was the consequence of the cumulative effects of all influencing factors. Soil texture had the greatest impact on both bacterial and fungal communities, with specific microbial taxa co-occurring with sand and gravel and rhizosphere and endosphere. Furthermore, the narrow composition of core-microbiome in different compartments

especially in bulk soil, shows passive selectivity of desert plants toward a large aggregation of microbial resources. This could be due to plant's ability to utilize less energy in the form of root exudation to allow or not mutualistic relationships. These plants already focus more effort on reducing the impact of abiotic stress such as high heat, low moisture, and lack of nutrients. It has also been argued that desert plant species establish a large rhizo-sheath – a feature that may be an important adaptation to water-stressed environments (Marasco et al., 2012; Ndour et al., 2020). However, we have not seen any visible sign of rhizosheat around the roots of *Z. qatarensis*. The selectivity of microbial diversity and function is one of the major challenges to identify stress tolerance traits in desert plants. Hence, isolation and identification of culturable rare species can be essential to enhance functional roles in water-stressed environments, where mobilization of nutrients such as Si could essentially improve plant growth performance of not only their natural host, but several crop species. Utilizing rare players from the plant microbiome may be vital in reducing crop stress tolerance and productivity during harsh environmental conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under BioProject PRJNA771947 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA771947) and PRJNA767523 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA767523) for bacteria and fungi, respectively.

AUTHOR CONTRIBUTIONS

AK designed the study and wrote the manuscript. SB extracted gDNA from samples. LL and AK analyzed the sequence data and prepared graphs. SA, VB, and KC cleaned the sequence data and edited the manuscript. AA-H, AA-R, and DS supervised the work and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.841217/full#supplementary-material

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Corrigendum: Microbiome variation across populations of desert halophyte *Zygophyllum qatarensis*

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In the published article, there was an error in **Materials and Methods**, *'DNA Extraction, Library Preparation, and MiSeq Sequencing'*, paragraph two. The accession numbers in the sentence "All quality reads related to the study are available at NCBI under BioProject PRJNA337739, 16S Accessions (KDUM00000000, KDUL00000000, and KDUK00000000), and ITS Accessions (KDUJ00000000, KDUI00000000, and KDUH00000000)" were incorrect. The sentence has been updated as follows:

"All quality reads related to the study are available at NCBI under BioProject PRJNA771947 (SRP341951) and PRJNA767523 (SRP339516) for bacteria and fungi, respectively."

There was also an error in the **Data Availability Statement**. The accession number was incorrectly given as "PRJNA337739". This section has been updated to read:

"The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under BioProject PRJNA771947 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA771947) and PRJNA767523 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA767523) for bacteria and fungi, respectively."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way.

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Overhauling the Effect of Surface Sterilization on Analysis of Endophytes in Tea Plants

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Increasing evidence shows that plant Endophytes play a crucial role in the fitness and productivity of hosts. Surface sterilization is an indispensable process before high-throughput sequencing (HTS) and tissue separation of plant endophytes, but its potential impact on the composition and diversity of endophytes has rarely been investigated. In the present work, the influence of sodium hypochlorite (NaClO) on the diversity of endophytic bacteria and fungi in leaves and stems of tea plants was investigated. We found that the diversity of bacterial endophytes was significantly affected by the concentration of NaClO as well as the pretreatment time. Pretreatment with 0.5% NaClO for 8 min and 2.0% NaClO for 3 min were suitable for the tea plant leaves and stems, respectively, but the effects of NaClO on the diversity of fungal endophytes were limited according to the results from HTS. Regardless of NaClO sterilization, most of the endophytes in tissues, such as the dominant taxa, could not be Isolated by using the regular culture-dependent approaches. Collectively, our results demonstrated that the pretreatment with NaClO should be modified to precisely understand the diversity of endophytes from different tissues of tea plants and also indicate that more attention should be paid to establish specific culture-dependent protocols for the isolation of plant endophytes.

Keywords: endophytes, surface sterilization, *Camellia sinensis*, sodium hypochlorite, diversity

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INTRODUCTION

As microorganisms inhabiting internal tissue of plants with no negative impacts on hosts, endophytes, the important components of plant microbiomes (Porras-Alfaro and Bayman, 2011), have attracted more attention around the world in the last few decades (White et al., 2019). The colossal biological diversity and the potential for biosynthesizing phytochemicals have made endophytes microbial resources with great research value and prospects (Jamwal and Gandhi, 2019). In terms of the symbiotic relationship, endophytes exert positive effects on the host plant in the following ways: (1) Modulating the growth of the host (Saravanan et al., 2008; Shi et al., 2010; Beltran-Garcia et al., 2014; Verma et al., 2017); (2) Enhancing the abiotic and biotic stress tolerance of hosts (Redman et al., 2002; Irizarry and White, 2018; Zhang et al., 2019); (3) Influencing the production of secondary metabolites in the host (Koskimäki et al., 2009; Verginer et al., 2010); and

(4) Increasing the resistance of the host against pathogens and pests (Arnold et al., 2003; Hartley and Gange, 2009; Gond et al., 2015; Busby et al., 2016; Xie et al., 2020). From an individual perspective, the various secondary metabolites produced by endophytes not only enrich the chemical diversity of biologically active molecules, but also make the endophytes a bioengineering tool for drug discovery (Alvin et al., 2014; Li et al., 2018; Sarsaiya et al., 2019).

As a worldwide economic woody crop, tea plants (*Camellia sinensis*) play essential roles in the forest ecosystem in which endophytic microorganisms have been a research focus. According to previous reports, endophytic fungi isolated from tea plants cover 3 phyla, 5 classes, 14 orders, 24 families, and 34 genera, and endophytic bacteria include 4 phyla, 7 classes, 13 orders, 24 families, and 32 genera (Xie et al., 2020). Research on biological functions of tea plant endophytes has found that some tea endophytes not only show antagonism toward tea plant pathogens (Rabha et al., 2014), but they also have antagonistic effects on other plant pathogens. In addition, endophytes have the capacity to promote the growth of tea plants (Nath et al., 2015; Yan et al., 2018; Borah et al., 2019) as well as to produce or modify tea plant metabolites (Agusta et al., 2005; Wang et al., 2014; Sun et al., 2019).

The previous research has shown that endophytes isolated from tea plants are worth studying in depth. The most frequently utilized method to isolate endophytes is the tissue separation with surface sterilization, and the factors of surface sterilization have a significant influence on the results. Theoretically, the only condition to determine the exposure duration is complete surface sterilization. However, because of the agents' penetration, it is difficult to eliminate the influence agents have on endophytic diversity and composition (Hallmann et al., 2006). Underexposure to the agent leads to the contamination of cultivable microorganisms and amplifiable nucleic acid, but overexposure may cause damage to the endophytes (Lundberg et al., 2012) by inducing DNA mutations (Tosi et al., 2021). Therefore, it is of importance to established a specific protocol for endophyte isolation from tea plants.

This study was set out to make a thorough exploration in the influence of the surface sterilization method based on the sodium hypochlorite (NaClO), the most commonly used sterilizing agent (Tosi et al., 2021), on diversity and composition of tea plants' foliar and cauline endophytes by comprehensively analyzing the results from high-throughput sequencing (HTS) and tissue separation and then to find an efficient and specific endophytic microorganism isolation method for *Clonorchis sinensis*.

MATERIALS AND METHODS

Sample Collection and Preprocessing

Symptomless mature leaves and stems with no disease or damage were collected from two varieties of *Camellia sinensis*, such as cultivar Longjing 43, from Yuhang county, Zhejiang Province, China, and cultivar Jiukeng, from Chun'an County, Zhejiang Province, China. All samples were placed under running tap water to remove adherent dust and soil particles and most microbial Surface impurities before further processing (Hallmann et al., 2006).

Sterility Examination of Surface Sterilization Combinations

The surface sterilization method was based on five concentrations of NaClO (0.25, 0.5, 1.0, 2.0, and 4.0%) and different durations for tissue immersion in the agent (1–15 min) (Xie et al., 2020). After the pretreatment, samples were sterilized with different combinations, followed by rinsing with sterile water three times. The last rinse was taken at the scale of 100 μl/dish onto Luria-Bertani (LB) medium and potato dextrose agar (PDA) medium, and samples were incubated in dark at 30°C for 7 days to confirm whether sterilization was complete.

DNA Extraction and High-Throughput Sequencing

However, the agent may permeate into the tissue and create conditions lethal for some endophytic microorganisms (Hallmann et al., 2006), HTS was employed to study the influence of the agent on Endophytes. DNA was extracted from the surface-sterilized samples according to the ALFA-SEQ Plant DNA Kit (mCHIP, China) and concentration and purity of which were determined by using the NanoDrop One (Thermo Scientific, Wilmington, United States). The V4 region of the Bacterial 16S rRNA genes was amplified by using 515f (5′ GTGYCAGCMGCCGCGGTAA 3′)/806r (5′ GGACTACNVGGGTWTCTAAT 3′) primers (Parada et al., 2016; Liu et al., 2018; Matsumoto et al., 2021), and the blocking primers peptide nucleic acids (mPNA and pPNA) were added to prevent the amplification of mitochondrial and chloroplast DNA (Lundberg et al., 2013). The ITS hypervariable region was amplified by using ITS1f (5′ CTTGGTCATTTAGAGGAAGTAA) and ITS2r (5′ GCTGCGTTCTTCATCGATGC) (Nilsson et al., 2019). For 16S PCR amplification, the thermocycler program was set for initial denaturing at 95°C for 5 min, followed by 30 cycles of denaturing at 96°C for 1 min, PNA annealing at 78°C for 5 s, primer annealing at 54°C for 1 min, extension at 74°C for 1 min, and a final extension at 74°C for 10 min. The ITS PCR procedures were as follows: pre-denaturation at 95°C for 5 min, followed by 30 cycles of deformation at 94°C for 30 s, annealing at 52°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were extracted on 1.0% agarose gels, the concentrations of which were examined by the GeneTools Analysis Software (Version 4.03.05.0, SynGene). The fragments purified with an E.Z.N.A. Gel Extraction Kit (Omega, United States) were sequenced under by PE250 based on the Illumina Nova 6000 platform (Illumina, San Diego, United States) at Guangdong Magigene Biotechnology Co., Ltd., (Guangzhou, China).

Isolation of Endophytic Fungi by Tissue Separation

The surface-sterilized samples were cut aseptically into segments (2 mm × 2 mm for leaf tissue (Liu et al., 2015) and 3 mm

for debarked stem samples (Win, 2018)) with a sterilized blade before being plated on malt extract agar (MEA) medium and PDA medium in 9-cm diameter plastic Petri dishes (10 segments per Petri dish). The Petri dishes were incubated at 26°C in dark for 7 days. Each isolate was inoculated on corresponding medium in 6-cm diameter plastic Petri dishes for pure culture and further processing.

Isolation of Endophytic Bacteria by Tissue Separation

After the surface sterilization, samples were homogenized at the ratio of 20 ml sterile water to 6 g leaf tissue and 15 ml sterile water to 6 g stem tissue, followed by the graded dilution to 10^{-1}, 10^{-2}, and 10^{-3} with sterilized water (Yan et al., 2018). Then, 200 μl diluted homogenate was removed and spread on the LB agar medium and nutrient agar (NA) medium in 9-cm diameter Petri dishes. The Petri dishes were placed in the incubator at 30°C and in the dark for 5 days. The streak plate method was used for the purification.

Molecular Identification of Isolated Endophytes

The sequence analysis of the 16S rRNA gene and the ITS rDNA region was used for the species-level identification of isolated endophytic bacteria and fungi, respectively. The ITS region was amplified with primers of ITS1 (5′ TCCGTAGGTGAACCTGCGG)/ITS4 (5′ TCCTCCGCTTATTGATATGC) (Pryce et al., 2003; Kehelpannala et al., 2018) and 16s rRNA genes were amplified with primers of 27f (5′ AGAGTTTGATCMTGGCTCAG)/1492r (5′ ACGGTTACCTTGTTACGACTT) (Wei et al., 2018). The sequences of isolates were subjected to a BLAST search[1] and compared with representative sequences in the NCBI database to determine the corresponding species (Vu et al., 2019).

High-Throughput Sequencing Analysis

The raw reads transformed from original data were quality-filtered by FASTP (version 0.14.1.). The paired-end reads were processed using the USEARCH (version 10.0.240). The operational taxonomic units (OTUs) that had a 97% similarity level using UPARSE (Edgar, 2013) were processed by utilizing QIIME2 (version 2020.11.0). The taxonomy of each 16S rRNA gene sequence was analyzed against the SILVA (v123) 16S rRNA gene database at a confidence threshold of 80%. The taxonomy of each ITS rRNA gene sequence was analyzed against the RDP (v2) ITS rRNA gene database at a confidence threshold of 80%.

The alpha diversity based on the R vegan package was conducted to reveal the within-habitat diversity and principal component analysis (PCA) was performed to examine dissimilarities in the community composition among samples based on an Euclidean metric. Venn diagrams showed the number of common or unique OTUs in multiple groups.

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

Statistical Analysis

The statistical analysis and graphic illustration were performed using the GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, United States) and SPSS Statistics version 26.0 (IBM Corporation., Armonk, NY, United States). Results were expressed as mean ± standard error of the mean (SEM). The statistical significance was indicated by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant and the statistical differences are indicated by superscripts.

RESULTS

Preliminary Screening of Surface Sterilization Methods

The sterilization efficiency of each combination was shown by the number of microbes after 7-day incubation (**Figure 1**). To achieve complete surface sterilization, the lower the concentration of NaClO used, the longer the effect time needed; conversely, when using an agent with higher concentration, the reaction time could be slightly shorter. Meanwhile, the sterilization efficiency was different in leaf tissue and stem tissue. Mature stem tissue required a higher concentration or longer duration. The results showed that the environmental bacteria of tea plants were more plentiful than fungi, and their survival appeared to be more related to concentration than duration. Considering both efficiency and feasibility, three combinations of each kind of tissue were screened for further study: for leaf tissue, 0.5% NaClO for 8 min (LL), 1.0% NaClO for 4 min (ML), and 2.0% NaClO for 2 min (HL); for stem tissue, 0.5% NaClO for 10 min (LS), 1.0% NaClO for 5 min (MS), and 2.0% NaClO for 3 min (HS).

Effects of Surface Sterilization on Endophytic Diversity

Diversity Analysis of Fungal Endophytes

After removing chimeric, plastids, and mitochondrial sequences, raw reads were drawn flat by minimum sequence. There were 104,605 reads per leaf sample and 184,954 reads per stem sample from cultivar Longjing 43, 394,335 reads per leaf sample and 390,126 reads per stem sample from cultivar Jiukeng. The alpha diversity (**Figures 2A–D**) index (observed OTUs) revealed that the differences were not significant (analysis of significant differences in two groups is shown in **Supplementary Table 1**), indicating that the within-habitat diversity of fungal endophytes was not noticeably influenced by the surface sterilization methods. Venn diagrams (**Figures 2E–H**) of fungal endophytic OTUs displayed the number of shared and unique OTUs in samples treated with different surface sterilization methods. The numbers of OTUs for leaf samples ranged from 151 to 258, and for stem samples ranged from 531 to 829. Among them, the ratios of shared OTUs were 86 (28.76%) in leaf samples of cultivar Longjing 43, 91 (21.77%) in leaf samples of cultivar Jiukeng, 298 (30.79%) in stem samples of cultivar Longjing 43, and 439 (35.32%) in stem samples of cultivar Jiukeng. Each

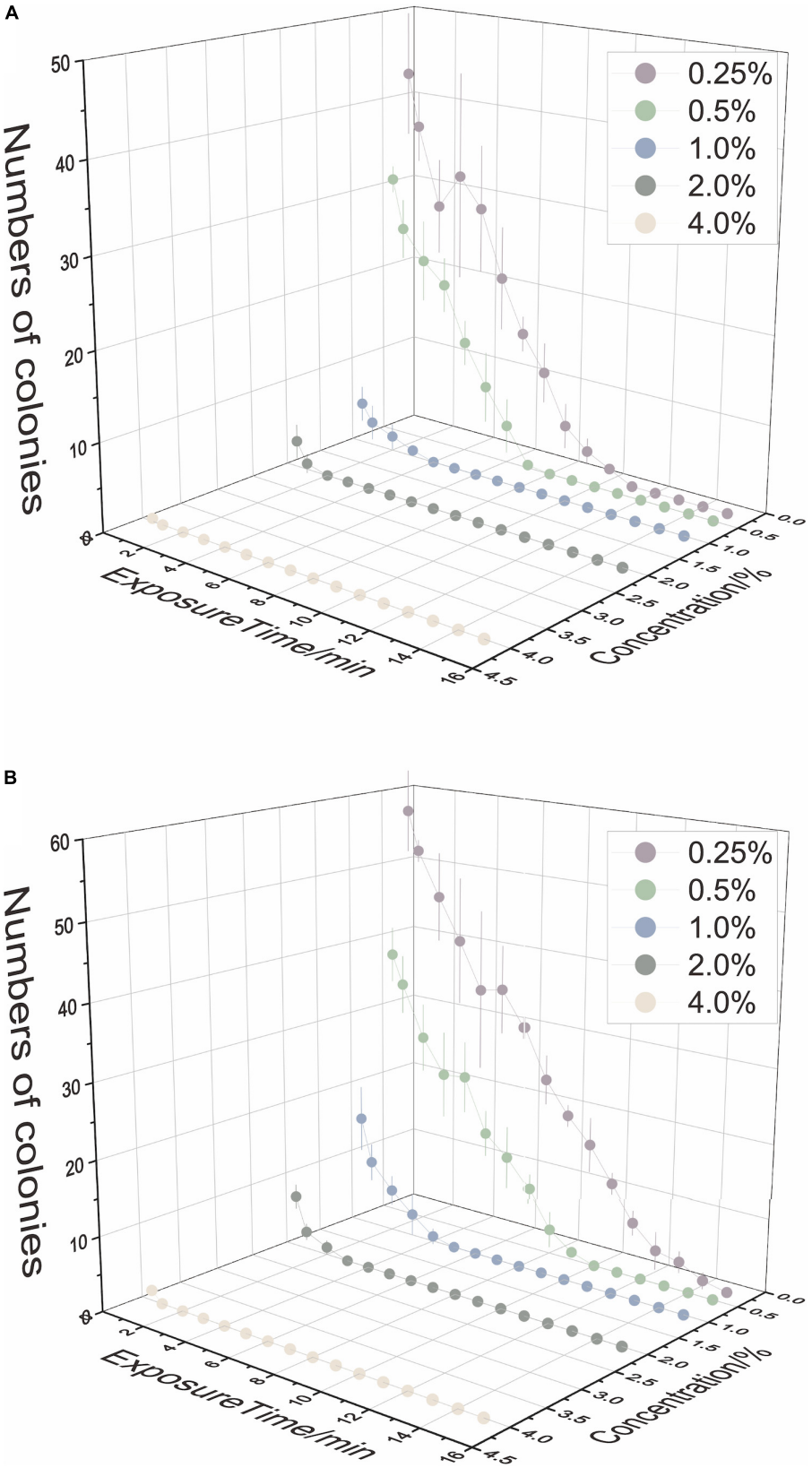

FIGURE 1 | The disinfection ability of sodium hypochlorite (NaClO) concentrations and exposure time on tea plant leaves **(A)** and stems **(B)**.

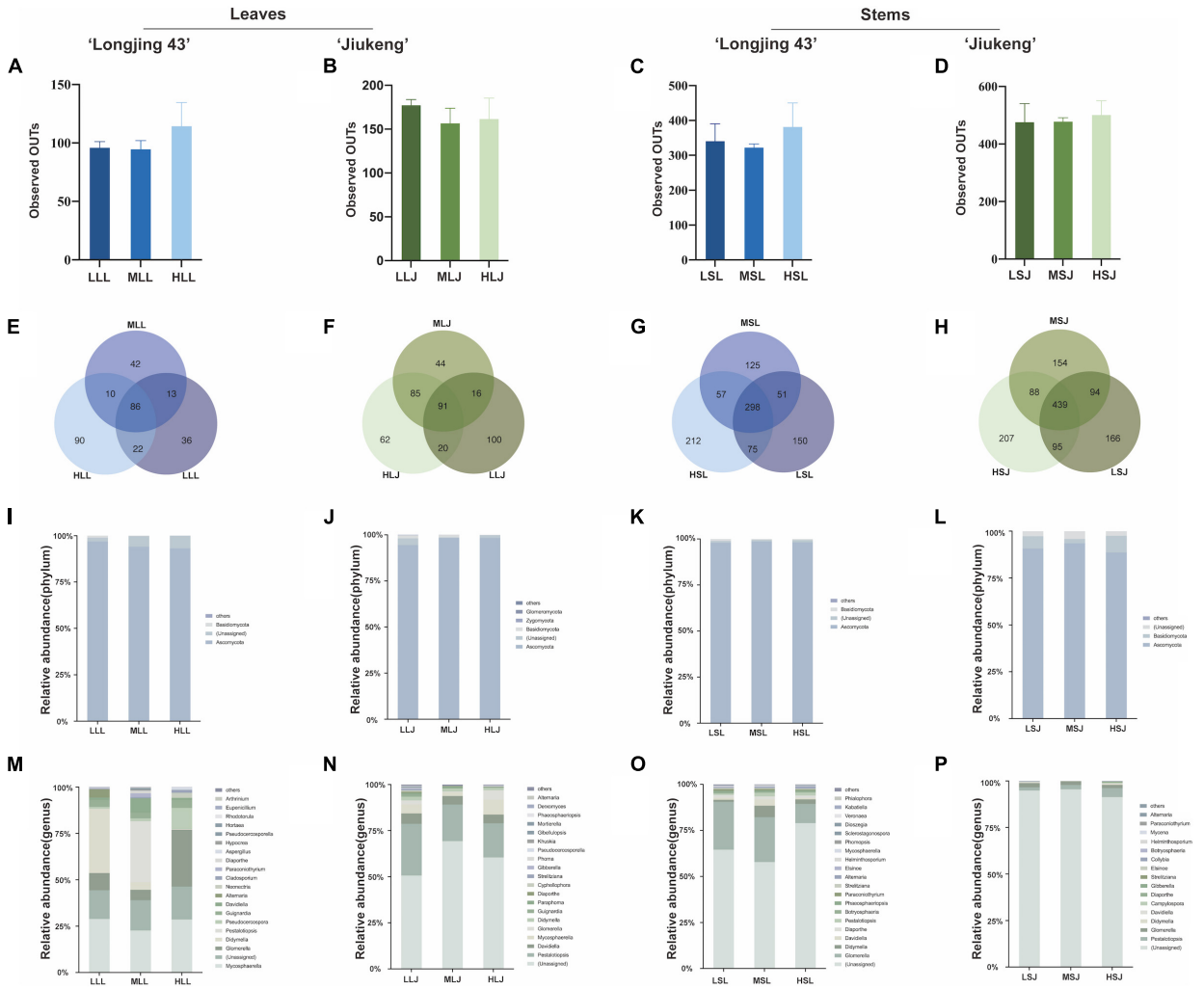

FIGURE 2 | Effects of sterilization treatments on the diversity of fungal endophytes from tea plant leaves and stems. **(A–D)** Alpha diversity of the fungal endophytic community; **(E–H)** Venn diagram showing the number of shared and unique operational taxonomic units (OTUs) (at 97% similarity); **(I–L)** relative abundance of fungal endophytes at the phylum level; **(M–P)** relative abundance of fungal endophytes at the genus level. LLL, MLL, and HLL represent the leaf samples from cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% of NaClO. LLJ, MLJ, and HLJ represent the leaf samples from cultivar Jiukeng treated with 0.5, 1.0, and 2.0% of NaClO. LSL, MSL, and HSL represent the stem samples from cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% NaClO. LSJ, MSJ, and HSJ represent the leaf samples from cultivar Jiukeng treated with 0.5, 1.0, and 2.0% NaClO.

group had unique OTUs, the differences of which indicated the sterilization methods had a significant impact on Fungal endophyte composition.

Analysis at phylum level (**Figures 2I–L**) showed high proportions of Ascomycota (93.0–98.3%) for fungal endophytes in leaves. Ascomycota (88.6–98.5%) was dominant and Basidiomycota (0.52–8.9%) was abundant for fungal endophytes in stems. The proportion of Basidiomycota in leaf samples declined as the concentrations increased. In two cultivars' leaves, (**Figures 2M,O**) common genera showed the same reaction to different surface sterilization methods: both *Mycosphaerella* and *Colletotrichum* had the lowest content in medium concentration; *Didymella*, *Pestalotiopsis*, *Diaporthe*, and *Pseudocercosporella* content were reduced by high concentrations. In the stems, (**Figures 2N,P**) *Glomerella* decreased sequentially with increasing concentrations; *Pestalotiopsis*, in contrast to *Helminthosporium*, was affected most by low concentration. Compared with consistency of endophytic bacteria in leaves and stems, trends in the common fungi in two tissue types were more uniform: *Davidiella*, *Pestalotiopsis*, *Didymella*, and *Diaporthe* had the highest percentages in medium concentration and *Glomerella* declined as dose increased in cultivar Longjing 43.

Diversity Analysis of Bacterial Endophytes

After qualified filtering the raw reads and normalizing the read counts, there were 3,945 reads per leaf sample and 55,804 reads per stem sample from cultivar Longjing 43, and 6,811 reads per leaf sample and 36,446 reads per stem sample from cultivar Jiukeng were retained. The alpha diversity (**Figures 3A–D**) index (observed OTUS) showed that the within-habitat diversity declined when the concentration was increased, indicating that

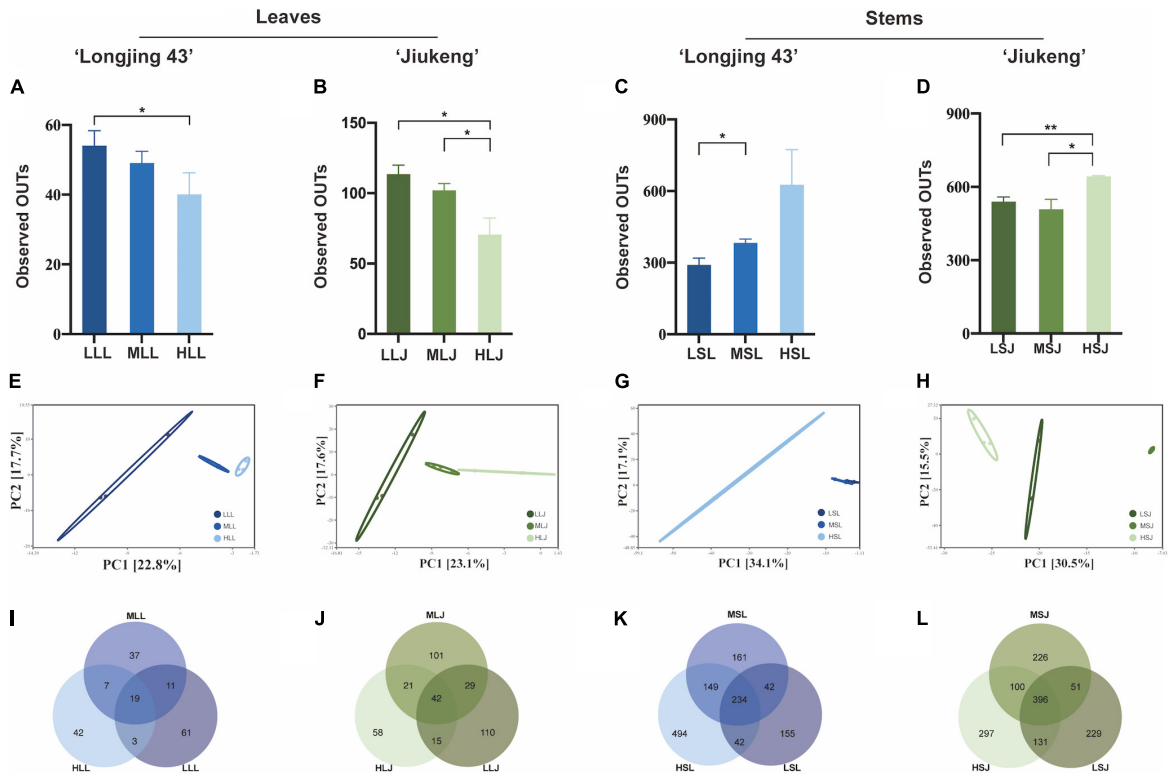

FIGURE 3 | Effects of sterilization treatments on the diversity of bacterial endophytes from tea plant leaves and stems. **(A–D)** Alpha diversity of the bacterial endophytic community; **(E–H)** principal component analysis (PCA) of the bacterial community structure; **(I–L)** Venn diagram showing the number of shared and unique OTUs (at 97% similarity). LLL, MLL, and HLL represent the leaf samples from cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% of NaClO. LLJ, MLJ, and HLJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% of NaClO. LSL, MSL, and HSL represent the stem samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% NaClO. LSJ, MSJ, and HSJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% NaClO. Asterisks indicate significant differences (*$p < 0.05$ and **$p < 0.01$).

the diversity of bacterial endophytes in leaves is influenced by the concentration of the agent. The results were opposite in stem samples in that the group treated with the highest concentration for the lowest exposed duration maintained the best richness. Bacterial endophytes in stem tissue were better tolerant of high concentrations compared to those in leaf tissue. Beta diversity analysis revealed clustering of differently treated samples when calculating PCA plots based on Euclidean distances (**Figures 3E–H**) and PCoA plots based on Bray–Curtis distances (**Supplementary Figure 1**). Venn diagrams (**Figures 3I–L**) of bacteria endophytic OTUs in samples of leaf tissue and stem tissue of each cultivar demonstrate the number of shared and unique OTUs in samples treated with different surface sterilization methods. At a 97% similarity level, the numbers of OTUs for leaf samples ranged from 49 to 196, and stem samples ranged from 473 to 924. The ratios of shared OTUs were not high in each group according to tissue types and cultivar types, respectively; there were 19 (10.56%) in leaf samples of cultivar Longjing 43 and 42 (11.48%) in leaf samples of cultivar Jiukeng. There were 234 (18.32%) in stem samples of cultivar Longjing 43 and 396 (27.69%) in stem samples of cultivar Jiukeng. In addition, each group had unique OTUs, the proportions of which indicated that, to some extent, differences in sterilization methods had an important impact on the bacterial endophyte composition.

Bacterial endophytic structure and composition (**Figure 4**) showed that the abundant phyla (≥0.5% of all sequences across all samples) in leaves were Proteobacteria (35.2–72.5%), Firmicutes (1.4–5.9%), Bacteroidetes (2.8–49.9%), Actinobacteria (0.8–5.0%), and Euryarchaeota (0.6–2.9%). It was also observed that the abundant bacterial phyla in two cultivars showed similar trends under different treatments: *Firmicutes* showed the highest abundances in medium concentration treatment (ML) compared to the other two treatments; *Actinobacteria* and *Euryarchaeota* declined trend as the concentration increased. The common genera in two cultivars appeared to have same response to different treatments. Relative abundance of *Acinetobacter* increased while *Methylobacterium* decreased with a higher concentration agent. *Bacillus*, *Paracoccus,* and *Massilia* took up the highest proportion under the secondary dosage and processing time. In terms of the relative abundance of cauline bacterial endophytes, the dominant phyla (≥0.5% of all sequences across all samples) were Proteobacteria (59–76.4%), Actinobacteria (5.3–16.5%), Bacteroidetes (3.2–11.3%), and Firmicutes (0.6–4.6%). Actinobacteria had an advantage with 2.0% NaClO treatment. At the genus level, *Massilia* showed the same response, in that medium combination negatively affected the relative abundance. *Jatrophihabitans* and *Burkholderia* were identified as the most abundant among the three treatments in both cultivars. Although most endophytic

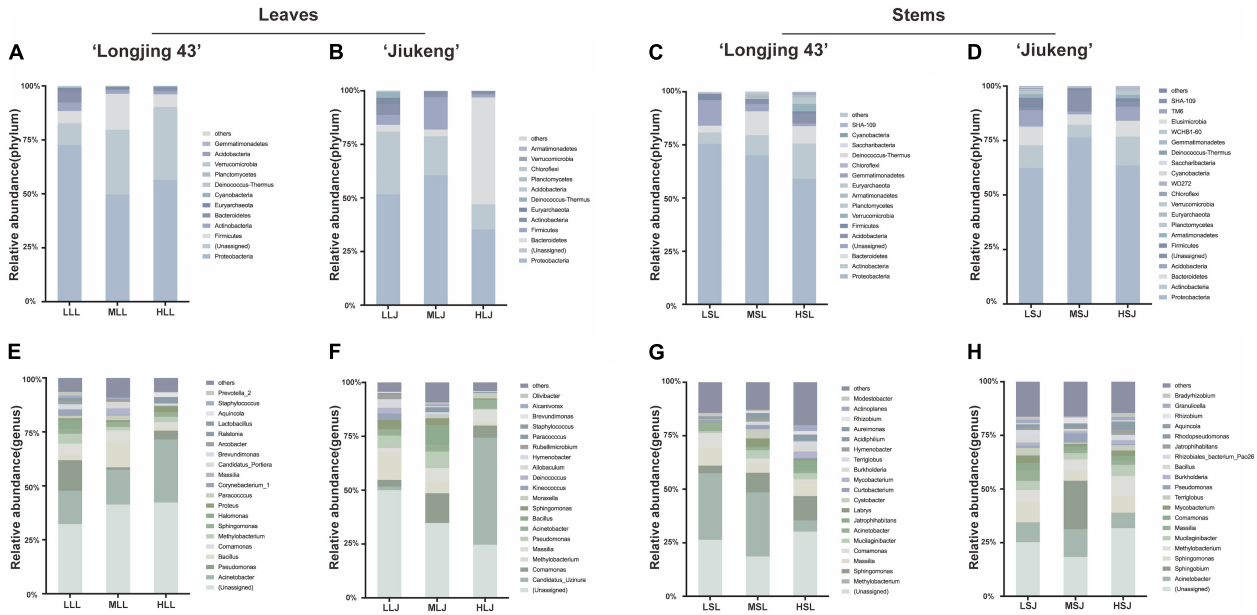

FIGURE 4 | Effects of sterilization treatments on the composition of bacterial endophytes from tea plant leaves and stems at the phylum level **(A–D)** and genus level **(E–H)**. LLL, MLL, and HLL represent the leaf samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% of NaClO. LLJ, MLJ, and HLJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% of NaClO. LSL, MSL, and HSL represent the stem samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% NaClO. LSJ, MSJ, and HSJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% NaClO.

bacteria had different characteristics in different tissue type, some of the common genera were found to have similarities: *Comamonas* in cultivar Longjing 43 showed intolerance to high concentration, *Pseudomonas* in cultivar Jiukeng accounted most in medium treatment group compared with other two groups and *Sphingomonas* was dominant in the low treatment group.

Analysis of Cultured Endophytes

Endophytes isolated from tea plants are summarized in the **Supplementary Table 2**. There were 1,040 strains of fungal endophytes isolated in total, 43 strains per group on average, which were categorized by surface sterilization methods, cultivars, tissue types, and medium types. Two phyla and 46 genera were involved. As shown in **Figure 5**, statistics were consistent with the results of HTS at the phylum level; the proportion of Ascomycota (99.4%) was significantly higher than Basidiomycota (0.58%). At the genus level, non-conformity was found between the results of HTS and tissue isolation. Genera such as *Mycosphaerella* and *Davidiella,* which were abundant according to the results of HTS, were not isolated; *Diaporthe* accounted for up to 30.5% of isolated endophytic fungi but measured only 0.02–1.7% in the HTS results. However, *Botryosphaeria*, *Didymella*, *Alternaria*, and *Pestalotiopsis* were not only enriched in testing results, but also in abundance after isolation and cultivation. Based on a single factor comparison, medium types showed little influence on the diversity and quantity of cultivable genera, and MEA medium was slightly better than PDA medium. For leaf samples, treatment with 1.0% NaClO for 4 min (ML) gave better results than did the other two concentrations, while higher concentration combined with lower exposure time was more suitable for stem samples.

There were 337 strains of bacterial endophytes isolated, covering four phyla and 34 genera (**Figure 6**). Proteobacteria (59.1%) and Actinobacteria (32.3%) were the dominant phyla, and Firmicutes and Bacteroidetes accounted for 6.2 and 2.4%, respectively. At the genera level, *Curtobacterium* (18.99%), *Sphingomonas* (15.73%), and *Herbaspirillum* (11.87%) were the top three with the highest percentages. Similar to fugal endophytes, statistical results at the genus level showed differences in cultivable ability and non-comformity between the results of HTS and tissue isolation. Relative consistency was maintained in sequencing and isolation in *Curtobacterium*, *Sphingomonas*, *Burkholderia,* and *Rhizobium*. The surface sterilization methods showed little influence on the diversity of isolation methods, and lower concentrations of the agent were more suitable for isolating the scarce strains. Meanwhile, endophytic bacteria species preferred tissue types in this study. For the diversity, cultivable bacterial endophytes from stems included 30 genera and 20 genera from leaf samples covered. *Bosea*, *Herbaspirillum*, and *Sphingomonas* were isolated from both leaf samples and stem samples, and all *Burkholderia* strains and *Caballeronia* strains were isolated from stem samples.

DISCUSSION

During the long period of co-evolution, a mutually beneficial relationship was gradually established between endophytes and their host plants (Saikkonen et al., 2004; Wang and Dai, 2011;

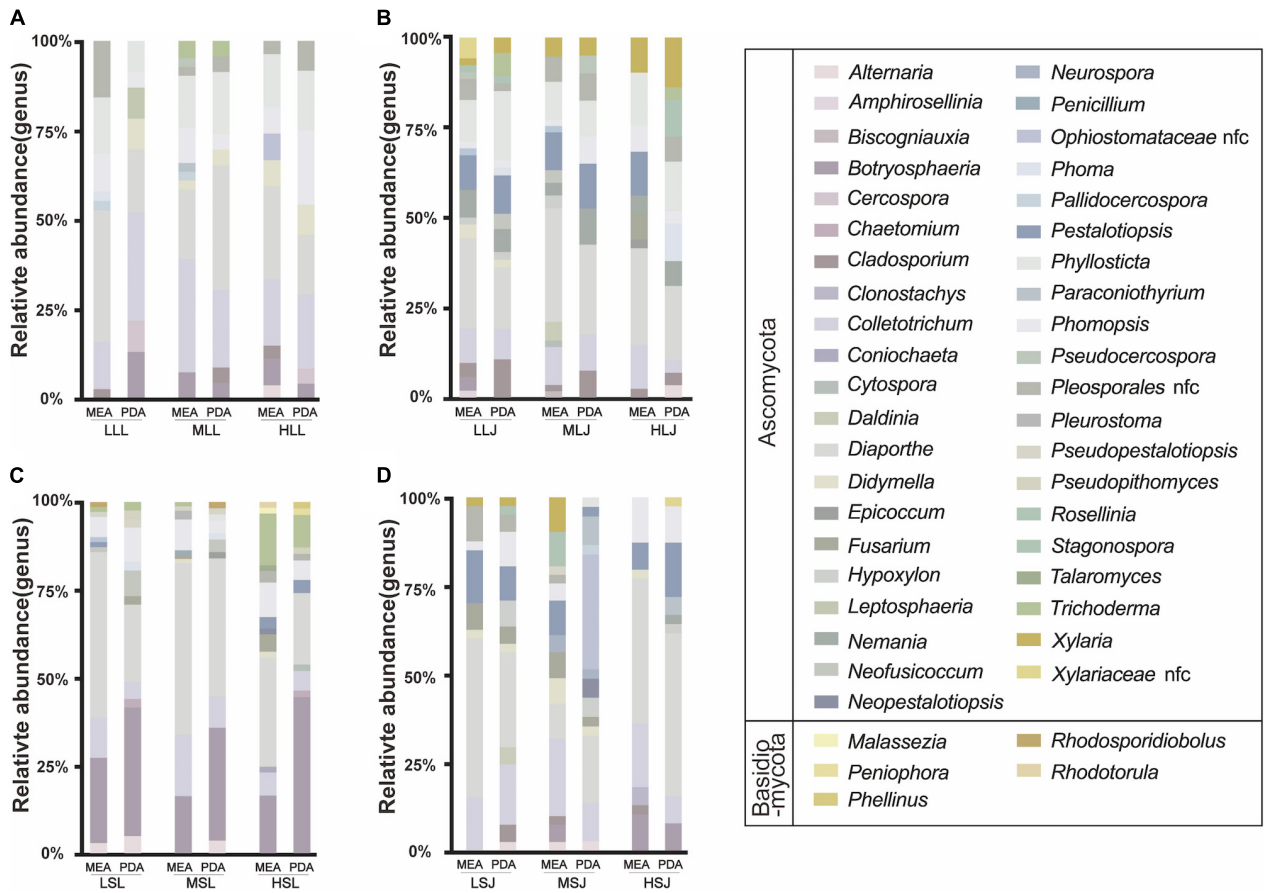

FIGURE 5 | Effects of sterilization treatments on the composition of cultured fungal endophytes from tea plant leaves **(A,B)** and stems **(C,D)** in MEA and PDA medium. LLL, MLL, and HLL represent the leaf samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% of NaClO. LLJ, MLJ, and HLJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% of NaClO. LSL, MSL, and HSL represent the stem samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% NaClO. LSJ, MSJ, and HSJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% NaClO.

Wani et al., 2015; Baltrus, 2017). The tea plant is one of the oldest domesticated plants in the world, and its endophytes play an important role in resisting biotic and abiotic stress (Rabha et al., 2014; Zhao et al., 2014), as well as participating in secondary metabolite production (Agusta et al., 2005). Surface sterilization is used to eliminate the non-endophytic microorganisms, which is essential for precisely exploring the endophytes (Shan et al., 2018; Jia et al., 2021). Here, we found that the diversity and composition of bacterial endophytes were influenced significantly by the NaClO concentration, while the diversity of fungal endophytes was relatively stable under higher concentrations of NaClO or a longer exposure time.

Consistent with the observations in previous studies (Fukuzaki, 2006; Hallmann et al., 2006; Gonzaga et al., 2015; Egamberdieva et al., 2017), NaClO showed similar dose-dependent and exposure time-dependent effects in our study. To figure out the detailed profiles underlying the effect of NaClO on endophytes in tea plants, the high-throughput sequencing was subsequently employed. Both diversity and composition of fungal endophytes were not significantly affected by the concentration of NaClO. More specifically, Ascomycota took up the most fungal endophytes while Proteobacteria, Bacteroidetes, and Actinobacteria had dominant proportions of bacterial endophytes. NaClO causes biosynthetic alterations in cellular metabolism and phospholipid destruction and hypochlorous acids (HClO-) present in the NaClO solutions may act as solvents in contact with organic tissue to release chlorine. Chlorine is a strong oxidant in solution that forms chloramines when combined with the protein amino group that then disrupt the cell metabolism and inhibit essential bacterial enzymes, leading to irreversible oxidation of SH groups (Estrela et al., 2002; Fukuzaki, 2006; Sahu et al., 2022). Bacteria may be more sensitive than fungi upon treatment with NaClO.

In addition to surface sterilization, the culture medium also has a vital effect on the isolation of endophytes. *Bacillales* of Bacilli was abundant in endophytes isolated and identified from tea plants (Xie et al., 2020), which was further confirmed by our results. Previously, the selective effect of mediums on the isolation of endophytic fungi from an Indian neem plant *Azadirachta indica A. Juss.* was investigated. This study found that not only the quantity, but also the variety and growth rate of endophytic fungi were affected by the culture medium, indicating

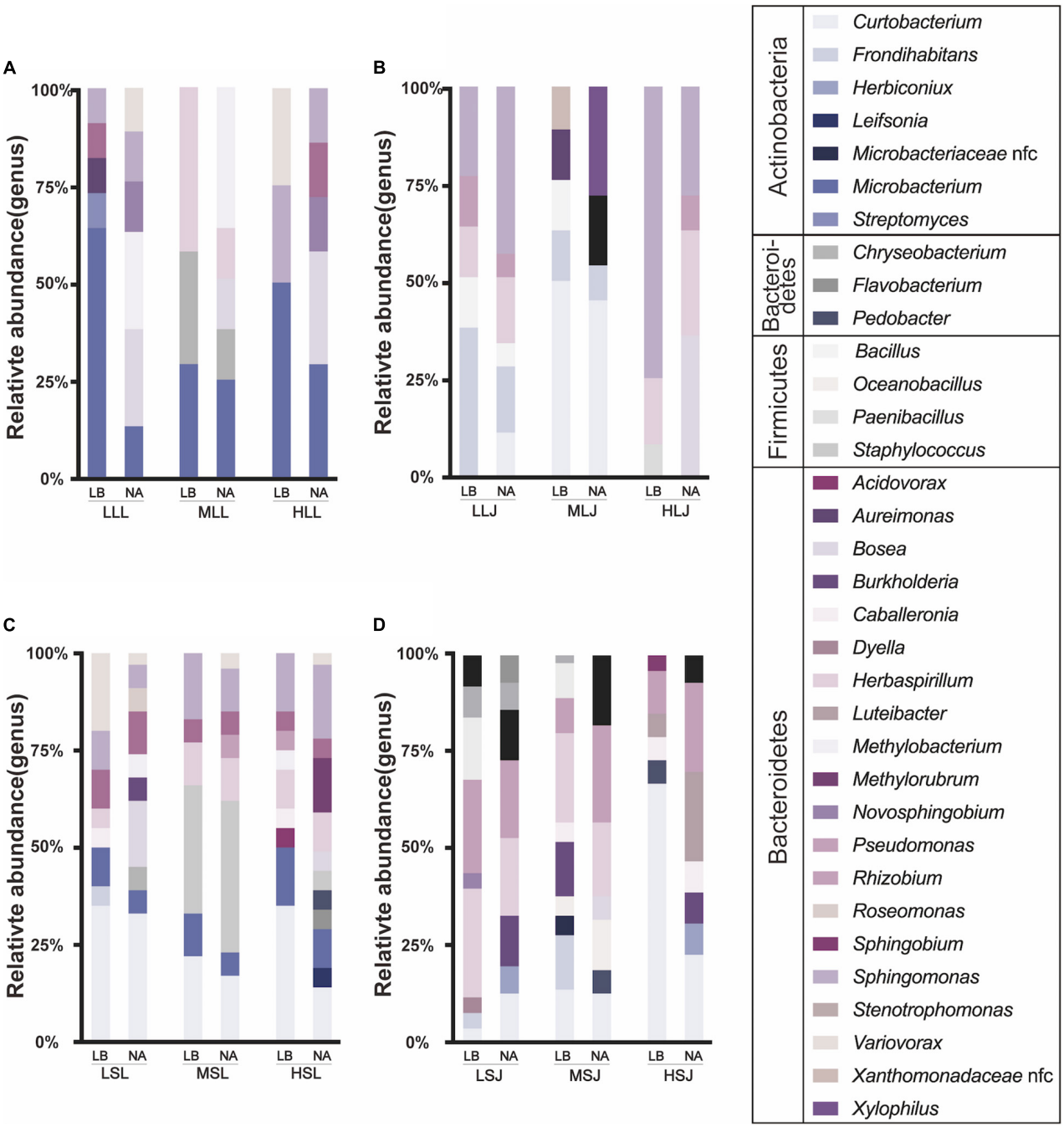

FIGURE 6 | Effects of sterilization treatments on the composition of cultured bacterial endophytes from tea plant leaves **(A,B)** and stems **(C,D)** in LB and NA medium. LLL, MLL, and HLL represent the leaf samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% of NaClO. LLJ, MLJ, and HLJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% of NaClO. LSL, MSL, and HSL represent the stem samples from the cultivar Longjing 43 treated with 0.5, 1.0, and 2.0% NaClO. LSJ, MSJ, and HSJ represent the leaf samples from the cultivar Jiukeng treated with 0.5, 1.0, and 2.0% NaClO.

media preference was one of the critical factors that can directly influence the endophytic isolation (Verma et al., 2011). Meanwhile, the flora preference of the cultivable endophytic bacteria influenced by the culture medium was also observed in *Dendrobium*, wheatgrass, and *Passiflora incarnata* (Ringelberg et al., 2012; Goulart et al., 2019; Wang et al., 2019). Furthermore, differences exist between endophytic bacteria in the results from HTS and those from culture-dependent methods, especially at the genus level (Wang et al., 2019). In our work, *Diaporthe* was found to the dominant genus in cultured endophytes, while *Mycosphaerella* and *Davidiella*, the most abundant in HTS results, were not cultured successfully. Similar results were also observed

in endophytic bacteria when comparing the results from culture-dependent methods and HTS.

Moreover, the potential functions of endophytes from tea plants were previously studied. For instance, *Alternaria alternata,* a species from the genus *Alternaria,* was found to produce bioactive metabolites that could inhibit the pathogenic microorganisms (Wang et al., 2014). *Diaporthe* sp. isolated from tea plants could stereoselectively oxidize the C-4 carbon of two R-substituted flavans to a 3-hydroxy structure from the same direction (Agusta et al., 2005). *Bacillus* and *Pseudomonas* had positive effects in growth promotion and disease prevention (Chakraborty et al., 2006; Morang and Dutta, 2012). However, in the present work, *Staphylococcus* and *Bacillus,* belonging to the order of Bacillales, were found to be susceptible to high concentrations of NaClO, indicating that NaClO sterilization increases the false-negatives in exploration of the functional endophytes in tea plants, and the effect of NaClO on microbiome research should be studied more in the future.

CONCLUSION

The diversity of bacterial endophytes in leaves and stems of tea plants was significantly affected by the concentration of NaClO as well as the sterilization time. In comparison, composition of fungal endophytes in tea tissues was less susceptible to NaClO. Thus, it is suggested that sterilization with NaClO should be modified to precisely understand the diversity of the bacterial endophytes from different tissues in tea plants.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Sequence Read Archive of NCBI repository, accession number PRJNA794727.

AUTHOR CONTRIBUTIONS

PX designed the study. YY and ZC collected the samples. YY, ZC, HX, and XF performed the laboratory experiments and analyzed the data. YY wrote the manuscript. PX and YW critically reviewed the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.849658/full#supplementary-material

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Genetic, Phenotypic and Metabolic Diversity of Yeasts From Wheat Flag Leaves

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The phyllosphere, the aboveground part of a plant, is a harsh environment with diverse abiotic and biotic stresses, including oscillating nutrient availability and temperature as well as exposure to UV radiation. Microbial colonization of this dynamic environment requires specific adaptive traits, including tolerance to fluctuating temperatures, the production of secondary metabolites and pigments to successfully compete with other microorganisms and to withstand abiotic stresses. Here, we isolated 175 yeasts, comprising 15 different genera, from the wheat flag leaf and characterized a selection of these for various adaptive traits such as substrate utilization, tolerance to different temperatures, biofilm formation, and antagonism toward the fungal leaf pathogen *Fusarium graminearum*. Collectively our results revealed that the wheat flag leaf is a rich resource of taxonomically and phenotypically diverse yeast genera that exhibit various traits that can contribute to survival in the harsh phyllosphere environment.

Keywords: yeast ecology, phyllosphere, (a)biotic stresses, antagonism, culturomics, functional characterization, biofilm formation, carbon utilization

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INTRODUCTION

The phyllosphere is a reservoir of yet unknown microorganisms with intriguing interactions. Microorganisms colonizing the aboveground plant surfaces and tissues, including floral and vegetative parts, are referred to as the phyllosphere microbiome. The phyllosphere is considered to be a harsh environment due to the microbial exposure to limited nutrient sources, UV radiation, temperature oscillations and toxic compounds (Vorholt, 2012). Phyllosphere microorganisms display a wide range of adaptations and antagonistic activities, which are gaining interest for sustaining plant health (Legein et al., 2020; Kavamura et al., 2021). Among the phyllosphere-inhabiting microorganisms, yeasts are found to be abundant as endo- or epiphytes, reaching on average 10^3–10^5 colony forming units (CFU) per gram of leaf (Glushakova and Chernov, 2007). The majority of studies on the phyllosphere microbiome, so far, focuses on bacteria and filamentous fungi, especially plant pathogens. Currently, little is known about the diversity and ecological roles of phyllosphere yeasts (Kavamura et al., 2021).

Previous culture-dependent and independent approaches have shown a predominance of the Basidiomycete yeasts *Sporobolomyces*, *Cryptococcus* (often reclassified as *Papiliotrema*) and

Pseudozyma (Nasanit et al., 2015a,c, 2016) in the phyllosphere of rice, corn, and sugarcane. Several biotic and abiotic factors impact the abundance and diversity of yeasts in the phyllosphere. These factors include plant genetics (e.g., plant species, genotype, and developmental stage) and environmental conditions such as UV radiation, moisture, geographic location and fungicides (Sapkota et al., 2015). For example, the abundance of Ascomycete yeasts, such as *Metschnikowia* and *Cryptococcus*, generally increased over time, particularly in nectar yielding flowers (Glushakova and Chernov, 2010). Additionally, an increase in nutrients found on damaged berries also increased the number of yeasts in the phyllosphere (Janakiev et al., 2019).

The phyllosphere is considered a resource-poor environment, thus microorganisms compete for nutrients and space. Nutrients leak from leaves or fruits into the environment, mainly consisting of carbohydrates such as fructose, glucose and sucrose, and also amino acids and methanol (Mercier and Lindow, 2000). Yeasts are known for their versatile substrate utilization (Żymańczyk-Duda et al., 2017), they can use different carbon sources (e.g., simple sugars, methanol, and methane) as well as amino acids and nitrogen sources (e.g., methylamine, ammonium salts and nitrate; Moliné et al., 2010; Chi et al., 2015; Shiraishi et al., 2015), allowing them to expand their ecological niche (Deak, 2006).

A key step to elucidate the ecological roles of phyllosphere yeasts lies in determining their ability to withstand harsh environmental conditions and their interactions with other phyllosphere members. A number of mechanisms have been proposed to facilitate the adaptations of yeasts to the phyllosphere environment. For example, the majority of phyllosphere yeasts are present in highly organized multicellular communities called biofilms (Váchová and Palková, 2018), which play a role in stress resilience. More specifically, biofilm formation has been implicated in resistance to fungicides and proposed as a physical barrier on plant surface injuries, preventing fungal hyphae from entering the plant tissue (Villa et al., 2017).

Currently, the majority of studies on environmental yeasts focusses on their biocontrol potential (Schisler et al., 2002a, 2015). Several studies have shown the ability of yeasts to inhibit plant pathogens and to protect against post-harvest diseases *via* the production of secondary metabolites, cell-wall-degrading enzymes, and so-called killer toxins (Freimoser et al., 2019). The mechanisms underlying these antagonistic activities have been identified only for a few species. For example, the production of pulcherriminic acid by *Metschnikowia pulcherrima* has been implicated in the growth inhibition of *Botrytis cinerea* (Sipiczki, 2020), whereas polymers (e.g., pullulan), volatiles (e.g., ethanol, phenylethanol and ethyl acetate) and secondary metabolites (e.g., aureobasidins) produced by *A. pullulans* have been shown to inhibit the growth of *Alternaria alternata* and *B. cinerea* (Contarino et al., 2019; Yalage Don et al., 2020; Di Francesco et al., 2021). For the majority of biocontrol yeasts, however, the mechanisms underlying the antagonistic activity are still unknown (Gore-Lloyd et al., 2019). Investigations of their lifestyles and adaptations to the phyllosphere environment will contribute to a better understanding of the interplay between yeasts and other phyllosphere members.

The present study aimed at investigating the taxonomic diversity of yeasts from wheat flag leaves, i.e., the last leaf before the ear emergence. The flag leaf can contribute up to 40% of the final photosynthetic capacity of wheat plants and therefore has a major impact on yield (Sylvester-Bradley et al., 1990). For a subset of taxonomically different flag leaf yeasts, we assessed their phenotypic and metabolic potential, including biofilm formation, substrate utilization spectrum, growth at different temperatures and antagonism toward the fungal pathogen *Fusarium graminearum*. Our results provide a first step toward characterizing the yeast diversity specifically found in wheat flag leaves and serve a foundation for further studies on the ecological roles of phyllosphere yeasts.

MATERIALS AND METHODS

Isolation of Yeasts From the Wheat Flag Leaf

Yeasts were isolated from the flag leaves of wheat (Triticum aestivum) cultivar Elixer. Samples were collected during the spring of 2020 at Taastrup, Denmark (55°38′46″N 12°17′53″E) on a fungicide untreated field plot. Plants were sampled at the flowering stage (growth stage 61–69 according to Zadoks code (Zadoks et al., 1974)). Three different methods were used to isolate both epiphytic and endophytic yeasts. For the first method a washing solution (0.5% Tween80 and 0.9% NaCl) was added to a 15 ml-tube with 33 flag leaves. Samples were vortexed for 1 min, sonicated for 2 min at 45 kHz, and vortexed again for 1 min. Leaves were transferred to a new tube and blended. The blended-washed leaves were stored in 15% (v/v) glycerol. In the second method, the washing solution was centrifuged for 5 min at 5.000 rpm, the supernatant was removed and the pellet was stored in 3 ml of 15% glycerol (v/v). The third method consisted of blending leaves directly (without the washing step) and storing in 15% glycerol (v/v). All samples were stored at −80°C.

For the selective isolation of yeasts, glycerol samples were 10-fold serial diluted (10^{-1}–10^{-7}) with 0.9% NaCl and plated on different media. Suspensions were plated on Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Agar (SDA), 869 media supplemented with wheat flag leaf extract (Eevers et al., 2015) and Yeast Extract Peptone Dextrose (YEPD), with and without 10% lactic acid to favor yeast growth. Bacterial growth was prevented by adding 50 µl/ml chloramphenicol and 50 µl/ml tetracycline. Plates were incubated for 5–7 days at 25°C. At least two of each yeast colony morphologies were picked and streaked on fresh plates (at least twice) to obtain pure cultures. Isolates were grown in YEPD broth for 2–3 days and stored in 15% glycerol (v/v). Isolates which did not grow well in liquid were streaked on plates and, after 3–5 days, cells were directly resuspended in glycerol. All samples were stored at −80°C for long-term storage.

Taxonomic Identification of Yeasts

All 175 yeast isolates were characterized by ITS rRNA gene sequencing. PCR amplifications were conducted using the

primers ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTT ATTGATATGC-3′), synthesized by Integrated DNA Technologies (IDT; White et al., 1990; Lane, 1991). Additionally, the selection of 51 isolates was also characterized by 28S rRNA gene sequencing (also known as D1/D2 sequencing) using the primers LR5 (5′-TCCTGAGG GAAACTTCG-3′) and LROR (5′-ACCCGCTG AACTTAAGC-3′). DNA template for colony PCR was obtained by disrupting the cells by heating in a microwave at 600 W for 60 s, followed by vortexing and again microwaving at 600 W for 60 s in sterile demineralized-water. Samples were centrifuged at 5.000 rpm for 5 min and an aliquot of 3 µl was used as DNA template. The reaction mixture contained Go Taq G2 Hot Start Green Master Mix 2×, 0.4 µm for both primers, DNA template and nuclease-free water. The PCR conditions in the Thermal cycler were: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min 30 s, with a final extension at 72°C for 5 min and stored at 12°C. The presence of the PCR products was verified on a 1% agarose gel in TBE buffer. PCR products were purified *via* ethanol precipitation or using a PCR purification kit (Zymo Research fungal/bacterial DNA Miniprep Kit). Samples were sequenced at BaseClear (Leiden, Netherlands) or Macrogen Inc. (Amsterdam, Netherlands) using the primer ITS1 and LR5. Taxonomic characterization was based on partial internal transcribed spacers (ITS) and the D1/D2 domains of the large subunit of 26S rRNA gene sequences using the NCBI BLAST database.[1] For the phylogenetic analysis, sequences were aligned using the MUSCLE algorithm in the MEGA software (version 7.0) and a phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model with a bootstrap of 1.000 replications (Kumar et al., 2016). The tree was visualized using iTOL (version 6). ITS and D1/D2 sequences are deposited at European Nucleotide Archive (ENA) under the project number PRJEB51687.

Culture Conditions

For all *in vitro* assays, the fungal isolate *F. graminearum* strain 8/1 (Miedaner et al., 2000) was grown on Potato Dextrose Agar (PDA, pH 7) plates for 5–7 days at 25°C. Yeast cultures were started from glycerol stocks. Isolates were plated on PDA plates and incubated at 25°C for 5–10 days. For all *in vitro* assays, a loop of the yeast cells was collected and inoculated in 0.9% NaCl. Cells were washed twice by centrifugation at 5.000 rpm for 5 min. An initial cell density (OD_{600nm}) of 0.1 was used for all experiments except the Biolog plate, where an OD_{600nm} of 0.01 was used. All assays were performed at 25°C, which was the temperature used for the isolation of yeasts, unless stated otherwise.

Metabolic Fingerprinting

The Biolog EcoPlate™ system (Biolog Inc., Hayward, CA, United States of America) were used to analyze differential utilization of carbon sources and thereby providing a metabolic fingerprint for each individual strain. Every plate contains 31 different carbon sources grouped into six categories (including amino acids, amines, carbohydrates, carboxylic acids miscellaneous, and polymer compounds) and a water control in three replicates. Yeast cells, described above, were adjusted to OD_{600nm} 0.01 and 100 µl per well was inoculated into the Biolog EcoPlate™. Plates were incubated at 25°C for 10 days at 180 rpm. Metabolism of specific substrates, and consequently growth of the cultures, resulted in a change in the tetrazolium dye. Cell density was measured after 2, 4, 7, and 10 days using a microtiter plate reader (OD_{590nm}), minus values were adjusted to zero. The average well color development (AWCD) was calculated by dividing the total of all values (excluding the water control) by 93. Additionally, the AWCD was calculated for each substrate group. MetaboAnalyst (Version 5.0) was used to visualize the 10 day-inoculation data. A heatmap was created following a Euclidean distance measure and a ward clustering method (Xia et al., 2009).

Growth at Different Temperatures

Yeast cells were collected as described above. The cell density was adjusted to OD_{600nm} 0.1 and 20 µl were added to 180 µl PDB pH 7 in a round-bottom microtiter 96-wells plate. Plates were sealed with plastic wrap and incubated at different temperatures, ranging from 4, 10, 25 to 37°C, at 200 rpm for 7 days. Cell density was measured after 2, 4, and 7 days using a microtiter plate reader (OD_{600nm}).

In vitro Biofilm Formation

Yeast cells were collected from plates and OD_{600nm} was adjusted to 0.1 as described above. A total of 20 µl of this suspension was added to 180 µl PDB in a flat-bottom microtiter plate. Plate was sealed and incubated statically in the dark for 3–4 days at 25°C. After incubation, cells were stained by adding 10 µl of 0.1% crystal violet to each well of the microtiter plate. Plate was incubated at room temperature for 15 min. Cells were washed three times with demineralized-water to remove platonic cells. After that, cells in biofilm were dissolved in 200 µl of 96% ethanol and incubated for 5 min. Cell density was measured using a microtiter plate reader at OD_{600nm}.

Antifungal Activity *via* Agar-Diffusible and Volatile Compounds

The effect of yeast diffusible compounds on the growth of the fungal pathogen *F. graminearum* was tested using *in vitro* dual culture assay. Yeast isolates and the fungal pathogen were previously grown as described above. An aliquot of 10 µl was streaked on one side of a 9-cm Petri dish (ϕ 9 cm) containing PDA at pH 7 (0.5 cm from the edge of the plate). Control treatments were inoculated with 10 µl 0.9% NaCl. A total of four replicates was prepared. Plates were incubated for 24 h at 25°C. After that, a fungal mycelial agar plug (ϕ 5 mm) was placed 24 h later at the opposite side of the Petri dish. Plates were incubated for 6 days at 25°C until the

[1]https://www.ncbi.nlm.nih.gov/

fungus on the control plates reached the edge of the plate. After 6 days, the inhibition zone was determined by measuring the area between the fungal hyphae and the yeast colony (area treatment) using ImageJ (FIJI). The inhibition percentage was calculated as

$$\left[\left(\left(\text{area control}\right)-\left(\text{area treatment}\right)\right)*100/\text{area control}\right].$$

The effect of volatile organic compounds (VOCs) on the growth of *F. graminearum* was investigated using two-compartment Petri dishes, which allowed the physical separation between the yeast and the fungal isolates. Yeast and fungal inocula were prepared as described above. An aliquot of 10 µl of the yeast isolates at OD_{600nm} 0.1 was inoculated on one of the compartments of the Petri dish containing PDA (pH 7) and spread evenly. Control treatments were inoculated with 10 µl 0.9% NaCl. A total of four replicates were prepared. Plates were incubated for 24 h at 25°C before inoculation of the fungal pathogen. A mycelium agar plug was placed (ϕ 5 mm) on the other compartment of the Petri dish. Plates were sealed three times with plastic wrap and incubated at 25°C for 4 days. Growth inhibition was calculated by measuring the mycelial growth (area treatment) after VOC exposure. The inhibition of the four replicates was calculated by

$$\left[\left(\left(\text{area control}\right)-\left(\text{area treatment}\right)\right)*100/\text{area control}\right].$$

Statistical significance was determined with one-way ANOVA, Tukey's HSD test ($p < 0.05$).

Scanning Electron Microscopic Analysis

Scanning electron microscopy (JEOL SEM 6400 equipped with Image Convert for windows) was used to visualize the influence of yeasts on the morphological changes in the fungal hyphae of *F. graminearum*. Samples from the dual culture confrontation assay (described above) were fixed with 1.5% glutaraldehyde in PBS for 1 h while shaking. Then, samples were dehydrated in an increasing percentage of acetone for 20 min each (70, 80, 90, 96, and 100% EtOH) and critical point dried (Baltec CPD-030). Afterward, the samples were sputter coated with platina and palladium to a 20-mm-thickness and stored in a vacuum until use.

RESULTS

Isolation and Phylogenetic Delineation of Phyllosphere Yeasts

A total of 175 yeasts were isolated from the surfaces and internal tissues of wheat flag leaves. ITS and D1/D2-amplicon sequencing revealed a total of 15 genera, representing 25 different species (**Figures 1**, **2**; **Supplementary Table S1**). Isolation on SDA and PDA media yielded the highest diversity of isolates, 10 and 9 out of 15 different genera, respectively, followed by YEPD (eight genera). The majority of the yeast isolates belonged to the phylum Basidiomycota (145 isolates; 82.9%), including the genera *Vishniacozyma* (46 isolates; 26.3%), *Sporobolomyces* (42 isolates; 24.0%) and *Papiliotrema* (19 isolates; 9.1%). Less frequent genera detected were *Pseudozyma*, *Anthracocystis*, *Dioszegia,* and *Rhodotorula*. Isolates belonging to the phylum Ascomycota (30 isolates; 17.1%) included *Aureobasidium* (28 isolates; 16.0%) and *Metschnikowia* (2 isolates; 1.1%). For further phenotypic and metabolic characterization, we selected 51 yeast isolates based on their phylogenetic delineation, with at least two isolates of the same genus (**Figures 1**, **2**; **Supplementary Figure 1**).

Metabolic Profiling of Phyllosphere Yeasts

To determine the metabolic diversity of the 51 selected phyllosphere yeasts, Biolog EcoPlate was used to screen for their ability to metabolize 31 nutrient sources of which at least 20 are of plant and/or microbial origin. Growth was measured spectrophotometrically, i.e., average well color development values (**Supplementary Table S2**). The results showed that isolates classified as *Vishniacozyma* (F300, F345), *Aureobasidium* (F359, F57) or *Papiliotrema* (F301) were able to utilize various carbon sources, while none of the *Sporobolomyces* and *Dioszegia* isolates grew on any of the substrates tested (**Figure 3A**). More specifically, *Vishniacozyma* isolate F345 had the broadest substrate utilization spectrum, followed by *Vishniacozyma* isolates F75, F300 and F66. Intriguingly, *F. graminearum* strain 8/1 was able to use 24 out of the 31 different carbon sources, indicating a broad substrate utilization spectrum for this prevalent fungal pathogen of wheat leaves. Among the different types of carbon sources tested, carbohydrates were the preferred substrates followed by polymers and carboxylic acid; none of the 51 yeast isolates were able to use amines under the tested conditions. The most frequently metabolized carbohydrates were D-Xylose, D-Mannitol and N-Acetyl-D-Glucosamine, the polymers were Tween 40 and 80, and the carboxylic acids were D-Galacturonic acid and D-Glucosaminic acid (**Figures 3B,C**).

Temperature Growth Range of Phyllosphere Yeasts

In the phyllosphere environment, yeasts are exposed to substantial temperature oscillations. To investigate the temperature range for growth, the selected 51 isolates were tested *in vitro* at 4, 10, 25 and 37°C. All yeast isolates were able to grow at 25°C, although the growth rate ranged considerably between the different isolates (**Supplementary Table S3**; **Supplementary Figure 2**). Most of the isolates (94%) grew well within 2 days of incubation at 25°C, whereas *Filobasidium* isolate F253 and *Aureobasidium* isolate F63 required up to 7 days of incubation at this temperature. When the temperature was reduced to 10°C, only 27 and 45 isolates were able to grow after 48 and 96 h, respectively. At 4°C, 13 isolates were able to grow after 4 days of incubation, and an additional 31 isolates showed growth with longer incubation. Isolates F225, F280 and F391 classified as *Cystobasidium* were unable to grow at 4°C, but grew at 10°C after 7 days. None of the 51 selected yeast isolates was able to grow at 37°C.

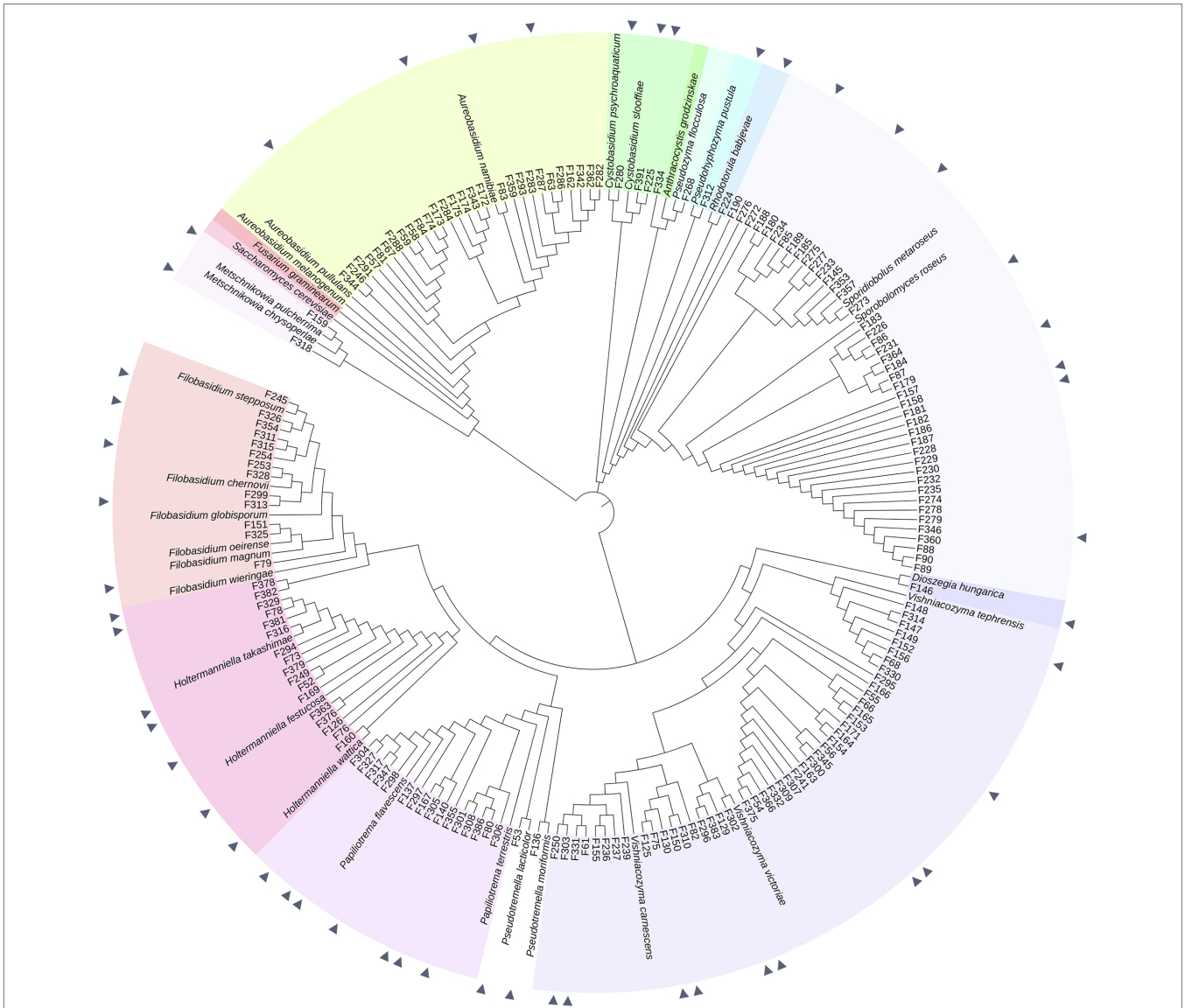


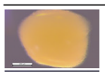
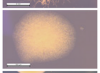
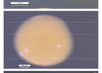
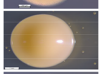
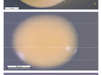
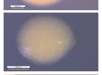
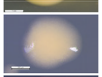
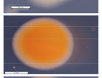
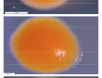
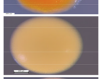
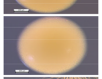
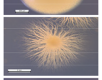
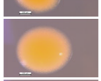
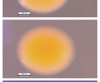
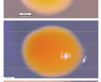
FIGURE 1 | Phylogeny of 175 phyllosphere yeasts isolated from the wheat flag leaf. Phylogeny is based on partial ITS sequences compared to NCBI database. Neighbor Joining tree was constructed with MEGA, MUSCLE for alignment. The iTol software was used to visualize and color-code the tree. Isolates indicated with a triangle are the 51 yeasts selected for the screening of different phenotypic and metabolic traits. *Fusarium graminearum* strain CBS 131786 was used as an outgroup. Type strains used are described in **Supplementary Table S1**.

Biofilm Formation by Phyllosphere Yeasts

Biofilms play a vital role in stress resilience and can protect yeasts against UV radiation and fungicides. Additionally, they can form a physical barrier on leaf surfaces, preventing penetration of the leaf surface by fungal pathogens. *In vitro* biofilm formation was detected by crystal violet staining of liquid cultures after 7 days of growth at 25°C with a threshold of OD_{600nm} at 0.05 to prevent any false positives due to background signal. A total of 19 yeast isolates (38%) were able to form a visible biofilm in at least 2 of the 3 screenings performed (**Supplementary Figure 3**). Biofilm formation was detected for all *Metschnikowia* (F159 and F318) and *Papiliotrema* (F297, F298, F301, F327, F347, F355, and F386) isolates, whereas no biofilm formation was detected for *Filobasidium, Dioszegia, Rhodotorula* and *Sporobolomyces* isolates under the tested conditions.

Interactions Between Phyllosphere Yeasts and *Fusarium graminearum*

To investigate the potential of phyllosphere yeasts to inhibit the growth of the leaf pathogen *F. graminearum*, different *in vitro* assays were performed. Dual-confrontation assays showed that several yeast isolates inhibited mycelial growth of the pathogen through the production of volatile and/or diffusible metabolites (**Figure 4**). Ten of the 51 isolates (19,6%)

Isolate	ID	ITS rRNA identity	D1/D2 rRNA identity
	F52	*Holtermanniella takashimae* (99.22%)	*Holtermanniella wattica* (98.45%)
	F53	*Pseudotremella lacticolor* (87.39%)	*Pseudotremella lacticolor* (94.11%)
	F57	*Aureobasidium namibiae* (97.39%)	*Papiliotrema terrestris* (84.39%)
	F63	*Aureobasidium melanogenum* (97.57%)	*Filobasidium oeirensis* (99.79%)
	F66	*Vishniacozyma carnescens* (96.69%)	*Vishniacozyma carnescens* (96.59%)
	F73	*Holtermanniella festucosa* (99.42%)	*Holtermanniella wattica* (96.68%)
	F75	*Vishniacozyma victoriae* (98.15%)	*Vishniacozyma carnescens* (97.81%)
	F79	*Filobasidium magnum* (99.10%)	*Filobasidium magnum* (99.33%)
	F86	*Sporobolomyces roseus* (98.35%)	*Sporidiobolus metaroseus* (99.89%)
	F129	*Vishniacozyma victoriae* (97.62%)	*Vishniacozyma carnescens* (98.57%)
	F130	*Vishniacozyma victoriae* (100.0%)	*Vishniacozyma carnescens* (98.77%)
	F136	*Pseudotremella moriformis* (86.53%)	*Pseudotremella moriformis* (96.84%)
	F145	*Sporobolomyces roseus* (99.10%)	*Dioszegia rishiriensis* (98.55%)
	F146	*Dioszegia hungarica* (99.77%)	*Diozegia rishiriensis* (95.96%)
	F159	*Metschnikowia pulcherrima* (93.00%)	*Metschnikowia chrysoperlae* (96.96%)
	F160	*Holtermanniella festucosa* (99.74%)	*Holtermanniella wattica* (94.27%)
	F174	*Aureobasidium namibiae* (98.49%)	*Aureobasidium pullulans* (89.64%)
	F179	*Sporobolomyces roseus* (98.37%)	*Sporidiobolus metaroseus* (99.66%)
	F181	*Sporobolomyces roseus* (99.63%)	*Sporidiobolus metaroseus* (99.66%)
	F182	*Sporobolomyces roseus* (99.63%)	*Sporidiobolus metaroseus* (96.47%)
	F185	*Sporobolomyces roseus* (98.24%)	*Sporidiobolus metaroseus* (98.43%)
	F188	*Sporobolomyces roseus* (99.10%)	*Dioszegia rishiriensis* (98.47%)
	F224	*Rhodotorula babjevae* (100.0%)	*Rhodotorula glutinis* (99.77%)
	F225	*Cystobasidium slooffiae* (97.43%)	*Cystobasidium slooffiae* (99.78%)
	F253	*Filobasidium chernovii* (98.82%)	*Filobasidium globisporum* (99.45%)
	F280	*Cystobasidium psychroaquaticum* (99.77%)	*Cystobasidium psychroaquaticum* (98.30%)

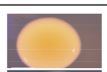
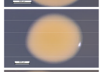
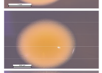
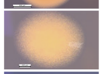
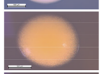
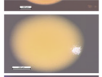
Isolate	ID	ITS rRNA identity	D1/D2 rRNA identity
	F294	*Holtermanniella festucosa* (99.41%)	*Holtermanniella takashimae* (95.33%)
	F297	*Papiliotrema flavescens* (99.14%)	*Papiliotrema terrestris* (96.81%)
	F298	*Papiliotrema flavescens* (98.53%)	*Papiliotrema terrestris* (97.15%)
	F300	*Vishniacozyma tephrensis* (93.15%)	*Vishniacozyma carnescens* (99.01%)
	F301	*Papiliotrema flavescens* (99.08%)	*Vishniacozyma carnescens* (98.00%)
	F303	*Vishniacozyma victoriae* (99.54%)	*Papiliotrema terrestris* (96.60%)
	F311	*Filobasidium chernovii* (99.29%)	*Filobasidium globisporum* (99.01%)
	F312	*Pseudohyphozyma pustula* (83.16%)	*Hamamotoa lignophila* (93.74%)
	F313	*Filobasidium chernovii* (98.84%)	*Filobasidium globisporum* (98.46%)
	F314	*Vishniacozyma tephrensis* (98.41%)	*Papiliotrema terrestris* (81.32%)
	F318	*Metschnikowia pulcherrima* (94.63%)	*Metschnikowia chrysoperlae* (96.04%)
	F326	*Filobasidium chernovii* (99.17%)	*Filobasidium globisporum* (98.31%)
	F327	*Papiliotrema flavescens* (98.31%)	*Papiliotrema terrestris* (98.17%)
	F331	*Vishniacozyma carnescens* (97.93%)	*Vishniacozyma carnescens* (97.51%)
	F345	*Vishniacozyma tephrensis* (93.95%)	*Vishniacozyma victoriae* (97.31%)
	F346	*Sporobolomyces roseus* (99.45%)	*Sporidiobolus metaroseus* (97.90%)
	F347	*Papiliotrema flavescens* (98.12%)	*Papiliotrema terrestris* (97.70%)
	F355	*Papiliotrema flavescens* (99.33%)	*Papiliotrema terrestris* (96.22%)
	F359	*Aureobasidium namibiae* (99.04%)	*Aureobasidium pullulans* (99.48%)
	F376	*Holtermanniella festucosa* (99.80%)	*Holtermanniella wattica* (96.73%)
	F378	*Filobasidium wieringae* (83.18%)	*Filobasidium magnum* (95.13%)
	F382	*Filobasidium wieringae* (83.58%)	*Filobasidium magnum* (95.13%)
	F383	*Vishniacozyma victoriae* (95.50%)	*Vishniacozyma carnescens* (95.91%)
	F386	*Papiliotrema flavescens* (99.33%)	*Papiliotrema terrestris* (98.23%)
	F391	*Cystobasidium slooffiae* (97.54%)	*Cystobasidium slooffiae* (91.68%)

FIGURE 2 | Phenotypic and taxonomic characterization of the selected yeast isolates. Pictures depict 5 to 10-day-old isolates grown on PDA at 25°C. Partial ITS and D1/D2 rRNA gene sequences were compared to the NCBI database for taxonomic identification.

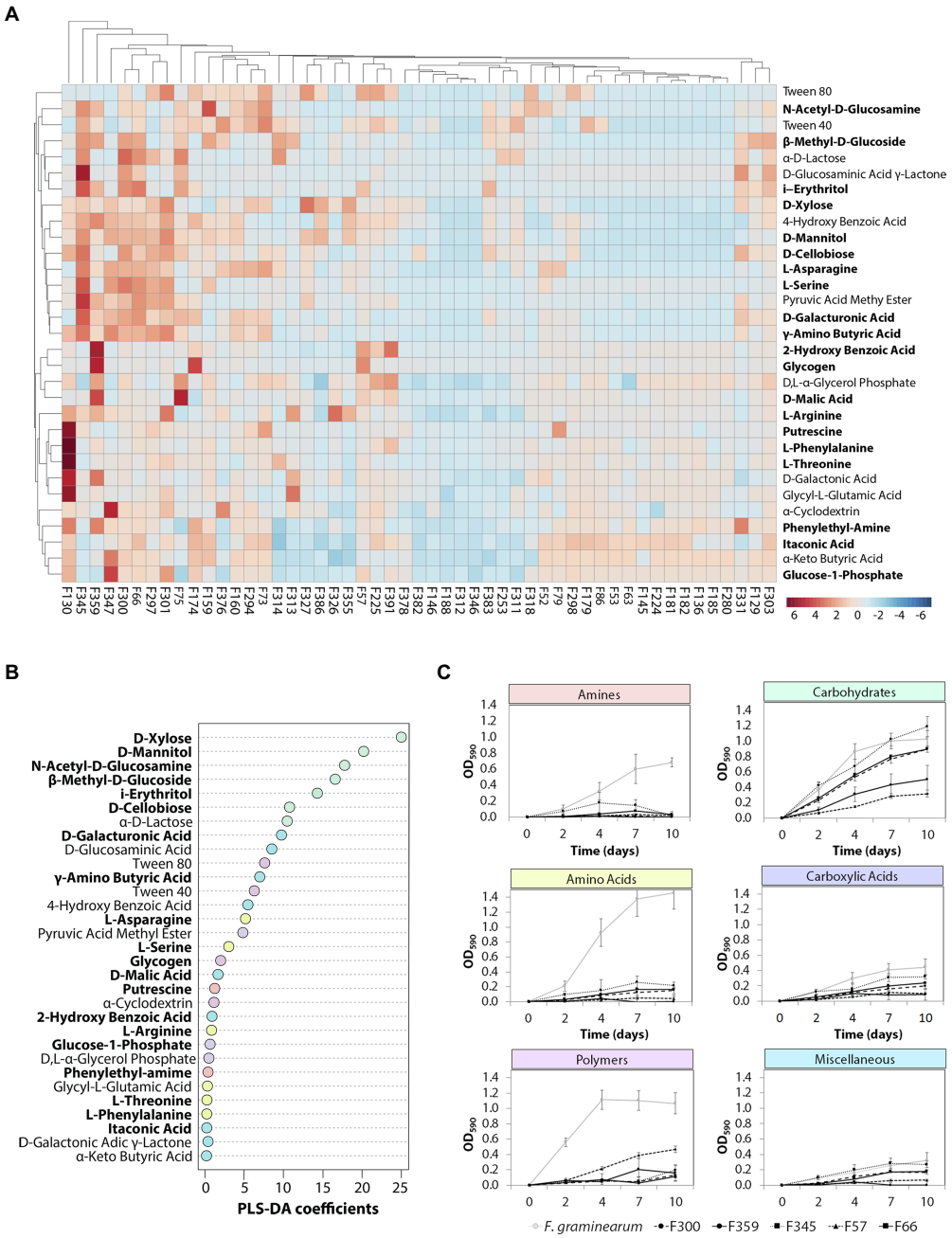


FIGURE 3 | Metabolic profiling of the phyllosphere yeasts. **(A)** Hierarchical cluster and heat-map analyses of nutrient sources utilized by the selected yeast isolates performed with MetaboAnalyst. Yeast isolates were inoculated (OD_{600} = 0.01) in the BIOLOG EcoPlates, incubated for 10 days at 25°C and 180 rpm, growth was measured with a plate reader at OD_{590nm}. Columns represent the average of three replicates of each of the 51 isolates. Rows represent the different carbon sources (blue: low abundance, red: high abundance). Compounds of plant and/or microbial origin are displayed in bold. **(B)** Partial least squares-discriminant analysis (PLS-DA) indicating the importance of each carbon source. **(C)** The dynamics of the top 5 isolates including *F. graminearum* with the most diverse metabolic profile per carbon type.

exhibited significant volatile-mediated antifungal activity. Two of these antagonistic yeast isolates (F318 and F159) that were classified as *Metschnikowia* inhibited the fungal growth by 66 and 56%, respectively. Also isolates classified as *Aureobasidium, Papiliotrema, Rhodotorula*, *Sporobolomyces* or *Vishniacozyma* were able to inhibit the growth of this fungal pathogen *via* volatiles. Two other *Papiliotrema* isolates and one *Metschnikowia* isolate also inhibited hyphal growth *via* agar-diffusible metabolites, by 55, 54 and 21%, respectively. Altogether, these results indicated that various, taxonomically

diverse phyllosphere yeasts can inhibit the growth of *F. graminearum via* volatile and diffusible metabolites.

To visualize the interaction between phyllosphere yeasts and *F. graminearum*, scanning electron microscopy showed that *Metschnikowia* isolate F318 and *Papiliotrema* isolate F386 both altered hyphal morphology compared to the control conditions, where smooth fungal hyphae were observed for *F. graminearum* (**Figure 5**). More specifically, direct confrontation between *Metschnikowia* and *F. graminearum* resulted in the production of an extracellular matrix (presumably extracellular polysaccharides (EPS)) on the hyphal surface as well as in between the hyphae (**Figure 5**). Confrontation with *Papiliotrema* resulted in shrunken and distorted hyphae (**Figure 5**).

DISCUSSION

Yeasts are well-known for their biotechnological and medical importance, with *Candida* and *Saccharomyces* spp. extensively explored. Plant-associated yeasts have been described for their biocontrol potential, especially in post-harvest disease management but knowledge on yeast ecology and the mechanisms underlying their interactions with other members of the phyllosphere microbiome are still limited. In this study, we examined the diversity of culturable yeasts colonizing wheat flag leaves, their adaptive traits and ability to inhibit the growth of the fungal pathogen *F. graminearum* (**Figure 6**).

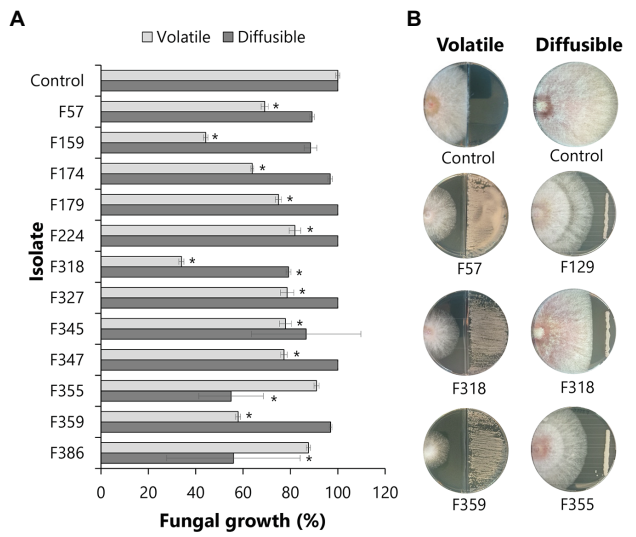


FIGURE 4 | Antagonistic activity of phyllosphere yeasts against *F. graminearum*. Each isolate was screened against *F. graminearum* for the production of volatile organic compounds (VOCs) or agar-diffusible compounds. **(A)** Bars represent the standard deviation of the mean growth percentage of 4 independent replicates. Statistical differences as compared to control (exposed to medium alone) were determined using Student's *t*-test ($p < 0.05$) and are indicated with an asterisk (*). **(B)** Pictures of the antagonistic activities by representative isolates made after 4 and 6 days of exposure for the volatile and agar-diffusible compounds, respectively.

The Wheat Flag Leaf Harbors a Taxonomically Diverse Reservoir of Yeasts

Previous studies have shown that yeasts colonize a wide range of natural environments, including soils, oceans, insects and plants (Boekhout et al., 2021). Plant-associated yeasts are receiving increased attention in the past two decades, in particular those colonizing fruits (Boekhout et al., 2021). In line with previous culture-dependent and independent studies of the wheat phyllosphere (Karlsson et al., 2014; Sapkota et al., 2015; Knorr et al., 2019), we found that Basidiomycetes were the most frequently isolated yeasts. Among these, yeasts belonging to the genera *Aureobasidium*, *Vishniacozyma* and *Sporobolomyces*, also found in phyllosphere of other crops, such as rice, sugarcane and corn (Nasanit et al., 2015b,c, 2016), were the most abundant. Additionally, we also detected *Pseudohyphozyma* and *Pseudotremella* species, which were described previously for litter and forest soil (Mašínová et al., 2017) but are rarely isolated from the phyllosphere (Elisashvili et al., 2009; Nguyen et al., 2021).

Here we characterized the yeast isolates based on the variable ITS region as well as the 28S domain (also known as D1/D2) for better taxonomic delineation. Yeast taxonomy has long been based on these phylogenetic markers, but the resolution

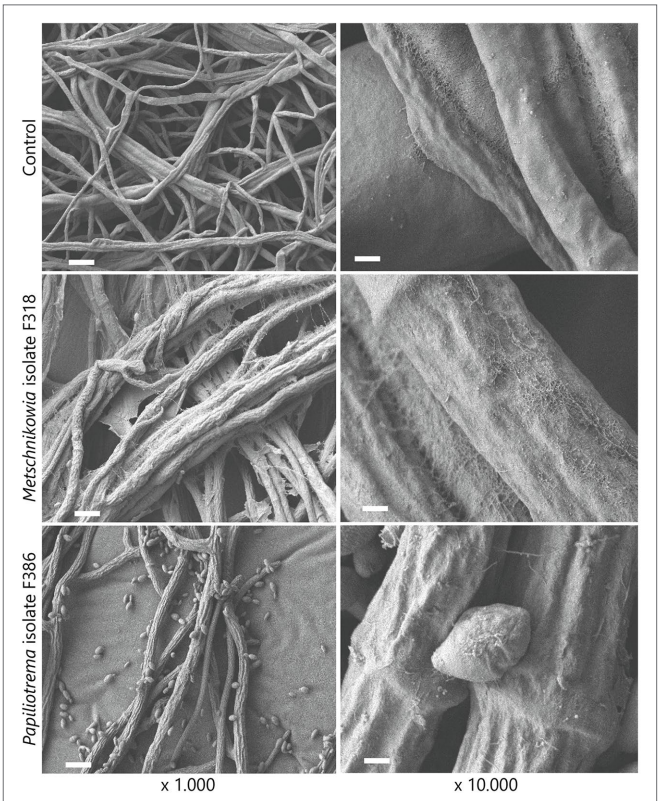


FIGURE 5 | Scanning electron microscopic (SEM) images of the interactions between phyllosphere yeasts and the fungal pathogen *F. graminearum*. Dual culture assays of *F. graminearum* growing alone (top panels) and exposed to *Metschnikowia* (middle panels) and *Papiliotrema* (bottom panels) isolates. Bars represent 10 μm and 1 μm for ×1.000 and ×10.000 magnification images, respectively.

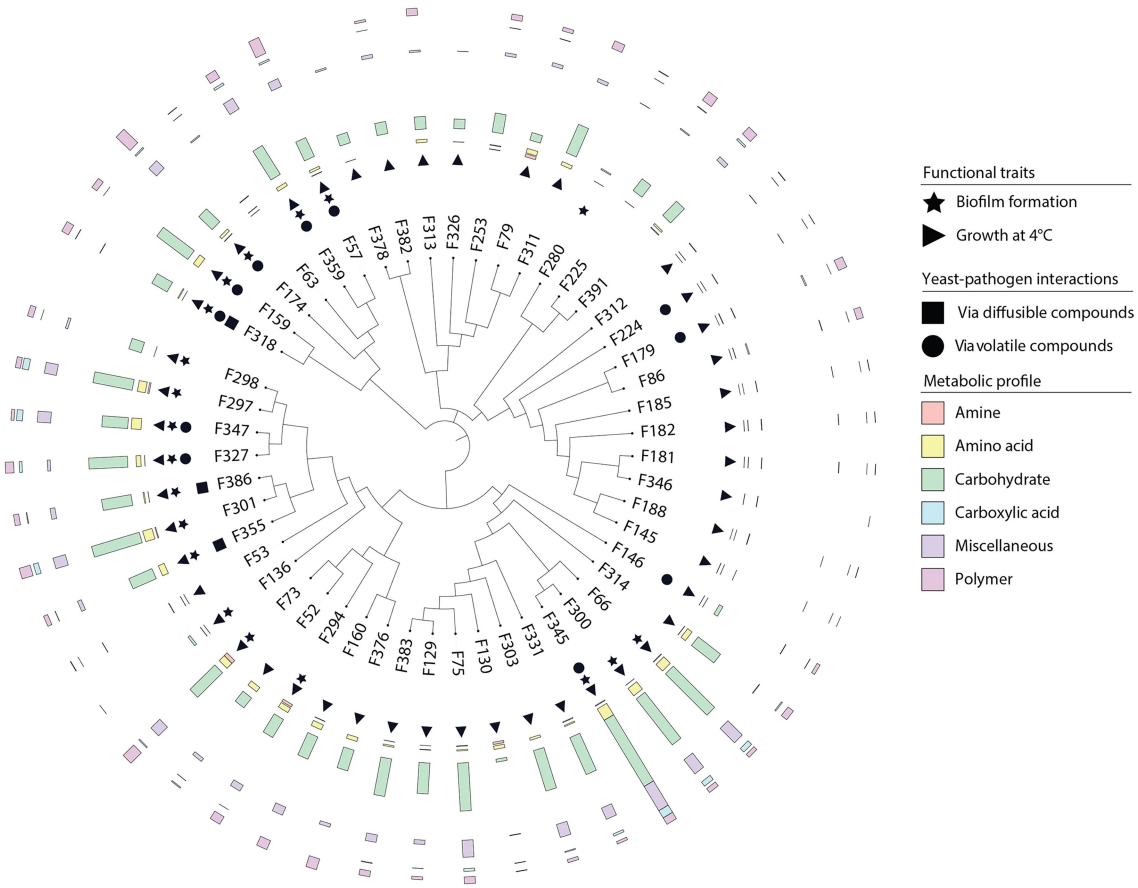


FIGURE 6 | Overview of the adaptive traits of yeasts inhabiting the wheat flag leaf. Neighbor-joining phylogenetic tree based on partial ITS sequences of yeast isolates. Isolates were screened for different traits including biofilm formation, growth at different temperatures, competition with the fungal pathogen *F. graminearum via* the production of agar-diffusible and volatile compounds, and substrate utilization.

is limited due to the highly conserved sequences in these regions, especially in basidiomycetous yeasts (Li et al., 2020). Therefore, a large number of yeast species are being reclassified (Yurkov et al., 2021). For example, *Cryptococcus* species are being reclassified as *Papiliotrema* and *Filobasidium*, whereas *Candida* species can be found in multiple teleomorphic taxa with different generic names. Hence, the yeasts described here will be subjected in the near future to whole genome sequencing for improved taxonomic delineation.

Adaptations of Yeasts to the Harsh Phyllosphere Environment

The phyllosphere is a heterogeneous environment where microorganisms are exposed to different (a)biotic stresses. Here we studied a number of traits that may contribute to the yeast's adaptation to the phyllosphere, including carbon utilization, growth at different temperatures, and biofilm formation. Nutrients, such as carbon compounds, are important for growth and the synthesis of secondary metabolites (Andrews, 1992). A diverse metabolic profile can offer an advantage to withstand the harsh conditions in the phyllosphere, allowing microorganisms to expand their ecological niche by making better use of the available carbon sources (Deak, 2006). Here we showed that the yeasts from the wheat flag leaf displayed diverse metabolic profiles with varying degradation rates. *Vishniacozyma* and *Aureobasidium* isolates, both highly abundant on the wheat flag leaf, displayed the most diverse substrate utilization profiles. Unexpectedly, *Sporobolomyces* isolates were unable to use any of the carbon sources tested under these conditions, despite their frequent and high abundances in wheat leaves found in the present and previous studies (Bashi and Fokkema, 1977; Sapkota et al., 2017). It is likely that *Sporobolomyces* species colonize the wheat flag leaves through the utilization of glucose, fructose or sucrose, sugars that are present in the phyllosphere and enable rapid colonization (Leveau and Lindow, 2000) and competition with leaf pathogens such as *Cochliobolus sativus*, partly by limiting glucose and amino acid availability. Also, *Metschnikowia* isolates have been shown to inhibit the post-harvest pathogen *Colletotrichum gloeosporioides* through competition for glucose and fructose (Tian et al., 2018; Sipiczki, 2020). To begin to understand ecological niche overlap or niche differentiation between the phyllosphere yeasts and the fungal pathogen *F. graminearum* strain 8/1, we also determined the metabolic

profile of this pathogen. We found that *F. graminearum* is not only able to utilize most of the nutrient sources tested, but also utilizes amino acids, amines and polymers that could not be utilized by the phyllosphere yeasts tested. This versatile carbon utilization profile likely contributes to the successful infection of a wide range of cereal crops by this pathogen.

In addition to oscillating nutrient availability, phyllosphere yeasts also need to adapt to oscillating temperatures, which can differ drastically between day and night, winter and spring. Yeasts from the genera *Sporobolomyces*, *Rhodotorula* and *Vishniacozyma* have been proposed to tolerate extreme environmental conditions and to help plants adapting to cold environments (Buzzini et al., 2018; Vujanovic, 2021). Our results showed that all isolates from these genera grew at different temperatures, including low temperatures. Also, biofilms play an important role in stress resilience and protection against harsh environmental conditions (Váchová and Palková, 2018), including temperature oscillations. Additionally, biofilms form a physical barrier on injuries of plant surfaces preventing the invasion by pathogenic fungi. We show that biofilm formation was wide-spread among the wheat phyllosphere yeasts, including all *Metschnikowia* and *Papiliotrema* isolates. Previous studies have proposed biofilm formation as one of the antagonistic mechanisms employed by biocontrol *Metschnikowia* isolates on grape wounds (Sipiczki, 2020). This mode of action is hypothesized to be based on growth inhibition of the pathogen by increasing colonization efficiency (e.g., competition for space). However, other biocontrol mechanisms, e.g., production of secondary metabolites, cannot be excluded to contribute to the observed antagonistic activity. Currently, data on biofilm formation by environmental yeasts is sparse (Cordero-Bueso et al., 2017) and our results suggest this may be a more common trait in the phyllosphere. Future investigations involving microscopy should address if these biofilms occur *in planta* and if they contribute to the phyllosphere competence of yeasts.

Interactions Between Yeasts and the Fungal Pathogen *Fusarium graminearum* in the Phyllosphere

We found that the production of secondary diffusible compounds with inhibitory activity against *F. graminearum* was not a wide-spread mechanism among the yeasts isolated from wheat flag leaves. Only two out of the 51 phyllosphere yeasts tested were able to significantly inhibit hyphal growth. However, it is still possible that these yeast isolates can inhibit the fungal pathogen by competing for nutrients and space, rather than *via* the production of secondary metabolites. Competition for nutrients and space have been proposed as the primary mechanism by which biocontrol yeasts inhibit pathogens (Freimoser et al., 2019). Isolates F355 and F386, which showed the strongest inhibitory effects against *F. graminearum*, are closely related to *Papiliotrema flavescens*, formerly known as *Cryptococcus flavescens/C. nodaensis*. Earlier studies on *P. flavescens* OH182.9, isolated from wheat heads, showed no antagonism in *in vitro* experiments, but this isolate was able to reduce disease incidence caused by *F. graminearum* by 56% in a bioassay (Khan et al., 2001). Field experiments where different winter wheat cultivars were inoculated with this strain also demonstrated reduced disease severity of *F. graminearum* by 60% (Schisler et al., 2002b; Khan et al., 2004).

Scanning electron microscopy further showed hyphal morphological changes on the hyphae of *F. graminearum* during the interaction with specific yeast isolates. The interaction with *P. flavescens* (isolate F386) not only reduced hyphal growth but also changed the hyphal morphology from smooth to shrunken and distorted. This morphological change has been previously observed for *F. graminearum* confronted by different *Paenibacillus peoriae* strains, bacteria with a high potential of biocontrol and plant growth promotion (Ali et al., 2021). Interestingly, the interaction of *F. graminearum* with *Metschnikowia* isolate F318, an isolate displaying little reduction of hyphal growth *in vitro*, led to considerable changes in hyphal morphology. These results suggest that *Metschnikowia* isolate F318 imposed stress to the fungal pathogen resulting in the production of extracellular polysaccharides (EPS). Biofilm formation by other *Fusarium* species, more specifically *F. oxysporum*, has been described to protect the fungal pathogen against several fungicides (Peiqian et al., 2014).

Contrary to diffusible secondary metabolites, volatile compounds with inhibitory effects on *F. gramineraum* were more frequently produced by the phyllosphere yeasts. These low molecular weight compounds serve as a means of interspecies communication from a distance and are proposed as an effective biological control strategy against multiple pathogens (Tilocca et al., 2020). The most promising volatile-producing antagonists belonged to *M. pulcherrima* (isolates F159 and F318), *Aureobasidium pullulans* (isolates F57, F174 and F359) and *Papiliotrema flavescens* (isolates F327 and F347), inhibiting hyphal growth by up to 65%. Volatile compounds produced by *Metschnikowia* and *Aureobasidium* species have been previously described for their biocontrol potential against several fungal pathogens, including *A. alternata* and *B. cinerea* (Oro et al., 2018). The volatile compounds ethanol, 2-phenylethanol and ethyl acetate have been proposed to be involved in this inhibitory activity (Oro et al., 2018; Contarino et al., 2019; Sipiczki, 2020; Yalage Don et al., 2020) and hypothesized to disrupt the fungal membranes leading to leakage and deformed hyphae (Yalage Don et al., 2020). To date, however, most of the knowledge on yeast volatiles stems from studies using yeasts of biotechnological importance, e.g., the production of volatiles by yeasts and their effects on wine aroma (Bordet et al., 2020). Further research efforts should be put on characterization of the volatile compounds produced by environmental yeasts and their ecological roles.

CONCLUDING REMARKS

The wheat flag leaf is a diverse reservoir of yeasts which can withstand the harsh conditions found in the phyllosphere through different mechanisms. Here we provide a first insight into the genetic and phenotypic diversity of yeasts living on and in the wheat flag leaf. Further research should focus on the molecular mechanisms underlying the interactions between yeasts, including the identification of genes and metabolites

involved in antifungal activity. To this end, current approaches and tools already in use for model yeasts can be employed for comparative genomics and functional analysis of the phyllosphere yeasts. Unraveling the mechanisms underlying the antagonistic activities would not only improve our understanding of the ecology of phyllosphere yeasts, but also contribute to the discovery of novel yeast-based biocontrol products.

DATA AVAILABILITY STATEMENT

Data was deposited at the ENA database under accession number PRJEB51687 (secondary accession ERP136341), available from June 19th, 2022.

AUTHOR CONTRIBUTIONS

LG and VC designed the study, processed and interpreted the data, and drafted the manuscript. LG, CV, and VC performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Community Structure of Phyllosphere Bacteria in Different Cultivars of Fingered Citron (*Citrus medica* 'Fingered') and Their Correlations With Fragrance

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In recent years, plant metabolomics and microbiome studies have suggested that the synthesis and secretion of plant secondary metabolites are affected by microbial-host symbiotic interactions. In this study, six varieties of fingered citron (*Citrus medica* 'Fingered') are sampled to study their phyllosphere bacterial communities and volatile organic compounds (VOCs). High-throughput sequencing is used to sequence the V5–V7 region of the 16S rRNA of the fingered citron phyllosphere bacteria, and the results showed that Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were the dominant bacterial phylum in the phyllosphere of fingered citron. There were significant differences in the phyllosphere bacteria community between XiuZhen and the remaining five varieties. The relative abundance of *Actinomycetospora* was highest in XiuZhen, and *Halomonas*, *Methylobacterium*, *Nocardioides*, and *Pseudokineococcus* were also dominant. Among the remaining varieties, *Halomonas* was the genus with the highest relative abundance, while the relative abundances of all the other genera were low. Headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) were used to analyze and identify the aroma compounds of six different fingered citron, and a total of 76 aroma compounds were detected in six varieties. Pinene, geraniol, and linalool were found to be the primary VOCs that affect the aroma of fingered citron based on relative odor activity value. The correlation analysis showed 55 positive and 60 negative correlations between the phyllosphere bacterial flora and aroma compounds of fingered citron. The top 10 genera in the relative abundance were all significantly associated with aroma compounds. This study provides deep insight into the relation between bacteria and VOCs of fingered citron, and this may better explain the complexity of the analysis of bacterial and metabolic interactions.

Keywords: phyllosphere bacteria, fingered citron, varieties, high-throughput sequencing, volatile organic compounds (VOCs)

INTRODUCTION

Plant volatile organic compounds (VOCs) are an important component of plant secondary metabolites (PSMs) and often influence plant aroma (Johnson et al., 2016; Tian et al., 2018). VOCs are one of the most important indicators used to determine fruit quality. Fingered citron (*Citrus medica* 'Fingered'), which belongs to Rutaceae, is a small evergreen tree. Its fruit is famous for its strong aroma and is widely used in the chemical, food, and medical industries as a raw material for extracting natural flavors. The differences in the fruit aroma of many plants, including fingered citron, are often thought to be related to variety, place of origin, and maturity (Nieuwenhuizen et al., 2015; Ji et al., 2019). Recent studies have shown that the fruit aroma is also related to the phyllosphere microbial community, such as *Methylobacterium*, *Bacillus*, and *Lactobacillus* (Nasopoulou et al., 2014; Gargallo-Garriga et al., 2016; Helletsgruber et al., 2017; Pang et al., 2021; Daniela et al., 2022).

The term "phyllosphere organisms" refers to organisms that are epiphytic or parasitic on the surface of plant leaves. Previous studies have shown that plants can synthesize or secrete various metabolites to affect their phyllosphere microbiome (Pang et al., 2021; Xu et al., 2021). There are significant differences in the concentration and composition of secondary metabolites, bacterial population size, and microbial community composition among different plants. Certain species of phyllosphere bacteria, such as *Methylobacterium extorquens* and the yeast *Candida boidinii*, can use VOCs such as methanol as growth substrates (Yurimoto and Sakai, 2009; Ochsner et al., 2015). This ability lends bacteria a selective advantage in the phyllosphere colonization of plants that emit large amounts of VOCs. Similarly, the terpenoids, phenylpropionic acids, and some aldehydes among the primary VOC components in plant leaves exhibit antibacterial activities that affect the colonization of microorganisms in the phyllosphere area (Devi et al., 2010; Gabriel and Zhang, 2020; Zhang et al., 2020). Burdon et al. (2018) found that in *Digitalis purpurea* pollinated by bees, volatile linalool in the nectar slowed the growth of bacteria in the phyllosphere environment.

Although many studies have provided insights into the community structure or dynamic basis of the phyllosphere microbiome, there are still many gaps in the study of the phyllosphere microbiome relative to the contribution of the rhizosphere microbiome to the secondary metabolites of the host plants. The study of the PSM–microbiome interaction has significance for crop cultivation, breeding, and other applied fields (Pang et al., 2021). In traditional crop breeding, breeders typically pay limited attention to the plant microbiome or PSM–microbiome interactions based on crop yield and other traits. In recent years, phyllosphere microorganisms have been found to regulate host stress resistance, and they are involved with VOC emissions (Matsumoto et al., 2021; Munir et al., 2022). Phyllosphere pathogenic microorganisms can alter plant VOC emissions by inducing plant defense responses or disrupting normal metabolism (Matsumoto et al., 2022). Toome et al. (2010) infected willow leaves with *Melampsora epitea* to study the influence of pathogens on the release pattern of VOCs from willow leaves. Compared with the control, the released amounts of sesquiterpenes and lipoxygenase products (LOX) in leaves infected with *Melampsora epitea* increased by 175 times and 10 times, respectively (Toome et al., 2010). However, the impact of commensal phyllosphere microbiota on the VOC emissions remains scarcely explored. Previously, the inhibition of their commensal phyllosphere microbiome while changing the composition and proportion of terpenes in the VOCs was found during the fumigation of *Sambucus nigra* with antibiotics that included the flowers and leaves (Peñuelas et al., 2014). This result suggests the potential link between the phyllosphere microbiome and VOCs in higher plants.

In this study, six different varieties of fingered citron are utilized to characterize their aroma compounds and phyllosphere bacteria communities. The aim of this study is to investigate the differences in the phyllosphere bacteria and VOCs of the different varieties of fingered citron and to explore the direct relationship between the phyllosphere bacteria and VOCs to provide a theoretical basis for cultivation and the breeding of fingered citron.

MATERIALS AND METHODS

Sample Source and Collection Methods

Samples were collected at The Fingered Citron Base in Qiaolifang Village, Chisong Town, Jindong District, Jinhua, China. The following varieties of *Citrus medica* 'Fingered' were used for experiments: YangGuang (YG), CuiZhi (CZ), QingYiTongZi (QYTZ), XiuZhen (XZ), DaYeQingYi (DY), and KaiXin (KX). The sampling method was as follows. Five fruit trees were randomly selected from each variety, and nine leaves and two fruits were selected from each tree. The leaves were used for the determination of the phyllosphere bacteria, with a total of 45 leaves sampled from each variety and eight pooled samples in total, considering all six varieties. The fruits were used for the determination of the aroma compounds, with 10 randomly selected fruits sampled for each variety and six pooled samples in total.

DNA Extraction

The total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions and stored at −20°C prior to further analysis. The quantity and quality of the extracted DNA were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and 1.0% agarose gel electrophoresis, respectively.

16S rRNA Gene Amplicon Sequencing

Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA genes in the V5–V7 region was performed using the forward primer 799F (5′-AACMGGATTAGATACCCKG-3′) and the reverse primer 1193R (5′-ACGTCATCCCCACCTTCC-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components

contained 5 μL of the reaction buffer (5×), 5 μL of the GC buffer (5×), 2 μL of dNTP (2.5 mM), 1 μL of each forward primer (10 μM) and reverse primer (10 μM), 2 μL of the DNA template, 0.25 μL of the Q5 DNA polymerase, and 8.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 2 min followed by 30 cycles consisting of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. The PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, the amplicons were pooled in equal amounts, and paired-end 2 × 250 bp sequencing was performed using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequence Analysis

Microbiome bioinformatics was performed with QIIME2 2019.4 (Bolyen et al., 2019) with slight modifications according to the official tutorials.[1] Briefly, raw sequence data were demultiplexed using the demux plugin, followed by primer cutting with the cutadapt plugin (Martin, 2011). Sequences were then quality filtered, denoised, and merged, and chimeras were removed using the DADA2 plugin (Callahan et al., 2015). Non-singleton amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) and used to construct a phylogenetic with fasttree2 (Price et al., 2009). Alpha-diversity metrics [Chao1 (Chao, 1984), observed species, Shannon (Shannon, 1948), Simpson (Simpson, 1949)], and the beta diversity metrics [Bray–Curtis dissimilarity (Bray and Curtis, 1957)] were estimated using the diversity plugin with samples rarefied to 345 sequences per sample. The taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin (Bokulich et al., 2018) against the SILVA database (release 132).

Bioinformatics and Statistical Analyses

The sequence data analyses were primarily performed using the QIIME2 and R packages (v3.2.0). The ASV-level alpha diversity indices, such as the Chao1 richness estimator, the Shannon diversity index, and the Simpson index, were calculated using the ASV table in QIIME2 and visualized as box plots. The beta diversity analysis was performed to investigate the structural variation in the microbial communities across samples using the Bray-Curtis metrics and nonmetric multidimensional scaling (NMDS) hierarchical clustering (Ramette, 2007). The taxonomic compositions and abundances were visualized using MEGAN (Huson et al., 2011) and GraPhlAn (Asnicar et al., 2015). The linear discriminant analysis effect size (LEfSe) was performed to detect differentially abundant taxa across groups using the default parameters (Segata et al., 2011).

[1]https://docs.qiime2.org/2019.4/tutorials/

Analysis of the Aroma Compounds in Fingered Citron

Hs-spme-gc-ms was used to detect and analyze the VOC_S in six varieties of fingered citron fruits, and the relative odor activity value (ROAV) analysis was used to determine the key aromatic compounds affecting the aroma. An HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The injection temperature was 250°C, the carrier gas was helium gas, the flow rate was 1.0 mL/min, and the solvent delay was 5 min. Heating process: The initial temperature was kept at 40°C for 3 min, the temperature was increased from 3°C/min to 100°C, and the temperature was then increased to 230°C at 5°C/min for 20 min. For the electron ionization source, the electron energy was 70 eV, the ion source temperature was 230°C, the interface temperature was 250°C, the full scanning mode was used, and the mass scanning range was 20–3350 m/z.

Qualitative and Quantitative Analysis of the Aroma Components

Qualitative: The volatile compounds in the fingered citron were identified using gas chromatography-mass spectrometry through the retention index (RI) and comparison with the NIST, and volatile compounds with positive and negative matching degrees greater than 800 were selected (Lee et al., 2018). The retention index was calculated using Equation 1:

$$\text{Retention index} = 100 \times n + 100\,(t_a - t_n) / (t_n + 1 - t_n) \quad (1)$$

where t_a is the retention time of the chromatographic peak a; and t_n and t_{n+1} are the retention time of C_n and C_{n+1}, respectively.

Quantification: The relative contents of the volatile compounds in fingered citron were calculated according to the peak area normalization method:

$$\text{Relative content } (‰) = M/N \times 1000 \quad (2)$$

where M is the peak area of the aroma substances of a single component; and N is the total peak area (Shui et al., 2019).

Determination of Main Flavor Substances of Finger Citron by ROAV Method

The contribution of volatile flavor substances in fingered citron was evaluated by referring to the ROAV method, and then the primary flavor substances were determined (Gao et al., 2014). The components that contributed the most to the total flavor of fingered citron samples were determined as follows:

$ROAV_{max}$ = 100, then other components (a):

$$ROAV_a \approx 100 \times C\%_a / C\%_{max} \times T_{max} / T_a \quad (3)$$

where $C\%_a$ is the relative content of the volatile components (%); T_a is the sensory threshold of the volatile components (μg/kg); $C\%_{max}$ and T_{max} are the relative content (%) and sensory threshold (μg/kg), respectively, of the volatile components with the largest contribution to the overall aroma of the sample.

RESULTS AND ANALYSIS

Diversity of the Phyllosphere Bacterial Community of Fingered Citron

According to the high-throughput sequencing of the 16S rDNA amplicon, this study conducted a diversity analysis of the phyllosphere microbial communities of different fingered citron cultivars (**Figure 1**). Only seven samples of DY were used for all analyses due to the poor DY-8 sequencing results. The α diversity Chao1, Shannon, and Simpson indices were utilized to measure the species richness and diversity in the group. Chao1 is the index of species richness, while the Shannon and Simpson indices are used to reflect the species diversity in samples (Jost, 2010). The Chao1 index showed that XZ had the highest microbial richness, and QYTZ had the lowest. The microbial richness of XZ was significantly different from those of QYTZ and CZ. The microbial richness of YG was significantly higher than that of QYTZ. There were no significant differences in the microbial richness among the other groups (**Figure 1A**). Both the Shannon and Simpson indices showed that XZ had the highest microbial diversity, while CZ had the lowest microbial diversity. The microbial diversity of XZ was significantly different from those of YG, CZ, KX, and QYTZ. The microbial diversity of DY was significantly higher than that of CZ. There was no significant difference in microbial diversity among other cultivars (**Figures 1B,C**).

The β diversity was used to compare the microbial community differences between varieties (Schloss et al., 2009). The NMDS analysis based on the Bray-Curtis distance was performed on the samples of different varieties of fingered citron. The results are shown in **Figure 1D**. The biological repeats of the same variety clustered together, indicating good repeatability. The difference between XZ and the other cultivars indicated that the microbial composition of XZ was significantly different from those of the other cultivars. QYTZ, DY, YG, CZ, and KX were clustered together, indicating similar microbial compositions among the five cultivars.

The Phyllosphere Bacterial Community Structure of Fingered Citron

The sequencing data showed that a total of 8,064,211 high-quality reads were obtained from six varieties of fingered citron. After splicing and quality control, a total of 2,760 ASVs were obtained. According to the ASV species annotation, there were 202 genera in 18 phyla. The top 10 phyla and genera with the highest relative abundances were selected for analysis. The top 10 phyla were as follows: Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Deinococcus-Thermus, Chloroflexi, Deferribacteres, Cyanobacteria, Synergistetes, and Spirochaetes (**Figure 2A**). Proteobacteria, Actinomycetes, and Firmicutes were present in all the samples with high relative abundances. Actinobacteria, Firmicutes, and Bacteroidetes were present in all six varieties of fingered citron. Proteobacteria reached a relative abundance of greater than 68% in all the varieties except XZ, and even 89.57% in CZ, while only 32.42% in XZ. Actinobacteria reached a relative abundance of 52.78% in XZ and less than 10% in the rest of the species. Firmicutes had the highest relative abundance in DY (10.05%), while it was only 0.88% in XZ. The relative abundances of Bacteroidetes in DY, KX, and QTYZ were 5.70, 2.02, and 1.79%, respectively, and less than 0.4% in the other three species. The relative abundances of the remaining phyla were low among the species.

At the genus level, *Halomonas* was present in all the samples and had a high relative abundance (**Figure 2B**). However, the relative abundance of *Halomonas* in XZ was significantly lower than that of the other varieties. The relative abundance of *Actinomycetospora* in XZ reached 25.28%, while in all the other species the relative abundance was less than 5%. The relative abundance of *Methylobacterium* in in QYTZ and CZ was 0.00%, and in XZ, KX, DY, and YG were 7.54, 4.36, 4.32, and 3.42%, respectively. The relative abundances of *Nocardioides* and *Pseudokineococcus* in XZ were significantly higher than those of the other species. The relative abundances of *Bacillus*, *Nesterenkonia*, and *Aliihoeflea* among all the samples were low, and the differences were not significant.

Phyllosphere Bacterial Biomarkers in the Different Varieties

The LEfSe analysis was used to analyze the enriched characteristic groups in the different varieties of fingered citron, and the LDA threshold was set to 2. The results are shown in **Figure 3**. XZ had the most biomarkers, involving 2 phyla, 3 classes, 9 orders, 10 families, and 16 genera, namely, *Actinomycetospora*, *Nocardiodies*, *Methylobacterium*, *Pseudokineococcus*, *Marmoriacola*, *Blastococcus*, *Agrococcus*, *Paracoccus*, *Quadrisphaera*, *Sphingomonas*, *Pseudonocardia*, *Phycicoccus*, *Belnapia*, *Truepera*, *Aureimonas*, and *Rhodococcus.* There were 15 biomarkers in CZ, involving two phyla, two classes, two orders, four families, and five genera. At the genus level, the relative abundances of *Halomonas*, *Nocardioides*, *Planifilum*, *KD4-96*, and *Aliihoeflea* in CZ were significantly higher than in the other varieties, and these high abundances were biomarkers of CZ. The biomarkers in DY involved two phyla, two classes, three orders, two families, and three genera. The genera included *Muribaculaceae*, *Lachnospiraceae_NK4A136_group*, and *Amnibacterium*. The biomarkers of QYTZ involved one family and one genus (Dysgonomonadaceae and *Proteiniphilum*, respectively). The markers of YG involved one order, one family, and one genus. The relative abundance of *Kineococcus* in YG was significantly higher than that in the other varieties. KX had no biomarker. All biomarkers were distributed in six phyla, except *Chloroflexi*, which was in the top five relative abundances of each variety.

Structure and Diversity of Volatile and Aromatic Components of Fingered Citron

The names and relative contents of the volatile flavor compounds in the different fingered citron varieties are displayed in **Supplementary Table 1**. A total of 76 types of volatile flavor compounds were detected in six varieties. The quantity and relative contents of the volatile flavor compounds in the different fingered citron varieties differed to some extent. As shown in **Figure 4**, the specific volatile substances in

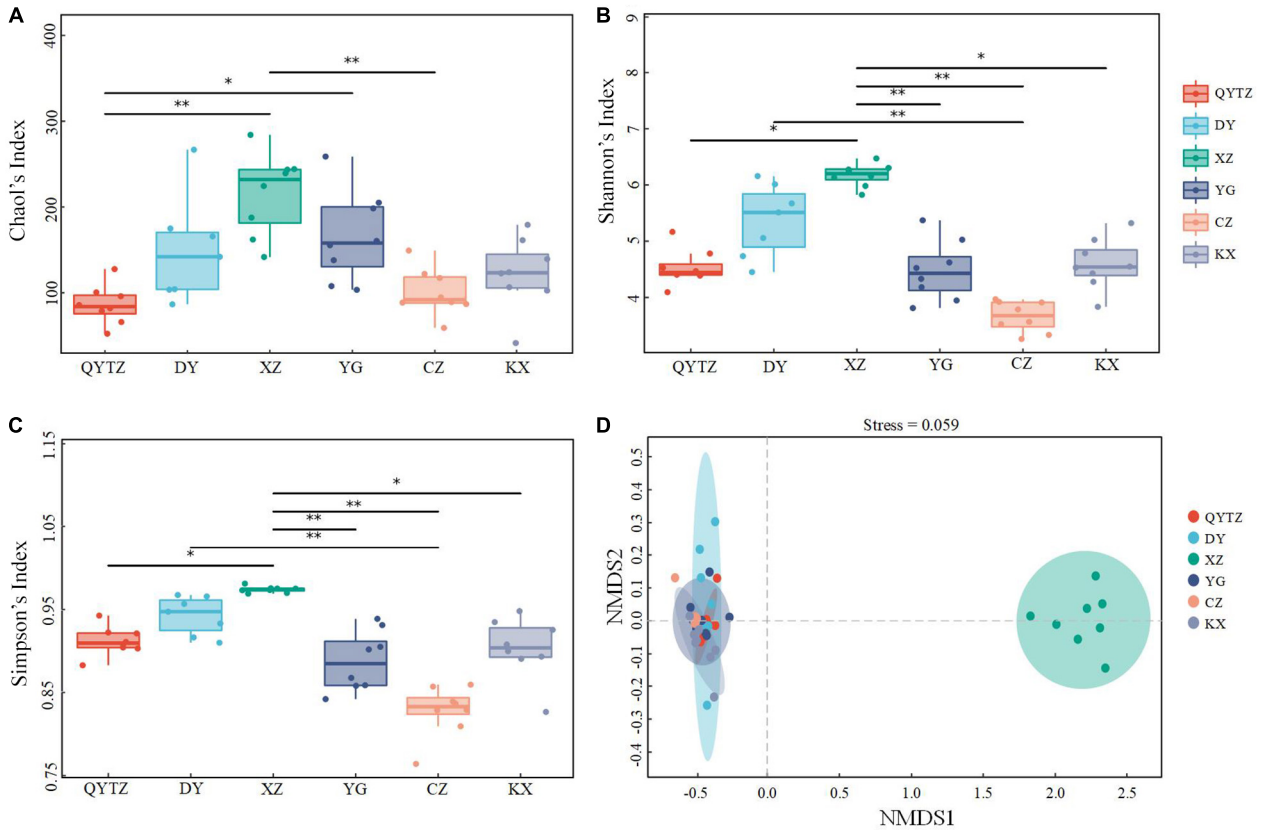

FIGURE 1 | Microbial diversity of the phyllosphere bacteria of the different varieties. **(A)** Chao1 index; **(B)** Shannon index; **(C)** Simpson index; **(D)** NMDS analysis. *$P < 0.05$; **$P < 0.01$. QYTZ, QingYiTongZi; DY, DaYeQingYi; XZ, XiuZhen; YG, YangGuang; CZ, CuiZhi; KX, KaiXin.

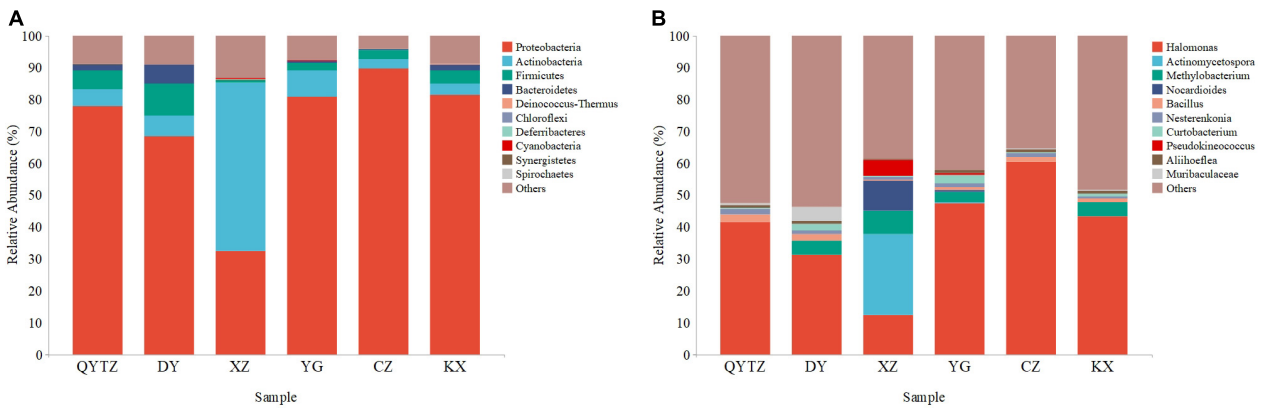

FIGURE 2 | The relative abundances of the phyllosphere bacteria of the different varieties: **(A)** Relative abundances at the phylum level; **(B)** relative abundances at the genus level. QYTZ, QingYiTongZi; DY, DaYeQingYi; XZ, XiuZhen; YG, YangGuang; CZ, CuiZhi; KX, KaiXin.

the variety DY included dodecane, hexamethyl-cyclotrisiloxane, benzenemethanol, α,α,4- trimethyl-, α-terpinene, elixene, and (+)-valencene. The characteristic volatile compounds of QY were 2-carene, cyclopentadecane, and heptadecane. The unique volatiles of CZ included 2-bornene. The KX characteristic volatile substances were alloocimene, 1,2,3,4-tetramethyl-benzene, and a-gurjunene. The unique volatile substances in XZ were 2,4(8)-p-menthadiene and naphthalene. The unique volatile substances in YG were γ-elemene and camphene. According to the cluster analysis of the volatile compounds in the heat map, the volatile components of CZ and XZ were the most similar and could be roughly divided into one category, while some components

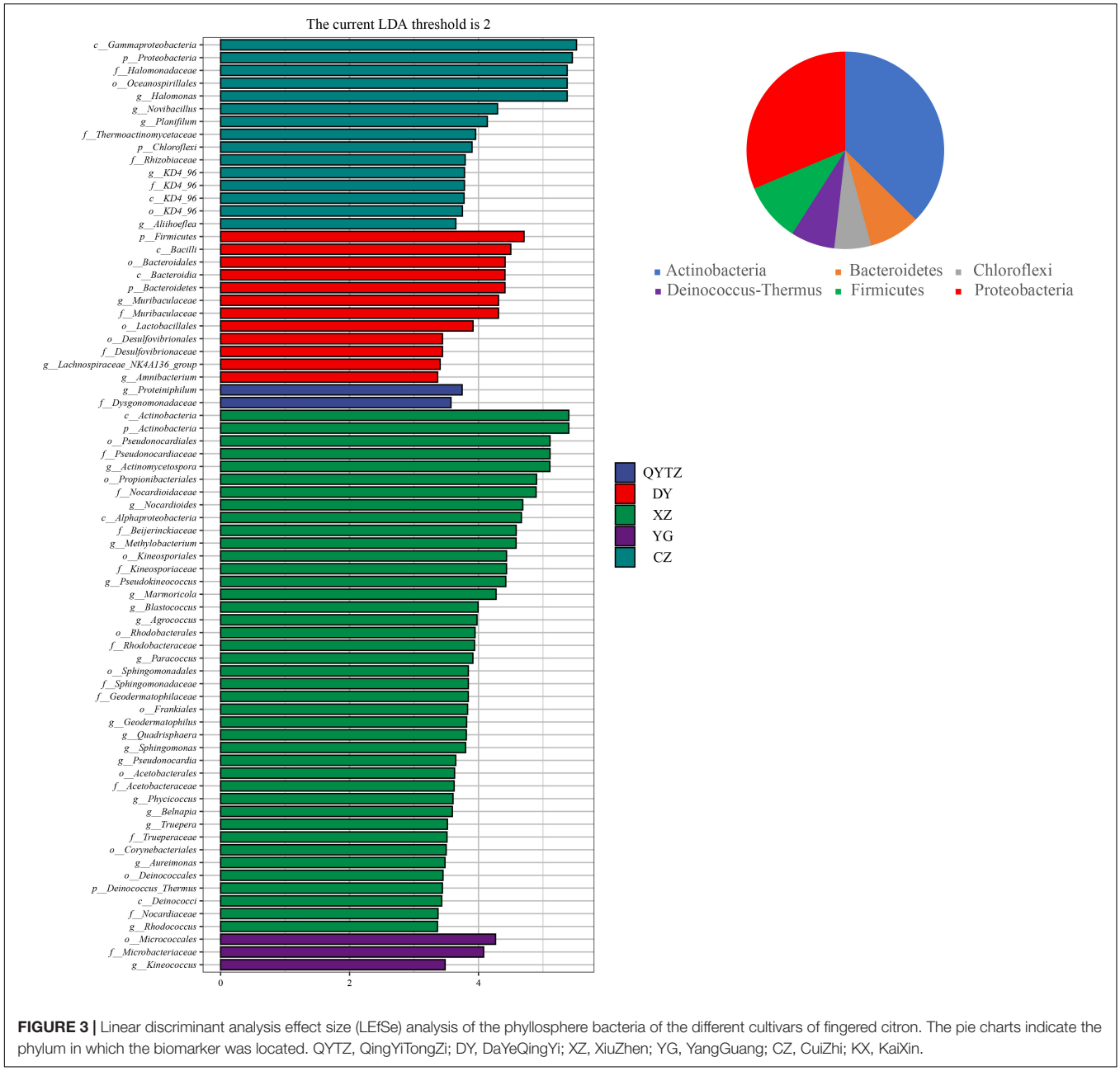

FIGURE 3 | Linear discriminant analysis effect size (LEfSe) analysis of the phyllosphere bacteria of the different cultivars of fingered citron. The pie charts indicate the phylum in which the biomarker was located. QYTZ, QingYiTongZi; DY, DaYeQingYi; XZ, XiuZhen; YG, YangGuang; CZ, CuiZhi; KX, KaiXin.

exhibited content differences. The volatiles of the other four varieties could be roughly divided into one category, and the volatile components of DY were the most different from those of the other five varieties.

A variety of volatile flavor compounds were detected in the different fingered citron cultivars, but only a subset of them contributed to the overall fruit aroma. The remaining ones only played a modifiable and synergistic role in the presentation of the overall flavor of fingered citron. The contributions of VOCs to the characteristic aroma of fingered citron were determined by their relative contents and the aroma threshold, which is the minimum smellable value of odor. Hence, the relative content of VOCs did not explain its contribution to the flavor of fingered citron. Therefore, the ROAV was analyzed based on the threshold values of each VOC. Substances with ROAV $\geq$ 1 contributed significantly to the aroma of samples and were considered to be key aroma compounds. The larger the ROAV value was, the greater the contribution to the total flavor of the sample was.

There were 22 key aroma compounds, that contributed significantly to the aroma of fingered citron, varying considerably between varieties (**Table 1**). DY had five key aroma compounds, with pinene having a greater impact on its overall flavor. QYTZ had six species, with pinene and geraniol having a greater impact on its overall flavor. CZ had four species, with pinene and linalool having a greater impact on its overall flavor. KX had three species, with linalool having a greater impact on its overall flavor. XZ had

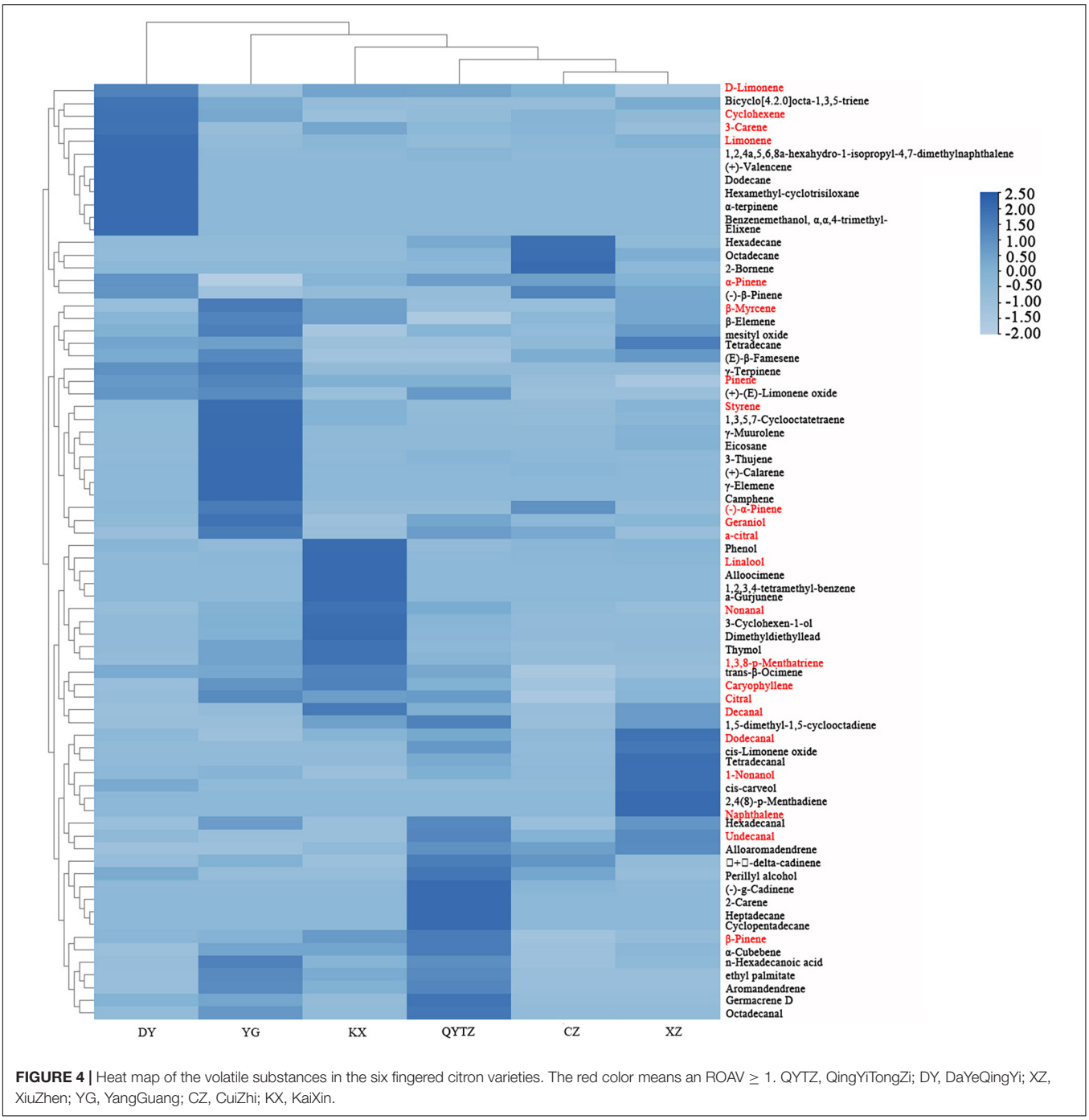

FIGURE 4 | Heat map of the volatile substances in the six fingered citron varieties. The red color means an ROAV ≥ 1. QYTZ, QingYiTongZi; DY, DaYeQingYi; XZ, XiuZhen; YG, YangGuang; CZ, CuiZhi; KX, KaiXin.

five types, with linalool and geraniol having a greater impact on its overall flavor. YG had 14 species, with pinene and linalool having a greater impact on its overall flavor.

Correlation Between Fruit Volatiles and Bacterial Microbiota

To explore the correlation between microbial components and volatile substances, a Spearman correlation analysis was conducted between the microorganisms with the top 10 relative abundances at the genus level and 22 key aroma substances with ROAV ≥ 1. The results are shown in **Figure 5**. Results with $P < 0.05$ and $R > 0.6$ were further screened, as shown in **Supplementary Table 2**. There were 55 positive correlations and 60 negative correlations between microbes and volatile compounds. The most volatile compounds were positively correlated with *Nocardioides*. These included styrene, β-pinene, β-myrcene, caryophyllene, decanal, undecanal, citral, dodecanal, linalool, geraniol, 1-nonanol, and naphthalene. *Curtobacterium* was positively correlated with pinene only. Twelve volatile

TABLE 1 | The aroma compounds of ROAV ≥ 1 in the different varieties of fingered citron and their thresholds and odor quality.

No.	Compounds	Threshold (μg/kg)	ROAV					
			DY	QY	CZ	KX	XZ	YG
A1	Styrene	0.0036	–	–	–	–	1.178	2.158
A2	Pinene	0.0022	921.6	401.9	465.6	396.4	280	1134
A3	α-Pinene	0.014	75.38	42.27	94.27	33.31	161.1	25.28
A4	β-Pinene	0.14	10.22	10.28	9.671	8.329	24.64	11.08
A5	β-Myrcene	0.0012	–	–	–	11.78	48.3	35.76
A6	D-Limonene	0.034	253.4	100	173.8	100	–	47.93
A7	Limonene	0.2	100	9.922	32.3	15.28	100	16.95
A8	(-)-α-Pinene	0.1	13.23	–	100	–	–	100
A9	Cyclohexene	0.2	14.21	1.073	5.65	–	7.083	6.614
A10	Caryophyllene	0.064	5.851	4.689	6.961	6.425	19.47	9.626
A11	3-Carene	0.77	6.193	–	1.964	1.663	–	–
A12	1,3,8-p-Menthatriene	0.015	–	–	–	1.238	–	–
A13	Nonanal	0.0011	–	11.29	7.116	29.15	–	14
A14	Decanal	0.003	1.768	5.705	3.724	12.37	37.78	3.514
A15	a-Citral	0.032	–	4.173	7.352	–	–	11.31
A16	Undecanal	0.0125	–	1.858	1.616	–	8.142	–
A17	Citral	0.17	–	1.007	–	–	2.691	2.023
A18	Dodecanal	0.01	–	–	–	–	6.798	–
A19	Linalool	0.00022	242.6	165.5	446.5	1174	998.3	320.3
A20	Geraniol	0.0011	177.9	282	271.6	0	712.3	955.9
A21	1-Nonanol	0.0455	–	–	–	–	1.347	–
A22	Naphthalene	0.006	–	–	–	–	2.441	–

species were negatively correlated with *Muribaculaceae*: styrene, β-pinene, β-myrcene, caryophyllene, decanal, undecanal, citral, dodecanal, linalool, geraniol, 1-nonanol, and naphthalene. *Actinomycetospora* and *Pseudokineococcus* were both negatively correlated with only two volatile compounds (D-limonene and nonanal). *Nocardioides* was positively correlated with D-limonene and 3-carene.

DISCUSSION

Similarities and Differences Between Different Fingered Citron Cultivars

Plant host-associated microbial communities may be formed by a variety of environmental and host-related factors, including geographic location, plant phenotypes and genotypes, soil nutrients, and seasonal effects (Lindow and Brandl, 2003; Vorholt, 2012). In this study, it was found that the phyllosphere bacteria of fingered citron were primarily Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. The first three types of microorganisms had high relative abundances in all of the fingered citron varieties, while Bacteroidetes only had high relative abundance in some samples of *Citrus medica* 'Fingered' (DaYeQingYi) and *Citrus medica* 'Fingered' (KaiXin) (**Figure 2A**). Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were the primary phyllosphere bacterial communities (Rastogi et al., 2012; Trivedi et al., 2020). Proteobacteria have a variety of functions, such as methyl nutrition, nitrification, and nitrogen fixation, in the phyllosphere bacterial community (Michael et al., 2008; Atamna-Ismaeel et al., 2012), and reports have indicated that Proteobacteria make up a high proportion of the phyllosphere bacteria of studied plants (Trivedi et al., 2020). Actinomycetes and Firmicutes are closely related to plant resistance and nitrogen fixation (Sasikumar et al., 2013). Bacteroidetes are considered a specific phylum in that biosphere that degrades complex organic matter (Huang et al., 2020). The differences in the secondary metabolites between the different plants of the same variety may be the reason for the large variation within the group.

There are adaptive matches between phyllosphere bacteria and their plant hosts (Lajoie et al., 2020; Whipps et al., 2008). As shown in **Figure 2B**, there were obvious differences between the phyllosphere bacteria of XZ and other varieties. Actinomycetes had the highest relative abundance in XZ, followed by *Halomonas*. *Halomonas* was a common genus among all samples, and it was the genus with the highest relative content in all the cultivars except for XZ. In addition, *Bacillus*, *Nesterenkonia*, and *Aliihoeflea* were found in all the cultivars, but their relative abundances were low.

The community structure and diversity of phyllosphere bacteria are influenced by cultivation conditions, plant species and genotypes, leaf age, and other factors (Zhiwei, 1995; Hirano and Upper, 2000; Finkel et al., 2012). XZ had the highest microbial richness and diversity, with biomarkers of 17 genera. The NMDS analysis clearly distinguished XZ from the other

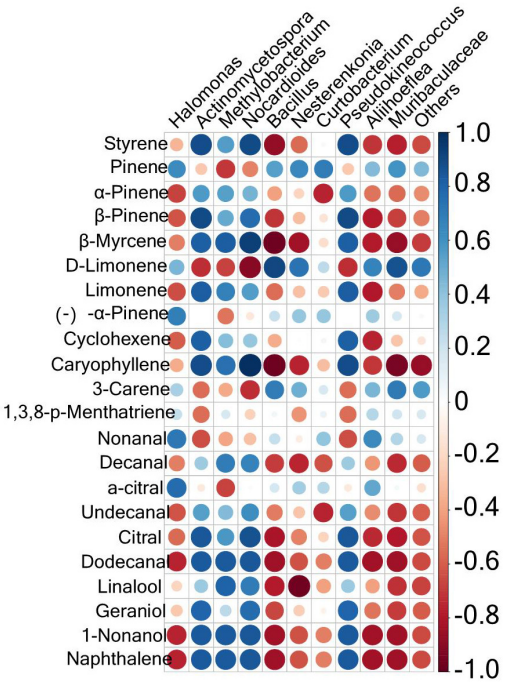

FIGURE 5 | Correlation analysis of the phyllosphere bacterial communities and the aroma compounds in the fingered citron. The circle size is positively correlated with the correlation coefficient.

varieties, indicating that variety was one of the conditions affecting the phyllosphere bacterial community structure.

Similarities and Differences in the VOCs Among the Different Varieties of Fingered Citron

Aroma is an important index used to measure fruit quality, and VOCs vary between varieties. A total of 76 VOCs were detected in six different varieties of fingered citron (**Supplementary Table 1**). The results were consistent with those obtained using steam distillation and SPME, which showed that alkenes, alcohols, and aldehydes were more predominant in the different species of fingered citron (Xia et al., 2018). α-cubebene, caryophyllene, and (+)-delta-cadinene were identified in all of the varieties.

Fingered citron fruit extract has anti-cancer, anti-oxidant, and anti-free radical effects and is a raw material for anti-bacterial agents, pharmaceuticals, and food additives (Wang et al., 2020). Volatile oils, also known as aromatic oils, and essential oils are an important part of the fingered citron extract. In this study, it was found that the fruits of different varieties of fingered citron possess their own specific volatiles. VOCs of the fingered citron are not only related to variety, but also are constantly changing as the fruit develops and matures. Aldehydes, esters, and acid compounds were the primary volatile substances in the fingered citrons harvested in Guangdong Province in June and July, while the primary volatile substances in the fingered citrons harvested in August and September included alcohols, aldehydes, esters, alkanes, and phenols at low levels (Tang et al., 2021). Therefore, when making further processed products of fingered citron, attention should be paid to the choice of raw material, especially the variety and the time of harvesting, so that more of the active substance can be obtained.

The Relationship Between VOCs and the Bacterial Microbiota

It has been demonstrated that microbial community structure affects fruit volatiles. For example, 2,5-dimethyl-4-hydroxy-2H-furan-3-one from strawberries is associated with *Methylobacterium* (Nasopoulou et al., 2014). Raspberries contain *Bacillus*, *Lactobacillus*, *Bacteroides*, and other bacteria that can increase the volatile substances in raspberries (Daniela et al., 2022). These microbes not only produce volatile compounds that enhance the fruit's flavor but also enhance its ability to defend against pests and pathogens (Daranas et al., 2019). *Bacillus* stimulates plant growth, and recent research has found that *Bacillus subtilis* in citrus trees can effectively resist the Huanglongbing pathogen (Daivasikamani, 2009; Yu et al., 2011; Munir et al., 2022). In strawberry and apples, lactic acid bacteria can be used as a biological control agent for disease (Trias et al., 2009; Tsuda et al., 2013; Zhou et al., 2017). In this study, it was found that *Halomonas* were significantly positively correlated with pinene, α-pinene, nonanal, and a-citral, among which, pinene and α-pinene were key volatiles in all of the fingered citron varieties. Actinomycetes were significantly positively correlated with styrene, β-pinene, β-myrcene, limonene, cyclohexene, caryophyllene, citral, dodecanal, geraniol, 1-nonol, and naphthalene, and the contents of these volatile compounds were higher in XZ. However, whether these volatiles are produced by bacterial enrichment requires further study. It was found that *Bacillus* could produce nonanol in raspberries (Daniela et al., 2022), but the correlation between *Bacillus* and nonanol was not significant in this study. It requires further investigation as to whether this phenomenon is caused by the difference in host plants or the weak correlation due to the small sample size.

In addition to the volatiles produced by some bacteria that increase fruit quality, the volatiles produced by fruits also cause changes in the bacterial community structure. Citrus plants produce a large number of volatile substances that affect the microbial community composition of the phyllosphere. The volatile oil of fingered citron had significant inhibitory effects on the growth of gram-positive bacteria, gram-negative bacteria, and yeast, especially on *Bacillus subtilis* and *Escherichia coli*, which reduced the diversity of microorganisms in fingered citron (Guo et al., 2009; Gao et al., 2020). In this study, it was found that there was a negative correlation between *Bacillus* and 11 key volatile compounds, and the relative abundance of *Bacillus* was low in the leaves, and this might have been caused by the bacteriostatic effect of the volatile oil of the fingered citron. Specific volatile substances with inhibitory effects on *Bacillus* need to be identified in further experiments.

Correlations between bacteria and volatiles may indicate direct bacterial production or the utilization of volatiles, the enhanced release of volatiles from plant tissues, the selective effects of VOCs on specific bacterial groups, or even indirect

non-causal relationships (Daniela et al., 2022). The interaction between the bacterial community and volatile compounds, the environment, host plants, and other factors may have resulted in the different aroma characteristics of the different varieties.

CONCLUSION AND PROSPECTS

In this study, the HS-SPME and ROAV combined analysis was used to determine the key aroma compounds of different fingered citron varieties. The contribution of bacterial microflora to the aroma of fingered citron fruits was investigated. The correlation analysis highlighted several significant associations of bacterial genera and aroma emissions that may explain the complexity of the analysis of the bacterial and metabolic interactions. This study revealed the characteristics of the core microbiota in the phyllosphere area of the fingered citron for the first time. Based on the results of this study, the naturally parasitic bacteria in the phyllosphere area of fingered citron may be associated with the production and emission of aromatic substances. In the future, to further determine the association between phyllosphere bacteria and VOCs of fingered citron, high-throughput sequencing will be used to further explore the composition of the phyllosphere bacterial community of fingered citron at the species level, and bacteria (such as Bacillus and Halomonas) with significant associations with VOCs on fingered citron leaves will be extracted, isolated, and cultured. The cultured bacteria will be inoculated onto the leaves of the fingered citron, and the changes in the VOCs of the fingered citron fruits will be measured. Hence, their correlations will be determined.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://ngdc.cncb.ac.cn/gsa/, PRJCA009805.

AUTHOR CONTRIBUTIONS

YW and PS designed the study. JW, CC, and YW conducted the experiment and collected the samples. JS, JW, and YW analyzed the data and wrote the manuscript. JS and YW critically reviewed the manuscript. All authors approved the final manuscript.

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Sphagnum capillifolium holobiont from a subarctic palsa bog aggravates the potential of nitrous oxide emissions

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Melting permafrost mounds in subarctic palsa mires are thawing under climate warming and have become a substantial source of N_2O emissions. However, mechanistic insights into the permafrost thaw-induced N_2O emissions in these unique habitats remain elusive. We demonstrated that N_2O emission potential in palsa bogs was driven by the bacterial residents of two dominant *Sphagnum* mosses especially of *Sphagnum capillifolium* (SC) in the subarctic palsa bog, which responded to endogenous and exogenous *Sphagnum* factors such as secondary metabolites, nitrogen and carbon sources, temperature, and pH. SC's high N_2O emission activity was linked with two classes of distinctive hyperactive N_2O emitters, including *Pseudomonas* sp. and *Enterobacteriaceae* bacteria, whose hyperactive N_2O emitting capability was characterized to be dominantly pH-responsive. As the *nosZ* gene-harboring emitter, *Pseudomonas* sp. SC-H2 reached a high level of N_2O emissions that increased significantly with increasing pH. For emitters lacking the *nosZ* gene, *an Enterobacteriaceae* bacterium SC-L1 was more adaptive to natural acidic conditions, and N_2O emissions also increased with pH. Our study revealed previously unknown hyperactive N_2O emitters in *Sphagnum capillifolium* found in melting palsa mound environments, and provided novel insights into SC-associated N_2O emissions.

KEYWORDS

***Sphagnum* moss, bacteria, N_2O emitters, N_2O-related genes, pH, permafrost peat**

Introduction

Arctic permafrost soils store ample nitrogen (N) reservoirs that may be subject to remobilization due to climate warming (Christensen et al., 2013), that leads to permafrost degradation and thawing (Borge et al., 2017). After permafrost thaws, increased nitrous oxide (N_2O) emissions are observed in arctic permafrost

peatlands (Voigt et al., 2017a,b). N_2O is a potent greenhouse gas and contributes to the disruption of the ozone layer (IPCC, 2007; Ravishankara et al., 2009). Therefore, urgency to understand the primary source of N_2O emissions in this arctic environment is crucial.

Peatlands store one-third of global soil carbon, and boreal peatlands account for 83% of the global peatland area (Eurola et al., 1984; Savolainen et al., 1994). Bare peat in permafrost peatlands has been identified as a hot spot for N_2O emissions due to low availability nitrogen (N) competition in subarctic tundra (Repo et al., 2009; Marushchak et al., 2011). *Sphagnum*-dominated bogs have low nutrient content, low primary production, low-quality plant litter, low litter decomposition rates, and low mineral content combined with a low pH (<4.5) environment, which is vital for carbon (C) sequestration (Chronáková et al., 2019). Mineral N deposition to *Sphagnum* bogs has progressed, with ammonification, ammonia oxidation, and denitrification playing a critical role in the emission of N_2O (Van Cleemput, 1998; Francis et al., 2007). In addition, the water table level also affects N_2O emissions in northern peatland, as lowering the water table leads to increased N_2O production (Regina et al., 1996). Once the peatlands are drained, *Sphagnum* vegetation and surface peat layers are exposed to the atmosphere, activating nitrification due to ammonium (NH_4^+-N) release in aerobic peat degradation, followed by denitrifier stimulation in N-enriched conditions to emit N_2O (Martikainen et al., 1995; Regina et al., 1999; Minkkinen et al., 2020). Palmer and Horn (2012) reported that palsa peatlands in the northwestern Finnish Lapland showed N_2O emissions *in situ* from −0.02 to 0.01 μmol N_2O m^{-2} h^{-1}. Emissions of N_2O may rise considerably during the thaw of permafrost, representing another ongoing change in northern peatlands. It was reported that a five-fold increase in N_2O flux from palsa mire peat in a permafrost thaw experiment (Voigt et al., 2017b). However, determining which active N_2O emitters in these northern ecosystems contribute to high emissions remains largely elusive.

Sphagnum mosses (non-vascular plants) dominate the vegetation of many northern mire ecosystems and harbor a high diversity of nitrifiers and denitrifiers (Dedysh et al., 2006; Gilbert et al., 2006; Opelt et al., 2007). In these moss communities, N_2O gas is mainly produced *via* nitrification, nitrifier denitrification, and denitrification pathways (Wrage et al., 2001). High hummocks in bogs and palsa mire permafrost mounds have relatively thick aerobic acrotelm layers and are the most potential microhabitats to N_2O emissions. These microhabitats are characteristically dominated by *Sphagnum fuscum* (SF) and *Sphagnum capillifolium* (SC) (Markham, 2009; Novak et al., 2015; Zhong et al., 2020), which are widely distributed throughout European and North American peat bogs. These keystone species develop climax-type, raised bog hummock vegetation. Upon exposure to high N inputs, polyphenol secondary metabolites produced by these *Sphagnum* mosses, such as caffeic acid, are often reduced (Bragazza and Freeman, 2007; Montenegro et al., 2009). These secondary metabolites may impact the activity and community composition of the microbiota within the holobiont and the associated N_2O emission rates (Wang and Cernava, 2020).

Our previous work has demonstrated that the N_2O source in southeastern Finland was mainly from *Sphagnum* moss rather than peat soil. However, this previous study only focused on the single keystone and dominant species of SF in Finnish temperate marine climate areas (Nie et al., 2015). The different contributions of N_2O emissions between several dominant *Sphagnum* species, especially in a typical subarctic permafrost peatland [hot-spots of N_2O emission (Voigt et al., 2017b)] in Finland, is largely unknown. This study uses SF as the control plants and aim to answer three questions: (1) Are the N_2O emission potentials between the two dominant *Sphagnum* species (SC and SF) similar or different in the subarctic palsa bog? (2) How does the culture-based N_2O assay for the bacterial community composition of the two *Sphagnum* species influence the N_2O emission potential? (3) What is the dominant process of N_2O production by active N_2O emitters under aerobic conditions of peat bogs? By investigating N_2O emission potential in SF and SC grown in drained palsa peat bogs of northwestern Finland, we aim to characterize the dominant N_2O emitters hidden in the microbiota of SF and SC in association with their N_2O emission traits in response to major holobiont factors.

Materials and methods

Sampling *Sphagnum* mosses

Composite samples of SF and SC (photos of them at one site are shown in Supplementary Figure S1) were collected from a plateau of a permafrost mound of a palsa mire near Kilpisjärvi (68°43′; 21°25′), northwestern Finland (Figure 1A). Each sample of SC/SF was formed from three random sampling sites with three replicates in August–September, 2014. SC and SF were collected from the same patch (within 50–100 cm) and the sampling sites were 50 to 100 m away from each other. From each sampling site, random 533 to 565 individual plants of either SC or SF were collected and mixed for each sample in order to guarantee the sample's representation. Both SC and SF were collected from large homogenous stands with a 40 cm thaw layer above the permafrost surface. The region has a low annual mean temperature (−2.3°C) and moderate mean annual precipitation (487 mm). The growing season is one of the shortest in continental Europe (~100 d when the mean daily temperature is ≥5°C). The *Sphagnum* samples stored in Ziploc® bags at 4°C were used for further culture-based N_2O emission measurements.

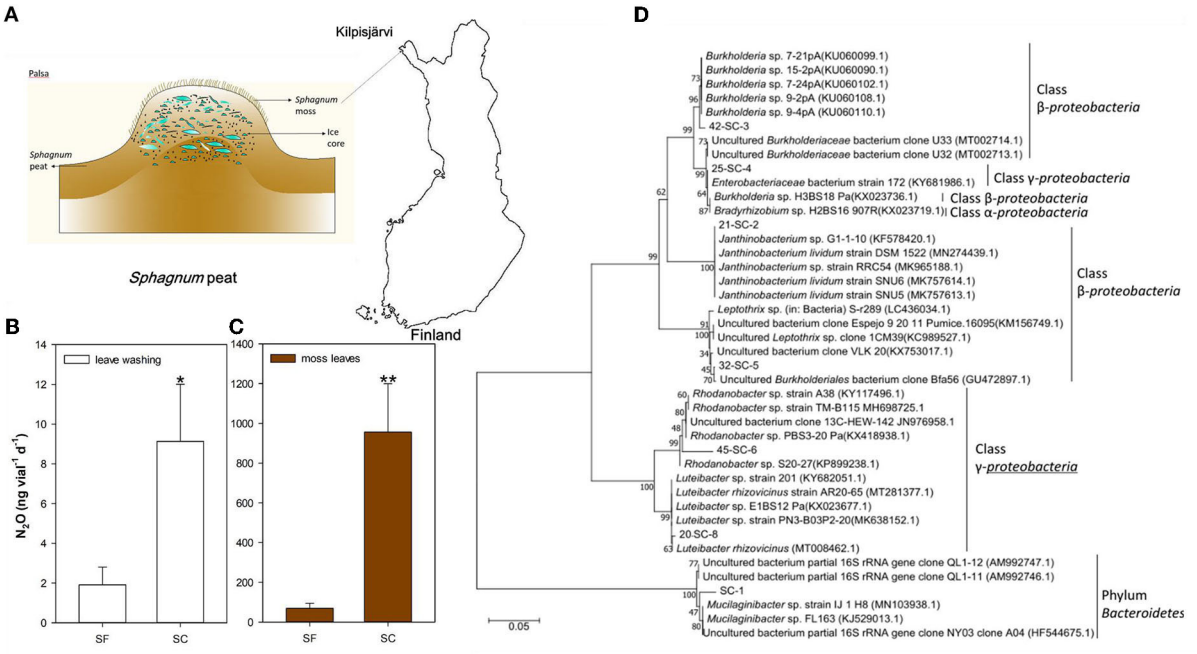

FIGURE 1
N_2O emission potential and microbial communities in the *Sphagnum* mosses grown in Finland's plateau of a permafrost mound. Sample site of *Sphagnum* mosses **(A)**. 100 μL *Sphagnum* leaves washing (100 mg/10 ml) as inoculants **(B)**. *Sphagnum* mosses as the inoculants **(C)**. The community structure of bacteria revealed by the PCR-DGGE profile was subjected to phylogenetic analysis of SC **(D)**. Incubation conditions of **(B,C)**: pH = 5, incubated at 15°C, 7 days, $n = 3$, with 0.05% sucrose. $^*P < 0.05$ *and* $^{**}P < 0.01$.

Comparison of N_2O emission potentials in two *Sphagnum* mosses

To evaluate the potential for N_2O emission of the two *Sphagnum* mosses under an experimental nitrogen load, we took three *Sphagnum* mosses plants (~0.1 g in dry weight) randomly from the respectively, composite sample of SC and SF using sterilized tweezers. At the same time, we standardized the dry weight for the N_2O assay. Either 100 μL of *Sphagnum* moss leaf extract (100 mg/10 ml) or 3 plants were added to N_2O assay medium [10 ml of modified Winogradsky's Gellan (MWG) medium containing 0.005% yeast extract and solidified with 3% gellan gum with 22.6 ml of headspace in each vial (30 ml gas-chromatographic vial with a butyl rubber plug) (Nichiden-Rika Glass Co., Kobe, Japan)] with 0.05% sucrose diluted with sterilized Milli-Q water (the solution was adjusted to pH = 5.0 with 2 M H_2SO_4) (three replicates in each case) (Hashidoko et al., 2008). After incubation at 15°C (according to the mean value of summer temperature of Finland) for 7 days in the dark, an N_2O assay was carried out by using an electron capture detector(ECD)-gas chromatograph (Shimadzu GC-14B, 125 Kyoto, Japan) connected to a Porapak N column (1 m long, Waters, Milford, MS, USA). In another treatment, 0.1 g L^{-1} of caffeic acid instead of 0.05% sucrose was added as the carbon source to the vials with three plants (~0.1 g in dry weight) randomly taken from the above composite samples (pH 5). A control for the assay, without any carbon source, was also performed simultaneously (three replicates in each case). After incubation at 15°C in the dark for 4, 8, and 15 days, an assay of N_2O was performed as mentioned above.

DGGE profiling of the bacterial communities in two *Sphagnum* species

Polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) was performed to observe the culture-based bacterial communities on the leaves of the two *Sphagnum* mosses. First, genomic DNA was extracted from the medium after the N_2O assay using an Isoplant II DNA Extraction kit (Nippon Gene, Toyama, Japan). The PCR steps and conditions were as follows: PCR denaturation for 5 min at 95°C, and 30 cycles of amplification (15 s at 95°C, 30 s at 55°C, 30 s at 72°C), and 10 min elongation at 72°C. Then PCR products for DGGE were obtained by using the common 16S rRNA primers GC-341F (CGC CCG CCG CGC CCC GCG GGG GTC CCG CCG CCC CCG CCC GCC T AC GGG AGG CAG CAG) and 907R (CCG TCA ATT CCT TTR AGT TT) (Ferris et al., 1996) and run on a 30–70% denatured gradient gel (6% w/v). The sequences of DGGE-cutting bands were obtained

using an ABI prism^{TM} 310 Genetic Analyzer and retained in the NCBI (BioProject No. PRJNA681491).

Culture-dependent screening and identification of N_2O emitters

100 μl of medium with three *Sphagnum* mosses (after incubation for 7 days) was diluted 1×10^4- and 10^6-Fold and inoculated onto MWG plates to screen N_2O emitters. After incubation for 5 days at 20°C in the dark, 13 distinguishable bacterial colonies characterized by colony characteristics were selected for streak cultivation on MWG plates and transferred to potato dextrose agar (PDA) plates until purified. Each Pure strain [a total of 108 isolates (13 bacterial colonies with 8 replicates), with 100 μl of each bacterial cell suspension (OD_{660nm} = 0.9–1.0)] was inoculated into an N_2O assay vial with 10 ml of modified MWG medium to test their N_2O emission ability. The three pure strains SC-K1, SC-L1, and SC-H2 (from SC) showed relatively higher N_2O production and were active N_2O emitters (Supplementary Table S2, data collected from six top active N_2O emission-bacterial colonies). The genomic DNA of each strain was extracted, and the 16S RRNA gene was amplified through PCR by using a series of primers 27F, 338R, 341F, 907R, 1080R, 1380R, 1492R, 1112F, and 1525R. Sequencing was performed with an ABI Prism^{TM} 310 Genetic Analyzer (Applied Biosystems, USA) (Nie et al., 2015). All the resulting 16S RRNA gene sequencing datasets were deposited in the NCBI database (accession nos. MW301596–MW301598) and compared with sequences in the nucleotide basic local alignment search tool (BLASTN) database program provided by NCBI (National Center of Biotechnology Information, Bethesda, MD, USA; http://Blast.Ncbi.nlm.nih.gov/Blast.cgi).

N_2O emitters response to nitrogen sources, pH, and temperature

The pure isolates (SC-K1, SC-L1, and SC-H2) pre-cultured on PDA for 4 days at 15°C were separately scraped with a nichrome wire loop and suspended into 1.5 ml Milli-Q water (equal amounts of each pure strain was guaranteed). A 20 μl portion of the inoculant that showed an optical density of OD_{660nm} 0.9–1.0 was added to the N_2O assay vial and then was thoroughly vortexed for 30s. 1 mM NH_4NO_3, KNO_3, and NH_4Cl were tested and incubated at 15°C for 5 days with 0.05% sucrose (pH = 5.0) to determine the optimal nitrogen substrates for pure N_2O emitters. The pH was adjusted with 1 M H_2SO_4 and 1 M KOH solutions to 4.6, 5.0, 5.7, 6.8, and 7.3 before autoclaving and incubated at 15°C for 5 days with 0.05% sucrose to determine the optimal pH for N_2O emitters. Different temperatures (4, 10, 15, 20, 25, and 30°C) were set in separate incubators and incubated for 5 days with 0.05% sucrose to find the appropriate temperature. All experiments were performed with three replicates.

Carbon source- and polyphenol-supplementation assays

Sucrose and *E*-caffeic acid were applied as carbon sources and secondary metabolites (polyphenols), respectively, for the microbiota inhabiting *Sphagnum* moss (Nie et al., 2015). The inoculants were prepared as described in Nie et al. (2015). To observe the responses of the N_2O emitters (SC-K1, SC-L1, SC-H2) to sucrose, 0 (control), 0.05, and 0.5% sucrose were used for the separated/cultivated bacterial strains. To determine the optimal concentrations of *E*-caffeic acid for N_2O emitters (SC-K1, SC-L1, SC-H2), 0 (control), 0.005, 0.01, 0.05, 0.1, 0.5, and 1 g L^{-1} *E*-caffeic acid were used. Each treatment contained three analytical replicates incubated at 15°C for 5 days with inoculants for N_2O assays. Their N_2O emissions were separately measured.

Analysis of denitrification rates of N_2O emitters

We applied the acetylene inhibition assay, which is widely used to measure denitrification rates (Sørensen, 1978). The activity of N_2O reductase was inhibited by adding acetylene (C_2H_2) at pH 5.0 and 7.0, and 10% C_2H_2 gas was injected into the headspace of vials inoculated with N_2O emitters (the same with above inoculation method) (Bollmann and Conrad, 1997). At the same time, treatments without injected C_2H_2 gas were carried out as controls to compare the N_2O reductase activity (three replicates in each case). Incubation conditions were the same as described above.

Detection of nitrogen cycling functional genes in N_2O emitters

Functional genes of nitrogen cycling, including *narG*, *nirK*, *nirS*, and *nosZ* (Supplementary Figure S4), were detected by using the PCR method. The target genes were amplified by using the primers *narG*F (TCG GGC AAG GGC CAT GAG TAC) and *narG*R (TTT CGT ACC AGG TGG CGG TCG), *nirS*Cd3Af (AAC GYS AAG GAR ACS GG) (Nie et al., 2015) and *nirS*R3cd (GAS TTC GGR TGS GTC T) (Throbäck et al., 2004), *nirK*-1F (GGM ATG GTK CCS TGG CA) and *nirK*-5R (GCC TCG ATC AGR TTR TGG) (Braker et al., 1998), *nosZ*-1111F (STA CAA CWC GGA RAA SG), *nosZ*-661F (CGG CTG GGG GCT GAC CAA), *nosZ*-1527R (CTG RCT GTC GAD GAA CAG),

and *nosZ*-1773R (ATR TCG ATC ARC TGB TCG TT) (Scala and Kerkhof, 1998). The exact reaction conditions of the PCR amplifications are presented in Supplementary Table S1.

Statistical analysis

The data were expressed as mean with standard error (SE). The data were examined for normality and homoscedasticity using the Shapiro-Wilk's and Levene's tests, respectively (SPSS, version 23.0). All data was found to fit the normal distribution and homogeneity of variances. Comparisons were made using a one-way analysis of variance (ANOVA) among two or more groups. One-way ANOVA was used to compare differences in N_2O emission with different inoculants (*Sphagnum mosses* or their leaves washing), physicochemical factors [pH, temperature, sucrose, nitrogen types, and secondary metabolite (*E*-caffeic acid)], and C_2H_2 inhibition assay. Using the Fisher's Least Significant Difference(LSD) method, multiple comparisons were carried out using IBM SPSS 23.0 software (Chicago, Illinois, USA).

Results

N_2O emission potential and microbial communities

After incubation for 7 days, we found that the average N_2O emissions of SF were 1.9 ng vial^{-1} d^{-1} in the leaf extract and 69.9 ng vial^{-1} d^{-1} in the leaf samples. The SC sample showed N_2O emissions of 9.1 in the leaf extract and 956.2 ng vial^{-1} d^{-1} in the leaf samples (Figures 1B,C).

The PCR-DGGE profile showed that the major culture-based bacterial communities in these *Sphagnum* mosses were similar. However, the SC sample harbored the family *Enterobacteriaceae* (Figure 1D, Supplementary Figure S2), while the SF sample contained the genus *Dyella* of Gammaproteobacteria (Supplementary Figure S2). N_2O production increased with 0.1 g L^{-1} caffeic acid addition in both samples, and the effect was significantly larger in the SC sample than in the SF sample ($p < 0.05$) (Figures 2A,B).

Major N_2O emitters in *Sphagnum* mosses

Compared to PCR-DGGE, the culture-based approach revealed distinctive profiles of N_2O emitters (Supplementary Figure S2). Two *Burkholderia* spp. were isolated from the SF sample, while three *Gammaproteobacteria* (one *Pseudomonas* sp., one *Serratia* sp., and an unidentified *Enterobacteriaceae* bacterium) and one *Burkholderia* sp. were isolated from the SC sample. Among them, *Serratia* sp. SC-K1, *Enterobacteriaceae* bacterium SC-L1, and *Pseudomonas* sp. SC-H2 showed the most efficient N_2O emissions, and the activity of N_2O emissions was the greatest in *Pseudomonas* sp. SC-H2, then *Enterobacteriaceae* bacterium SC-L1, and then *Serratia* sp. SC-K1 (pH 5) (Table 1, Supplementary Table S2).

Effects of substrate type, temperature and pH on microbial N_2O emissions

According to the N_2O production responses to different nitrogen sources, KNO_3 was the most efficient substrate for N_2O emission, followed by NH_4NO_3, while almost no N_2O emissions were found with NH_4Cl as the substrate. Active N_2O emissions from KNO_3 indicated that the three N_2O emitters were nitrate reducers (Figure 3). N_2O emissions increased as the pH increased from 4.6 to 7.3. *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1 showed a temporary increase at a pH value of 5 but no drastic increase in N_2O emissions, indicating adaptation to acidic environments (Figures 4A,B). At pH values over 6, *Pseudomonas* sp. SC-H2 emissions increased sharply, making it the most likely N_2O emitter (Figure 4C). For the three strains used, N_2O emissions also increased with increasing temperature from 4 to 30°C (Figures 4D–F).

Disparate responses of N_2O emitters to caffeic acid and sucrose

The three microbial strains exhibited disparate responses to sucrose and *E*-caffeic acid (Figure 5). In the absence of added sucrose (control treatment), *Serratia* sp. SC-K1 emitted more N_2O than *Enterobacteriaceae* bacterium SC-L1 and *Pseudomonas* sp. SC-H2, while these last two strains emitted N_2O at higher levels with 0.05% sucrose supplementation (Figures 5A,B). Notably, the response of *Pseudomonas* sp. SC-H2 to 0.05% sucrose was very drastic, resulting in emission $\sim$2x10^3 times higher than without sucrose (Figure 5C). This result demonstrated that *Serratia* sp. SC-K1 is an oligotrophic bacterium, whereas *Enterobacteriaceae* bacterium SC-L1 and *Pseudomonas* sp. SC-H2 are eutrophic bacteria.

For the pure strains of *Enterobacteriaceae* bacterium SC-L1 and SC-K1, a relatively lower concentration of *E*-caffeic acid ($\leq$0.1 g L^{-1}) increased N_2O emissions of these two strains, and the optimum concentration was 0.1 g L^{-1} (Figures 5D,E). Among them, *Serratia* sp. SC-K1 was very sensitive to 0.1 g L^{-1}, and 13-fold higher N_2O production was found than without *E*-caffeic acid (Figure 5E). For *Pseudomonas* sp. SC-H2, when the concentration of *E*-caffeic acid was above 0.01 g L^{-1}, N_2O emissions decreased significantly ($p < 0.01$) (Figure 5F).

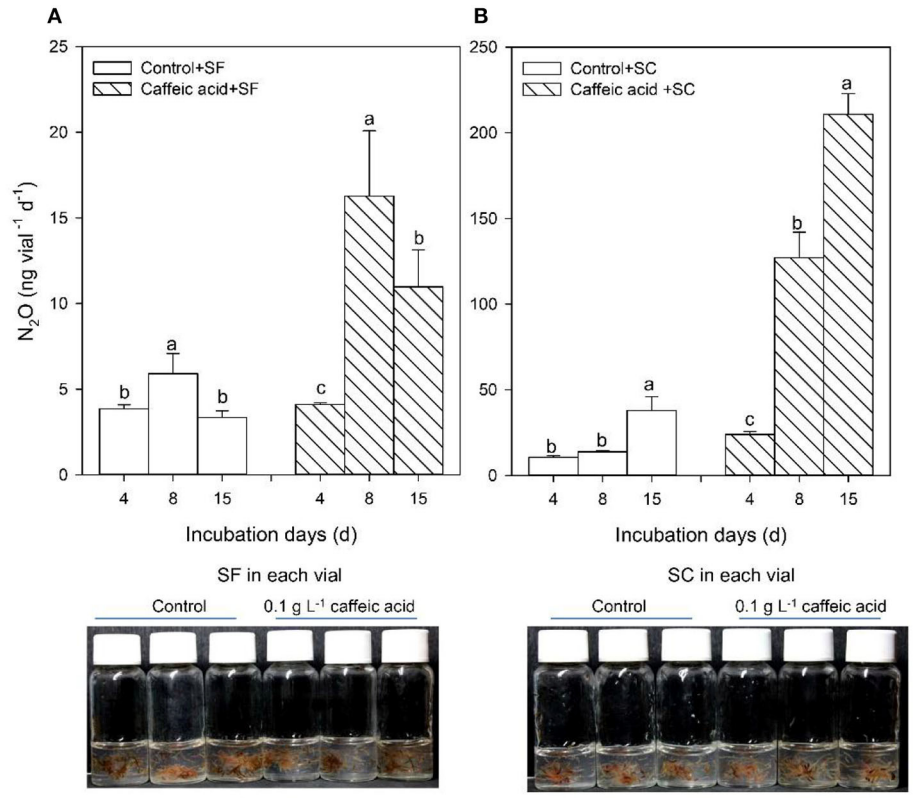

FIGURE 2
Response of N_2O production of *Sphagnum fuscum* (SF) and *Sphagnum capillifolium* (SC) to caffeic acid. Response of N_2O production of *Sphagnum fuscum* (SF) to caffeic acid **(A)**. Response of N_2O production of *Sphagnum capillifolium* (SC) to caffeic acid **(B)**. Incubation conditions: pH = 5, incubated at 15°C, 4, 8, 15 days, $n = 3$, without sucrose. Values are means ± s.e. (shown as error bars).

TABLE 1 Identification of the active N_2O emitters using 16s rRNA gene sequence.

Isolates	Length (bp)	Accession No.	Most aligned DNA (Accession No.)	Identities
SC-K1	1528	MW301598	*Serratia* sp. HC3-14(JF312984.1)	1515/1526(99%)
			Serratia sp. HC3-9(JF312979.1)	1513/1525(99%)
			Serratia sp. HC4-9(JF312995.1)	1512/1525(99%)
SC-L1	1165	MW301597	*Serratia liquefaciens* strain Noth_10 (MF716557.1)	1123/1153(97%)
			Enterobacteriaceae bacterium ENUB8 (JX162036.1)	1133/1167(97%)
			Serratia proteamaculans strain 336X(CP045913.1)	1132/1167(97%)
SC-H2	1514	MW301596	*Pseudomonas* sp. LH1G9(CP026880.1)	1513/1518(99%)
			Pseudomonas sp. 05CF15-5C (LC007966.1)	1513/1518(99%)
			Pseudomonas sp. Pi 3-62 (AB365063.1)	1512/1517(99%)

Modest responses of N_2O emitters to acetylene

There was no detectable difference between the 10% C_2H_2 and control treatment emissions at a pH value of 5.0. However, in *Pseudomonas* sp. SC-H2 cultured at a pH value of 7.0, N_2O emissions upon exposure to C_2H_2 were drastically increased to four-fold higher than that of the control. Without 10% C_2H_2, the production level of N_2O at a pH value of 7.0 was higher than that at a pH value of 5.0 (Figure 6). This result suggested that the peat ecosystem was highly disturbed at a pH value of 7.0, denitrification was greatly accelerated, and the final denitrification step to reduce N_2O to N_2 was driven by N_2O reductase.

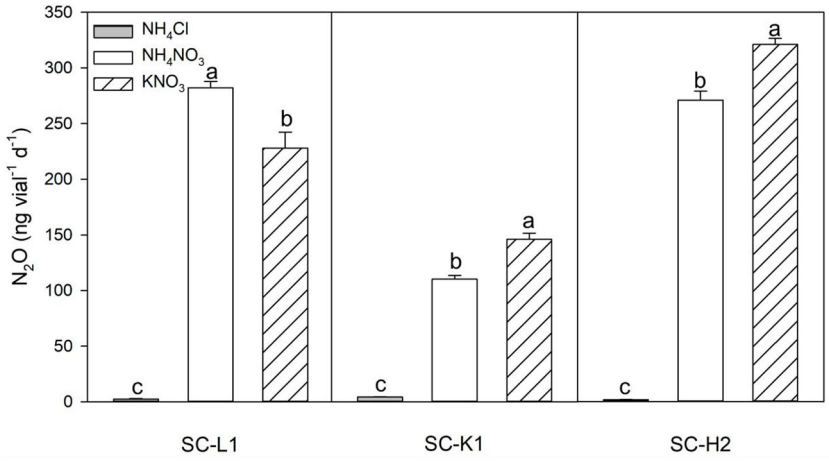

FIGURE 3
N_2O emission by three pure N_2O emitters (SC-L1, SC-K1, SC-H2) upon exposure to different nitrogen substrates (1 mM NH_4Cl, NH_4NO_3, KNO_3). Incubation conditions: pH = 5, incubated at 15°C for 5 days with 0.05% sucrose (n = 3). Values are means ± s.d. (shown as error bars).

Functional genes involved in N_2O emission

PCR assays detected the *narG* gene in the three N_2O emitter strains, but only *Pseudomonas* sp. SC-H2 contained *nirS* and *nosZ* genes (Table 2; Supplementary Figure S3). In combination with the results of the C_2H_2 assay, these results suggested that *Pseudomonas* sp. SC-H2 is a complete denitrifier. The *nir*K gene was not detected within *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1.

Discussion

Cultured bacterial communities in the leaves distinguishable between two *Sphagnum* species

Increased atmospheric N deposition can reduce the growth of some *Sphagnum* species, such as *Sphagnum magellanicum* (Aerts et al., 2001; Limpens and Berendse, 2003). In contrast, the production of SF increased with elevated N deposition but decreased as N deposition reached 14.0 kg ha^{-1} yr^{-1} as reported by Vitt et al. (2003). SC can also tolerate a high N supply (Bonnett et al., 2010). Our study offered evidence that individual samples of the latter two *Sphagnum* species had N_2O emission potential reasonably associated with their bacterial communities. In particular, the SC sample harbored specific bacterial communities associated with high N_2O emission. Surprisingly, the N_2O emission of the SC sample was significantly greater than that of the SF sample (Figure 1B) ($p < 0.01$). Such a large difference in N_2O emission between the SF and SC species gives precedence to the hypothesis of potential N_2O emission differences in different *Sphagnum* species.

Based on the analysis of bacterial communities using culture-based PCR-DGGE and isolation of N_2O emitters, the major *Sphagnum*-associated bacterial communities of our samples were consistent with boreal mire and tropical peat forest and included *Burkholderia*, *Mucilaginibacter*, *Rhodanobacter*, and *Janthinobacterium* but their N_2O emission activity was different in varied sites due to differences in climate and habitat environments (Hashidoko et al., 2008; Sun et al., 2014). *Janthinobacterium* spp. did not show high N_2O emission potential in subarctic palsa bog unlike in the tropical peatland soil, which suggested that the N_2O emission functions of N_2O emitters were changing in different climate zones. Previous experimentation has shown that the *Sphagnum* microbiota supported the host plant and the entire ecosystem under environmental changes (Bragina et al., 2014). *Burkholderia* spp. were N_2O emitters, but their N_2O emission functions were significantly lower than the acid-tolerant *Janthinobacterium* sp. in a deforested tropical peatland soil, which was previously determined by soil pH (Hashidoko et al., 2010). The *Burkholderia* spp. isolates in SF were similar to another climate zone in Finland, showing the same species of *Sphagnum* although in a different climate zone (Nie et al., 2015). Within this study, some unique bacterial strains were found in the leaves of SC, including a *Pseudomonas* sp. and two *Enterobacteriaceae* family members. In numerous previous studies, *Pseudomonas* species (*P. denitrificans*, *P. perfectomarinus*, *P. fluorescens*, *P. stutzeri*, *P. aeruginosa*, and *P. nautica*) were found performing denitrification (Delwiche, 1959; Payne et al., 1971; Balderston et al., 1976; Sørensen et al., 1980; Dooley et al., 1987; Viebrock and Zumft, 1988;

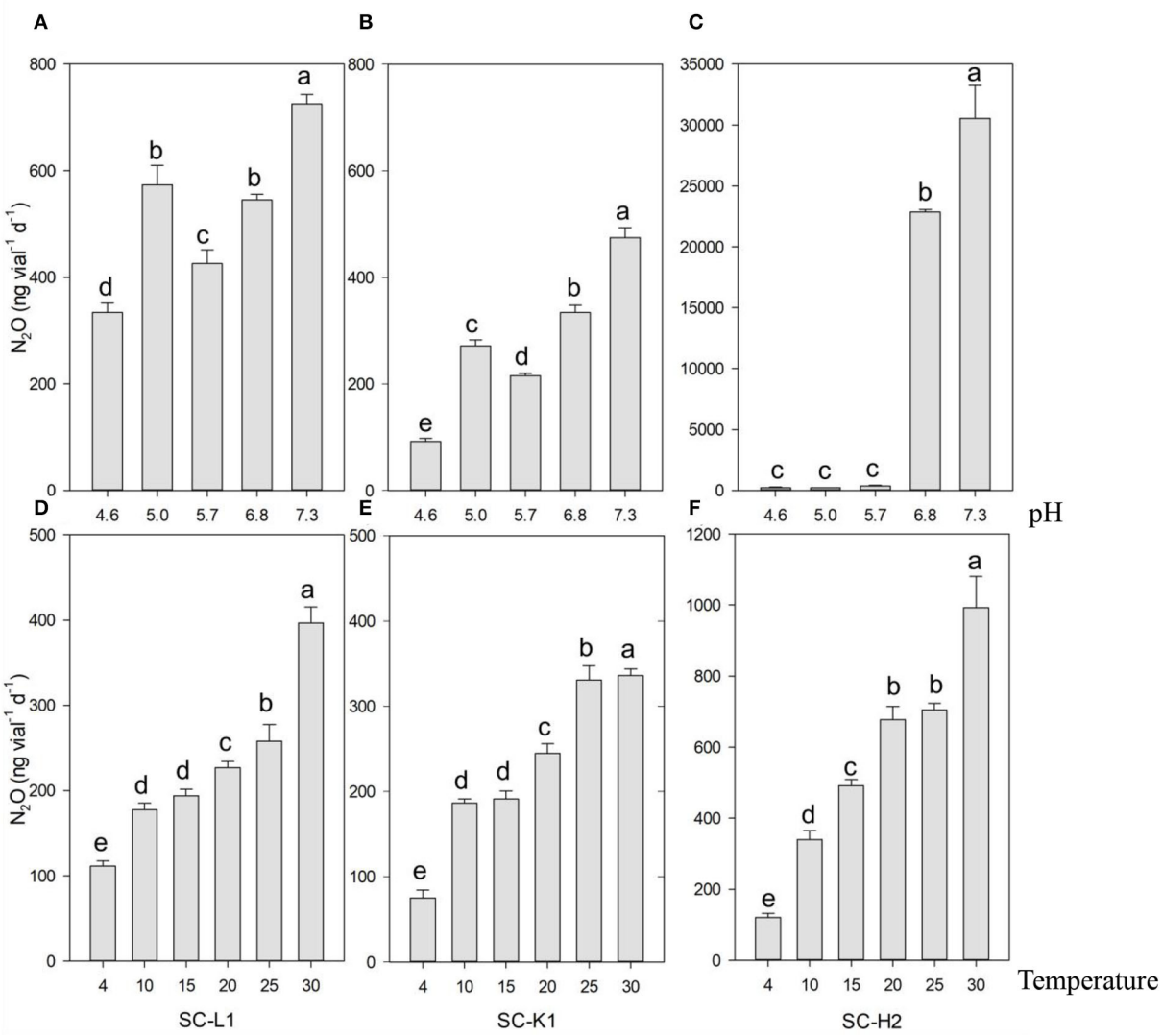

FIGURE 4
N_2O emission by three pure N_2O emitters (SC-L1, SC-K1, SC-H2) upon the gradient pH and temperature. N_2O emission by SC-L1 **(A,D)**, SC-K1 **(B,E)**, SC-H2 **(C,F)** upon exposure to different pH from 4.6 to 7.3 **(A–C)**, and different temperatures from 4 to 30 °C **(D–F)** was analyzed. For the impact of pH on N_2O emission, the N_2O emitters were incubated at 15°C for 5 days with 0.05% sucrose ($n = 3$). For the impact of temperature on N_2O, the N_2O emitters were incubated for 5 days with 0.05% sucrose ($n = 3$ and pH = 5).

SooHoo and Hollocher, 1991; Prudêncio et al., 2000). The isolated *Pseudomonas* sp. was not found in the bands of PCR-DGGE, possibly due to relatively low abundance under acidic conditions (pH 5) (Figure 4C). Anderson and Levine (1986) offered evidence that *Enterobacteriaceae* and *Serratia* sp.'s nitrate respiration produces N_2O, which was also found in our SC sample. *Enterobacter* sp. was also found as dissimilatory nitrate reduction to ammonium (DNRA) bacteria in agricultural soils (Heo et al., 2020). *Pseudomonas* sp. SC-H2, *Enterobacteriaceae* bacterium SC-L1, and *Serratia* sp. SC-K1 were responsible for N_2O emissions in our *Sphagnum* samples (SC). These findings suggest that the variation in the N_2O emission potential of *Sphagnum* found in peatlands is associated with species-specific bacterial communities, which are variable under different species and environments.

Complex environmental factors also impact N_2O production of active N_2O emitters

The top three active N_2O emitters (*Pseudomonas* sp. SC-H2, *Enterobacteriaceae* bacterium SC-L1, and *Serratia* sp. SC-K1) from SC increased N_2O production with increasing

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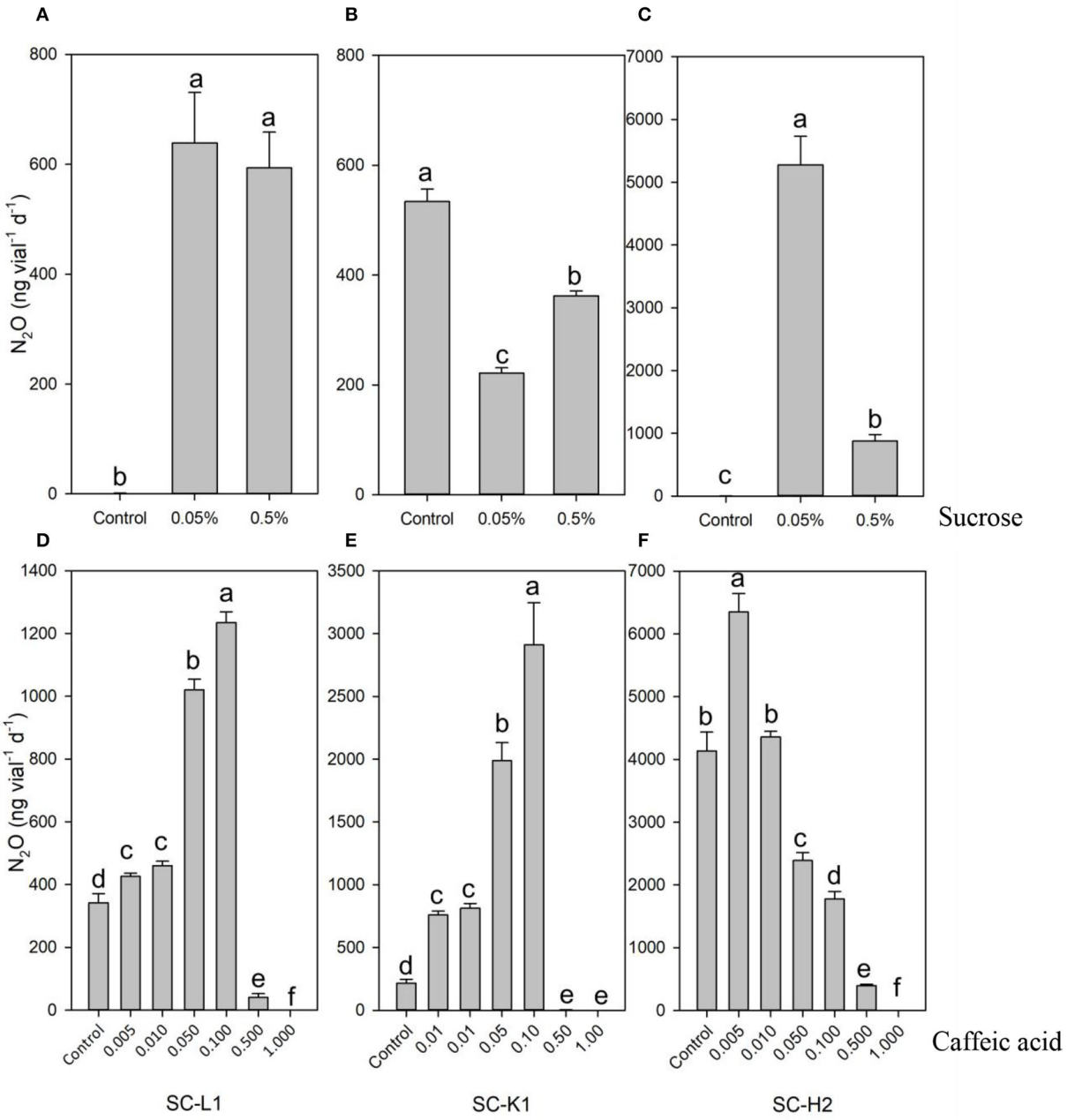

FIGURE 5
(A) N_2O emission by three pure N_2O emitters (SC-L1, SC-K1, SC-H2) exposure to the different concentrations of sucrose **(A–C)** and caffeic acid **(D–F)**. N_2O emission by SC-L1 **(A,D)**, SC-K1 **(B,E)**, SC-H2 **(C,F)** upon exposure to different concentration of sucrose from 0 to 0.5% **(A–C)** and different concentration of caffeic acid from 0 to 0.1 g L^{-1} **(D–F)** was analyzed. For the impact of sucrose on N_2O emission, the N_2O emitters were incubated at pH = 7 for 5 days ($n = 3$), and the control was without supplemented sucrose. For the impact of caffeic acid on N_2O emission, the N_2O emitters were incubated at pH = 7 for 5 days with 0.05% sucrose ($n = 3$), and the control was without supplemented caffeic acid.

temperature up to 30°C (Figures 4D–F), illustrating a potential rise in N_2O emissions following global warming (Pfenning and McMahon, 1997; Voigt et al., 2017a; Chen et al., 2020). For the three active N_2O emitters, N_2O production was relatively high at a pH value of 7.0 (Figures 4A–C), which is much higher than the naturally low pH of *Sphagnum* microhabitats (Tahvanainen and Tuomaala, 2003). Although N_2O reduction to N_2 by *Pseudomonas* sp. SC-H2 was obvious, the N_2O production was still high after 5 days of incubation (Figure 6). This result indicated that N_2O emission hotspots are inclined to be in neutral peatlands, as supported by Palmer and Horn (2015). Combining these results with acetylene inhibition assays at pH

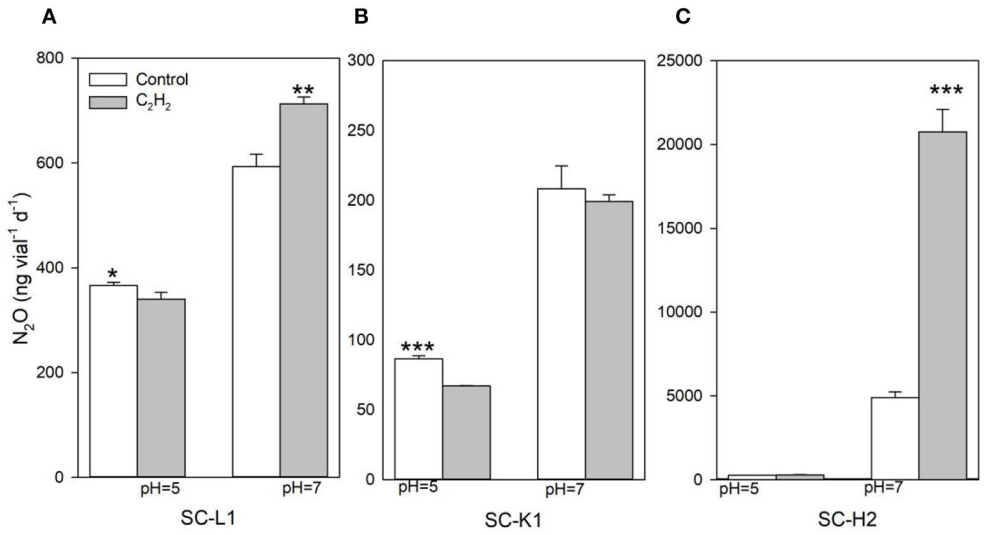

FIGURE 6
N_2O emission by three N_2O emitters (SC-L1, SC-K1, SC-H2) exposure to 10% C_2H_2 gas **(A–C)**. The three N_2O emitters were incubated at pH 5 and 7, under 15°C with 0.05% sucrose for 5 days ($n = 3$). Without C_2H_2 gas was used as a control. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

TABLE 2 Characteristics of the three active N_2O emitters isolated from SC and PCR assay to detect denitrification-related genes.

Isolates	Optimal pH	Optimal temperature (°C)	Optimal substrates	Sucrose %)	*E*-caffeic acid (g L^{-1})	*narG*	*nirS*	*nirK*	*nosZ*
SC-L1	7.3	30	NH_4NO_3	0.05/0.5	0.1	+	−	−	−
SC-K1	7.3	30	KNO_3	0	0.1	+	−	−	−
SC-H2	7.3	30	KNO_3	0.05	0.005	+	+	−	+

−; indicated the isolates without the functional genes.
+; indicated the isolates harboring the functional genes.

value of 5.0 and 7.0 showed that N_2O reduction to N_2 was almost negligible at a pH value of 5 for these three active N_2O emitters. This result is consistent with a previous study of the lack of N_2O reductase (nos) function at low pH (Liu et al., 2014). This result also suggested that N_2O reduction was inhibited in the acidic environment in the peat bogs. Since the *Sphagnum* microhabitats are very acidic, N_2O reductase activity is repressed, supporting that N_2O reduction is not a pathway decreasing N_2O emissions in the pristine *Sphagnum* bog system. Under low-pH conditions, N_2O production by *Pseudomonas* sp. SC-H2 was small, but N_2O could be accumulated. However, the palsa mounds are formed due to the ice core under the *Sphagnum* peat layer in the subarctic climate, and once they collapse after permafrost thawing, the peat acidity will be neutralized to some extent by mixing with mineral material and minerogenic water flow (Seppälä, 2011; Takatsu et al., 2022).

Sphagnum mosses are important for peat accumulation and form a carbon pool of global significance. Increasing atmospheric N deposition can activate phenol oxidase in peat bogs and destabilize peat carbon (Bragazza et al., 2006). Phenol oxidase requires bimolecular oxygen for its activity (Freeman et al., 2004), and drying increases aerobic conditions in peatlands (Swindles et al., 2019) and can degrade recalcitrant phenolic materials. Tahvanainen and Haraguchi (2013) showed that this phenolic mechanism is affected by pH. Such changes may reduce the generally high C:N ratio, which increases net N mineralization, nitrification, and denitrification rates, while subsequently increasing the potential of N_2O production in peat bogs, while lower C:N ratios (≤25–30) stimulate N_2O emissions (Huang et al., 2004; Klemedtsson et al., 2005; Maljanen et al., 2012). Connected mechanisms and the release of ice-trapped N_2O are further impacted by thawing permafrost (Voigt et al., 2017b). Our findings indicate that N_2O emissions are not exceptionally high under the naturally cold temperatures and low pH of *Sphagnum* habitats; rather, substantially high pH and temperatures, and perhaps a connected imbalance of microbial communities in such conditions, induced the highest N_2O emissions. The results warrant caution in interpretation and

against unexpected emission potential under rapidly changing conditions. It also calls for a need to monitor the *in situ* N_2O emissions from different permafrost *Sphagnum species* in the permafrost in future studies.

Responses of N_2O emitters to primary metabolites and secondary metabolites of *Sphagnum* mosses

Without sucrose, the N_2O emitters *Enterobacteriaceae* bacterium SC-L1 and *Pseudomonas* sp. SC-H2 could not emit N_2O because of their low growth. This result indicated that these two strains were heterotrophic microorganisms that needed to gain C sources from *Sphagnum* moss and form plant-microbial symbionts between plants and microbes. Interestingly, *Serratia* sp. SC-K1 grew well without sucrose and emitted much more N_2O; meanwhile, it could be significantly inhibited by adding a low concentration of sucrose (0.05%). This result indicated that this strain is an autotrophic microorganism adapted to nutrient-poor environments, using carbon dioxide (CO_2) as a C source. These autotrophic microorganisms contribute to CO_2 uptake and carbon sequestration. Drained peatland ecosystems have an immense potential for C sinks to maintain the C balance, even though droughts are occasionally caused by decreasing photosynthesis (Minkkinen et al., 2018).

Our study showed that N_2O emitters (*Serratia* sp. SC-K1 and *Enterobacteriaceae* bacterium SC-L1) could resist relatively higher concentrations of caffeic acid ($\leq$0.1 g L^{-1}), while the N_2O emitter (*Pseudomonas* sp. SC-H2) had low resistance to caffeic acid ($\leq$ 0.005 g L^{-1}) (Figures 5D–F). These results could explain why we could not find the *Pseudomonas* spp. using DGGE band sequencing. Polyphenol (caffeic acid) from *Sphagnum* moss inhibits growth and results in a low relative abundance of *Pseudomonas* spp. The more abundant *Serratia* sp. SC-K1 and *Enterobacteriaceae* bacterium SC-L1 were the dominant N_2O emitters due to their higher resistance to polyphenolic compounds. The stimulated N_2O production in the *Sphagnum* moss-microbe vial with 0.1 g L^{-1} caffeic acid confirmed *Serratia* sp. SC-K1 and *Enterobacteriaceae* bacterium SC-L1 were the dominant N_2O emitters. *Serratia* spp. are gram-negative bacilli and belong to the family *Enterobacteriaceae*. The interaction of polyphenolic compounds and *Enterobacteriaceae* bacteria might directly influence N_2O emissions in peatland ecosystems. High concentrations of polyphenols are likely to lower N_2O emissions. The response of phenol oxidase to N deposition differs by ecosystem type. In peat bogs, elevated N deposition decreased polyphenols' contents and decreased the polyphenol ratio to N, which may increase N_2O production due to an inverse relationship between N_2O emissions and the polyphenol to nitrogen ratio (Pimentel et al., 2015).

N_2O production of active N_2O emitters

The three N_2O emitters preferred KNO_3 as a substrate over NH_4Cl. This result suggested that these three isolates mainly use DNRA or denitrification to produce N_2O gas. For the *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1, the *nirS*, *nirK*, and *nosZ* genes were not detected, but the *narG* gene was, suggesting that they do not have nitrite reductase and are non-denitrifiers consistent with other *Enterobacteriaceae* bacteria emitting N_2O as a final product (Arkenberg et al., 2011). *Enterobacter* species are often reported as producing N_2O by DNRA (Smith and Zimmerman, 1981). This result indicated that they are also important sources for N_2O emissions in SC dominant bogs. *Pseudomonas* sp. SC-H2 harbored *nosZ*, *nirS*, and *narG*. Therefore, *Pseudomonas* sp. SC-H2 was a typical denitrifier. Microbial heterotrophic denitrification and DNRA compete for shared resources (Jia et al., 2020).

Although the N_2O potential was relatively high in the SC sample, the N_2O emissions *in situ* in the peat bogs were generally low in northern Finland, which might be impacted by the complexity of environmental conditions (Dinsmore et al., 2017). The potential N_2O emissions in the field (Repo et al., 2009; Voigt et al., 2017b) and laboratory incubations (Elberling et al., 2010) increase with increasing mineral N availability, permafrost thawing, and drainage. A previous study suggested that drainage of bogs alters nutrient cycling and microbial communities to increase N_2O emissions (Frolking et al., 2011). Unvegetated (free of vascular plants) peat surfaces resulting from wind erosion and frost action were hot spots for N_2O emission in subarctic permafrost peatlands due to the absence of plant nitrogen uptake, a low C:N ratio, and sufficient drainage (Marushchak et al., 2011; Voigt et al., 2017b). *Pseudomonas* sp. SC-H2 had negligible N_2O emissions at low pH (<4.5), while the other two N_2O-emitting *Enterobacteriaceae* bacteria from SC exhibited contrasting patterns in the *Sphagnum* bogs. Therefore, the contribution of denitrification and DNRA to N_2O emissions in boreal peat bogs should be considered in future studies.

Conclusion

In summary, our study identified several N_2O emitters in microbial communities of *Sphagnum* samples from the subarctic permafrost habitat of palsa mires. A composite sample of SC showed high potential to emit N_2O, and a composite of SF showed moderate potential to emit N_2O. The N_2O emission potential was attributed to distinctive bacterial communities inhabiting moss leaves in both cases. Two classes of hyperactive N_2O emitters hidden in the SC holobiont were revealed. *Pseudomonas* sp. SC-H2 was found to harbor *narG*, *nirS*, and *nosZ* genes. N_2O reduction to N_2 catalyzed by N_2O reductase was noteworthy in the neutral pH microenvironment. The other hyperactive N_2O emitters, *Enterobacteriaceae* bacterium SC-L1

and *Serratia* sp. SC-K1 lacked the *nirS*, *nirK*, and *nosZ* genes but contained the *narG* gene and emitted NO/N_2O as the final product, possibly *via* the DNRA pathway. These findings provided some theoretical evidence for the future N_2O emission study of the *in situ* subarctic palsa under elevated N availability and global warming.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

YH and YN designed the research, experiments, and acquired the funds. YH, RI, and TT collected the samples in Finland. YN performed experiments and analyzed data. YN, SYL, XT, XL, SL, TT, RI, and QY wrote and edited the paper. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.974251/full#supplementary-material

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Effect of ozonated water, mancozeb, and thiophanate-methyl on the phyllosphere microbial diversity of strawberry

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Phyllosphere microorganisms are closely linked to plant health. This study investigated the effect of ozonated water, mancozeb, and thiophanate-methyl on phyllosphere microorganisms in strawberry plants of the "Hongyan" variety. Sequencing analysis of the phyllosphere bacterial and fungal communities was performed using 16S rRNA gene fragment and ITS1 region high-throughput sequencing after spraying ozonated water, mancozeb, thiophanate-methyl, and clear water. Proteobacteria, Actinobacteria, and Firmicutes were the dominant bacterial phyla in strawberry. The relative abundance of Proteobacteria (82.71%) was higher in the ozonated water treatment group than in the other treatment groups, while the relative abundance of Actinobacteria (9.38%) was lower than in the other treatment groups. The strawberry phyllosphere fungal communities were mainly found in the phyla Basidiomycota and Ascomycota. The relative abundance of Basidiomycota was highest in the ozonated water treatment group (81.13%), followed by the mancozeb treatment group (76.01%), while the CK group only had an abundance of 43.38%. The relative abundance of Ascomycota was lowest in the ozonated water treatment group (17.98%), 23.12% in the mancozeb treatment group, 43.39% in the thiophanate-methyl treatment group, and 55.47% in the CK group. *Pseudomonas*, *Halomonas*, and *Nesterenkonia* were the dominant bacterial genera on strawberry surfaces, while *Moesziomyces*, *Aspergillus*, and *Dirkmeia* were the dominant fungal genera. Ozonated water was able to significantly increase the richness of bacteria and fungi and decrease fungal diversity. However, bacterial diversity was not significantly altered. Ozonated water effectively reduced the relative abundance of harmful fungi, such as *Aspergillus*, and *Penicillium*, and enriched

beneficial bacteria, such as *Pseudomonas* and *Actinomycetospora,* more effectively than mancozeb and thiophanate-methyl. The results of the study show that ozonated water has potential as a biocide and may be able to replace traditional agents in the future to reduce environmental pollution.

KEYWORDS

strawberry, phyllosphere microorganisms, ozonated water, mancozeb, thiophanate-methyl

Introduction

The living environment of phyllosphere microorganisms is directly exposed to air, and the community structure is directly influenced by factors such as light, humidity, and cultivation measures and indirectly by plant secondary metabolites, resulting in an unstable community structure (Finkel et al., 2012; Vorholt, 2012). Studies have shown that phyllosphere microorganisms can improve resistance to pests and diseases through the production of antimicrobial compounds, microbial–microbial competition, or the activation of plant defenses (Chen et al., 2020). The microbial communities of tomato leaves have been found to protect plants from infection by *Pseudomonas syringae* (Berg and Koskella, 2018). The composition and diversity of the phyllosphere fungal communities of *Eucalyptus grandis* have also been shown to be affected by *Leptocybe invasa* infestation, with significant differences between different levels of infestation (Messal et al., 2022). *Arabidopsis thaliana* phyllosphere-symbiotic *Pseudomonas* strains have even been observed to induce selective inhibition of specific *Arabidopsis* pathogens, leading to plant protection (Shalev et al., 2022). In addition, pathogenic bacteria parasitic on leaves can enter the plant through stomata or wounds when the dynamic balance of the phyllosphere microbial community structure is disturbed, thus inducing disease. Therefore, maintaining the dynamic balance of the phyllosphere microbial community structure and reducing the relative abundance of pathogenic bacteria are of great importance for plant health.

Strawberry (*Fragaria* × *ananassa* Duch.) is a perennial herb of the genus *Fragaria* in the family Rosaceae, native to South America, and is now cultivated around the world. Strawberries are not only unique in flavor but also rich in compounds such as folic acid, ellagic acid, and flavonoids, making them popular among consumers (Hannum, 2004; Tulipani et al., 2009). In 2020, the global output of strawberries reached 8.86 million tons, but strawberry plants are often stressed by biotic or abiotic factors during the cultivation process, which seriously affects yield and quality (Olimi et al., 2022). Traditional chemicals can cause problems such as food safety and environmental pollution, and their use in production has been gradually limited (Sylla et al., 2013). Therefore, the search for new fungicides is imminent.

Ozone is a strong oxidant that is easily soluble in water and has antibacterial and antifungal effects (Inatsu et al., 2011). Ozone decomposes quickly under ambient conditions, thereby having a reduced impact on air and water pollution (Remondino and Valdenassi, 2018). In cultivation, ozone is often used to fumigate the soil to eliminate parasites and pathogenic bacteria, which can effectively reduce the incidence of soil-borne diseases (Nicol et al., 2011). After harvest, ozone can be used to destroy pathogenic bacteria on fruit surfaces and degrade residual pesticides (Wang and Chen, 2020). Ozonated water can inhibit the conidial dispersal of *Phaeoacremonium aleophilum* and reduce the infection rate in grapevine (Pierron et al., 2015). Ozone disinfection of rice seeds effectively inactivates the spores of *Fusarium fujikuroi* (the fungus that causes rice bakanae disease) (Kang et al., 2015). In barley storage, ozone is very effective in inactivating barley-associated fungi, and mycelium is less resistant to ozone than spores (Allen et al., 2003). The use of ozonated water irrigation can significantly increase the shelf life of strawberries, total sugar content, total protein content, and mineral content and reduce the incidence of gray mold, powdery mildew, and other diseases. However, the effects of ozonated water on microorganisms have not been studied in detail (Zhuo et al., 2017; Lu et al., 2018). In this study, strawberry leaves were sprayed with ozonated water, mancozeb, thiophanate-methyl, and clear water, and the effects of different treatments on the structure, composition, and diversity of strawberry phyllosphere microbial communities were investigated using high-throughput sequencing, comparing the differences between ozonated water and traditional agents, which is beneficial to strawberry disease control and provides theoretical support for green production.

Materials and methods

Sample source and collection method

The experiment was carried out at the scientific research base of Jinhua National Agricultural Science and Technology

Park (119°37′12″ E, 29°01′4.79″ N) on October 22, 2021, and all strawberry plants tested were of the "Hongyan" variety. Strawberry seedlings were preselected for comparable growth rates. Based on the results of the pre-experiment, the ozonated water concentration was chosen to be 3–4 mg/L. Ozonated water above this concentration inhibited strawberry growth, and below this concentration, the effect was not significant. The concentrations of mancozeb and thiophanate-methyl were set according to the product instructions. Four treatments were set up in the test: 3–4 mg/L ozonated water (ozonated water), 70% Mancozeb WP 600 times (mancozeb), 500 g/L Thiophanate-methyl 600 times (thiophanate-methyl), and unaltered water spray was used as the control (CK).

Using a randomized block group design, each treatment was replicated 3 times, with 15 strawberries per replicate, and sprayed once, and strawberry leaves (approximately 50 g) were randomly collected from each group 3 days after application. After the samples were collected, they were placed in sterile sample bags and sent to the laboratory for experimental analysis.

DNA extraction

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States), following the manufacturer's instructions, and stored at −20°C prior to further analysis. The quantity and quality of the extracted DNA were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and 1.0% agarose gel electrophoresis, respectively.

16S rRNA gene fragment and ITS1 region amplicon sequencing

Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA gene fragment in the V5–V7 region was performed using the forward primer 799F (5′-AACMGGATTAGATACCCKG-3′) and the reverse primer 1193R (5′-ACGTCATCCCCACCTTCC-3′). The fungal ITS1 region was amplified using forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and reverse primer ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of reaction buffer (5×), 5 μL of GC buffer (5×), 2 μL of dNTP (2.5 mM), 1 μL of each forward primer (10 μM) and reverse primer (10 μM), 2 μL of DNA template, 0.25 μL of Q5 DNA polymerase, and 8.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 2 min, followed by 30 cycles consisting of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2 × 250 bp sequencing was performed using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequence and statistical analysis

Microbiome bioinformatics was performed with QIIME2 2019.4 with slight modifications according to official tutorials[1] (Bokulich et al., 2018). Briefly, raw sequence data were demultiplexed using the demux plugin, followed by primer cutting with the cutadapt plugin (Martin, 2011). Sequences were then quality filtered, denoised, and merged, and chimeras were removed using the DADA2 plugin (Callahan et al., 2015). Sequence data analyses were performed using the QIIME2 and R packages (v3.2.0). Alpha diversity metrics (Chao1, Shannon, Simpson) and beta diversity metrics (Bray–Curtis dissimilarity) were estimated using the diversity plugin, with samples rarefied to 345 sequences per sample. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin against the SILVA (release 132) and UNITE databases (release 8.0) (Bokulich et al., 2018). A linear discriminant analysis of the effect size (LEfSe) was performed to detect differentially abundant taxa across groups using the default parameters (LSD > 2, $P < 0.05$) (Segata et al., 2011).

Results and analysis

Diversity analysis of phyllosphere microbial community in strawberry with different treatments

The alpha diversity indices of bacteria and fungi are shown in **Table 1**. The Chao1 index showed the highest bacterial abundance after ozonated water treatment (795.41 ± 92.67) and the lowest bacterial abundance after thiophanate-methyl treatment (557.18 ± 120.97), and there was a significant difference between them. The Chao1 index of the ozonated water treatment group was significantly higher than that of the CK group (573.09 ± 221.15). After spraying mancozeb, the Chao1 index was higher than that in the thiophanate-methyl group, but there was no significant difference between them. There was no significant difference in the Shannon and Simpson indices for each treatment group.

1 https://docs.qiime2.org/2019.4/tutorials/

TABLE 1 Bacterial and fungal alpha diversity indices.

	Bacteria			Fungi		
	Chao1 index	Shannon index	Simpson index	Chao1 index	Shannon index	Simpson index
Ozonated water	795.41 ± 92.67a	5.81 ± 1.24a	0.87 ± 0.12a	57.99 ± 19.1 a	2.10 ± 0.74b	0.45 ± 0.14b
Mancozeb	701.24 ± 85.17abc	6.03 ± 0.53a	0.93 ± 0.03a	44.64 ± 12.17a	3.42 ± 1.17a	0.75 ± 0.24a
Thiophanate-methyl	557.18 ± 120.97bc	5.67 ± 1.13a	0.90 ± 0.12a	44.04 ± 14.56a	2.26 ± 0.97ab	0.56 ± 0.21ab
CK	573.09 ± 221.15c	5.63 ± 1.23a	0.91 ± 0.10a	49.13 ± 11.60a	3.24 ± 0.59ab	0.75 ± 0.10a

The Chao1 indices of strawberry phyllosphere fungi in each treatment group were, in descending order, ozonated water treatment group, CK group, mancozeb treatment group, and thiophanate-methyl treatment group but there was no significant difference between them. The Shannon and Simpson indices were both descending ordered as the mancozeb treatment group, CK group, thiophanate-methyl treatment group, ozone treatment group. The Shannon index was significantly higher in the mancozeb treatment group (3.42 ± 1.17) than in the ozonated water treatment group (2.10 ± 0.74). The Simpson index was significantly higher in both the mancozeb treatment group (0.75 ± 0.24) and CK group (0.75 ± 0.10) than in the ozonated water treatment group (0.45 ± 0.14).

β-diversity analysis was used to compare microbial community differences between treatments (Figure 1). Both bacterial and fungal NMDS analyses showed that the four treatment groups clustered together and could not be distinguished. This indicated that the bacterial and fungal compositions of the four treatment groups were not significantly different. However, the points in the ozonated water treatment group were more concentrated than in the remaining three groups.

Analysis of phyllosphere bacterial community composition of strawberry plants with different treatments

The sequencing data showed that a total of 16,628,293 high-quality reads were obtained from the four treatments, and a total of 2,791 ASVs were obtained after splicing and quality control. After species annotation, the top 10 phyla and genera of relative abundance were selected for analysis (Figure 2), among which the top 10 phyla of relative abundance were Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Chlamydiae, Patescibacteria, Planctomycetes, Acidobacteria, Cyanobacteria, and Elusimicrobia. The relative abundance of Proteobacteria was the highest in the ozone treatment group (82.17%), while there was little difference among the other treatment groups. The relative abundances of Actinobacteria in the mancozeb treatment group, the thiophanate-methyl treatment group, and the CK group were all above 15%, with little difference, while the relative abundance in the ozonated water treatment group was lower (9.38%). The relative abundances of Firmicutes in each treatment group from large to small were as follows: CK group (2.27%), mancozeb treatment group (1.87%), thiophanate-methyl treatment group (1.61%), and ozonated water treatment group (1.53%).

At the genus level, the relative abundance of *Pseudomonas* in the ozonated water treatment group was significantly higher than that in the other groups. The relative abundance of *Halomonas* was lowest in the ozonated water treatment group (8.33%) and highest in the CK group (12.02%). The relative abundance of *Nesterenkonia* in the ozonated water treatment group (48.82%) was significantly lower than that in the thiophanate-methyl treatment group (11.43%). The relative abundance of *Pantoea* in the ozonated water treatment group (2.27%) was significantly lower than that in the other treatment groups. The relative abundance of *Sphingomonas* in the CK group (0.80%) was significantly lower than that in the ozonated water (2.60%) and mancozeb treatment groups (2.54%). The relative abundance of *Mitochondria* in the ozonated water treatment group (3.05%) was significantly higher than that in the other treatment groups.

Analysis of phyllosphere fungal community composition of strawberry plants after different treatments

The sequencing data showed that a total of 4,492,346 high-quality reads were obtained from the four treatments, and a total of 392 ASVs were obtained after splicing and quality control. After species annotation, at the phylum level, the strawberry phyllosphere fungal community was mainly comprised of Basidiomycota and Ascomycota, and the relative abundances of the other phyla were all less than 0.01% (Figure 3A). The relative abundance of Basidiomycota in the ozonated water treatment group was the highest, and the CK group was the lowest. The relative abundance of Ascomycota reached 55.47% in the CK group; the relative abundance in the mancozeb treatment group (43.39%) was significantly higher than that in the thiophanate-methyl treatment group (23.13%), and the ozonated water treatment group had the lowest relative abundance (17.89%).

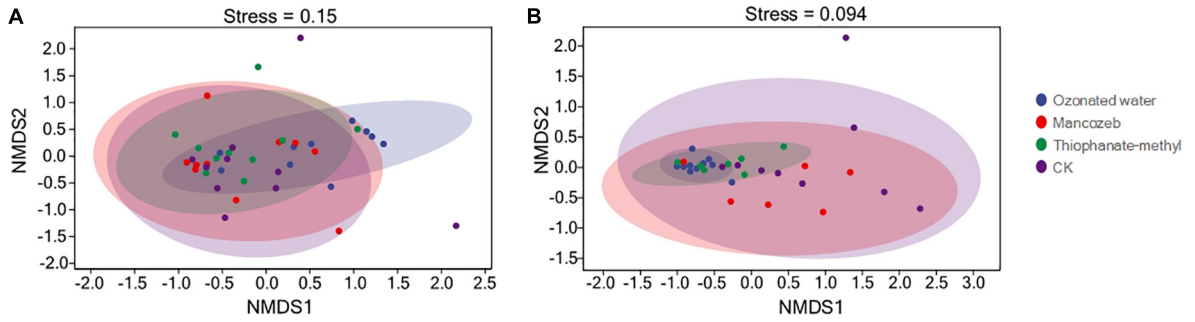

FIGURE 1
Analysis of microbial beta diversity among different treatment groups. **(A)** Non-metric multidimensional scaling (NMDS) analysis of bacteria; **(B)** NMDS analysis of fungi.

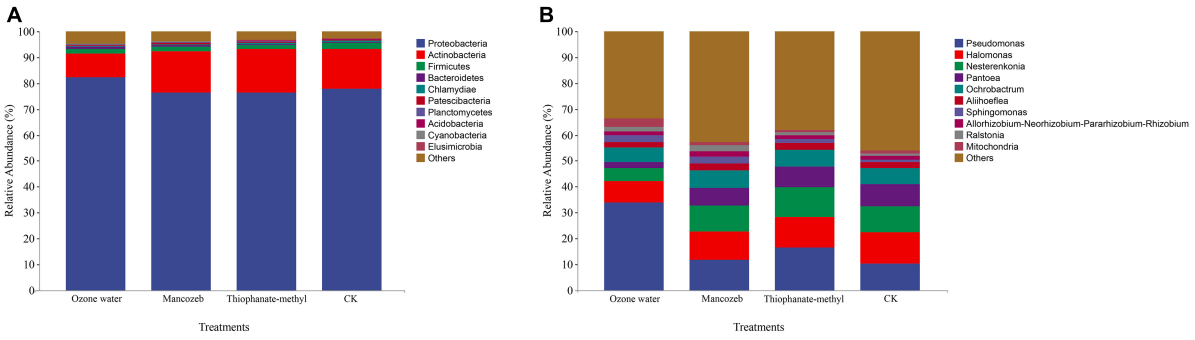

FIGURE 2
Relative abundance map of phyllosphere bacteria in different treatment groups. **(A)** Relative abundance at the phylum level; **(B)** relative abundance at the genus level.

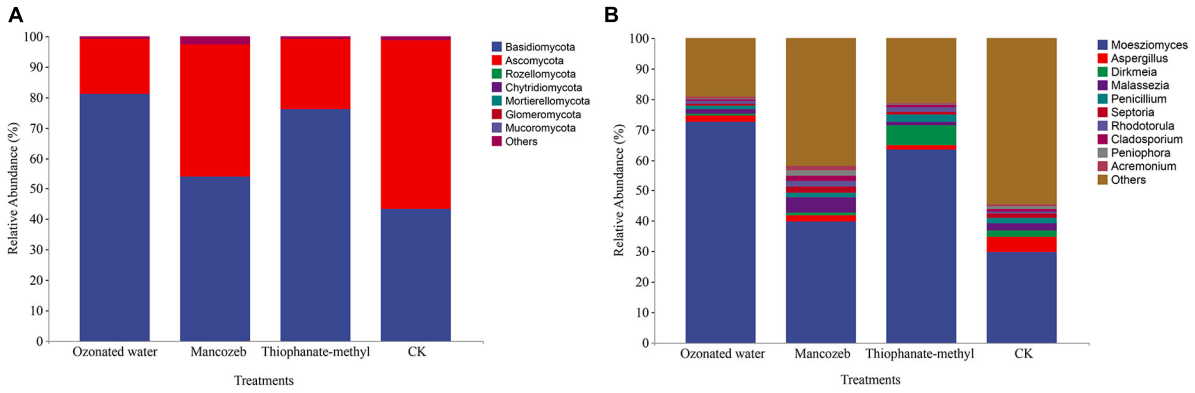

FIGURE 3
Relative abundance map of phyllosphere fungi in different treatment groups. **(A)** Relative abundance at the phylum level; **(B)** relative abundance at the genus level.

The fungal composition among the treatment groups was further analyzed at the genus level. The results are shown in Figure 3B. *Moesziomyces*, *Aspergillus*, *Dirkmeia*, *Malassezia*, and *Penicillium* were the top five genera in relative abundance in each group. The relative abundance of *Moesziomyces* significantly differed among the groups: from large to small, ozonated water treatment group (72.68%), thiophanate-methyl treatment group (63.52%), mancozeb treatment group (39.73%), and CK group (29.72%). The relative abundance of *Aspergillus* in the CK group (5.02%) was significantly higher than that

in the other treatment groups. The relative abundance of *Dirkmeia* was the highest (6.47%) in the thiophanate-methyl treatment group and the lowest (0.46%) in the ozonated water treatment group. The relative abundance of *Malassezia* in the mancozeb treatment group reached 4.89%, which was higher than in the other three treatment groups. The relative abundance of *Penicillium* in the thiophanate-methyl treated group was relatively high (2.48%), while the relative abundance of *Penicillium* in the ozonated water treatment group was low (1.11%).

Comparison of the relative abundance of strawberry pathogens in different treatment groups

The genera of the pathogenic bacteria of the main strawberry diseases in southern China were selected for analysis. The results are shown in **Table 2**. *Xanthomonas* had the highest relative abundance in the CK group and the lowest relative abundance in the mancozeb treatment group. The relative abundance of *Pseudocercospora* was zero in the mancozeb group and 0.000027 in the ozonated water treatment group. The relative abundance of *Colletotrichum* was 0.001747, 0.005743, and 0.001838 in the ozonated water, mancozeb, and thiophanate-methyl treatment groups, respectively. The relative abundance of *Pestalotiopsis* was 0 in the ozonated water and thiophanate-methyl treatment groups and 0.003497 in the mancozeb treatment group. The relative abundance of *Botrytis, Podosphaera,* and *Sphaerotheca* was 0 in all treatment groups.

Differential analysis of phyllosphere microorganisms on strawberry plants after different treatments

LEfSe analysis was used to analyze the enriched characteristic groups in different treatments, with the linear discriminant analysis threshold set to 2. The bacterial results are shown in **Figure 4A**. The ozonated water treatment group had the most biomarkers, including Bacteroidia, Thermoanaerobaculia, Erysipelotrichia, and KD4_96; Pseudomonadales, Rickettsiales, Bacteroidales, and seven other orders; Pseudomonadaceae, Mitochondria, Leptotrichiaceae, and 12 other families; and *Pseudomonas*, *Mitochondria*, *Leptotrichia*, and 19 other genera. The mancozeb treatment group had nine biomarkers, including Ruminococcaceae Marinobacteraceae, Parvularculaceae, Methylophilaceae, and five genera. *Chthoniobacterales* is a biomarker for the thiophanate-methyl treatment group. *Lactococcus and Pajaroellobacter* were biomarkers for the CK group.

When the relative abundance of fungal communities in the different treatment groups was compared, the threshold was set to 2. The results are shown in **Figure 4B**. A total of 15 biomarkers were present in the different treatment groups. The highest number of biomarkers was found in the CK group, including Dothideomycetes, Capnodiales, Ganodermataceae, Bolbitiaceae, Bolbitius, Ganoderma, and Emericellopsis. Botryosphaeriales, Phyllostictaceae, Periconiaceae, and Periconia were included in the thiophanate-methyl treatment group. The mancozeb treatment group included Sordariomycetes, Hypocreales, and Pleosporales. The ozonated water treatment group had the fewest biomarkers, only Agaricales.

Discussion

Effects of different treatments on the diversity of the phyllosphere microbial community of strawberry plants

The living environment of phyllosphere microorganisms changes rapidly, and factors such as agricultural cultivation measures, plant genotypes, and developmental stages affect the abundance and diversity of phyllosphere microorganisms (Whipps et al., 2008; Bringel and Couee, 2015; Lajoie et al., 2020). Bacteria and fungi are two types of phyllosphere microorganisms that researchers have focused on. Microbial–biological and microbe–host-plant interactions affect the fitness of plants in nature, the yield of crops, and the safety of horticultural products (Whipps et al., 2008). Bacteria and

TABLE 2 Relative abundance of strawberry pathogens.

	Genus	Ozonated water	Mancozeb	Thiophanate-methyl	CK
Bacteria	*Xanthomonas*	0.000081	0.000007	0.000448	0.000560
Fungi	*Pseudocercospora*	0.000027	0.000000	0.000257	0.000981
	Colletotrichum	0.001747	0.005743	0.001838	0.000895
	Pestalotiopsis	0.000000	0.003497	0.000000	0.000026
	Botrytis	0.000000	0.000000	0.000000	0.000000
	Podosphaera	0.000000	0.000000	0.000000	0.000000
	Sphaerotheca	0.000000	0.000000	0.000000	0.000000

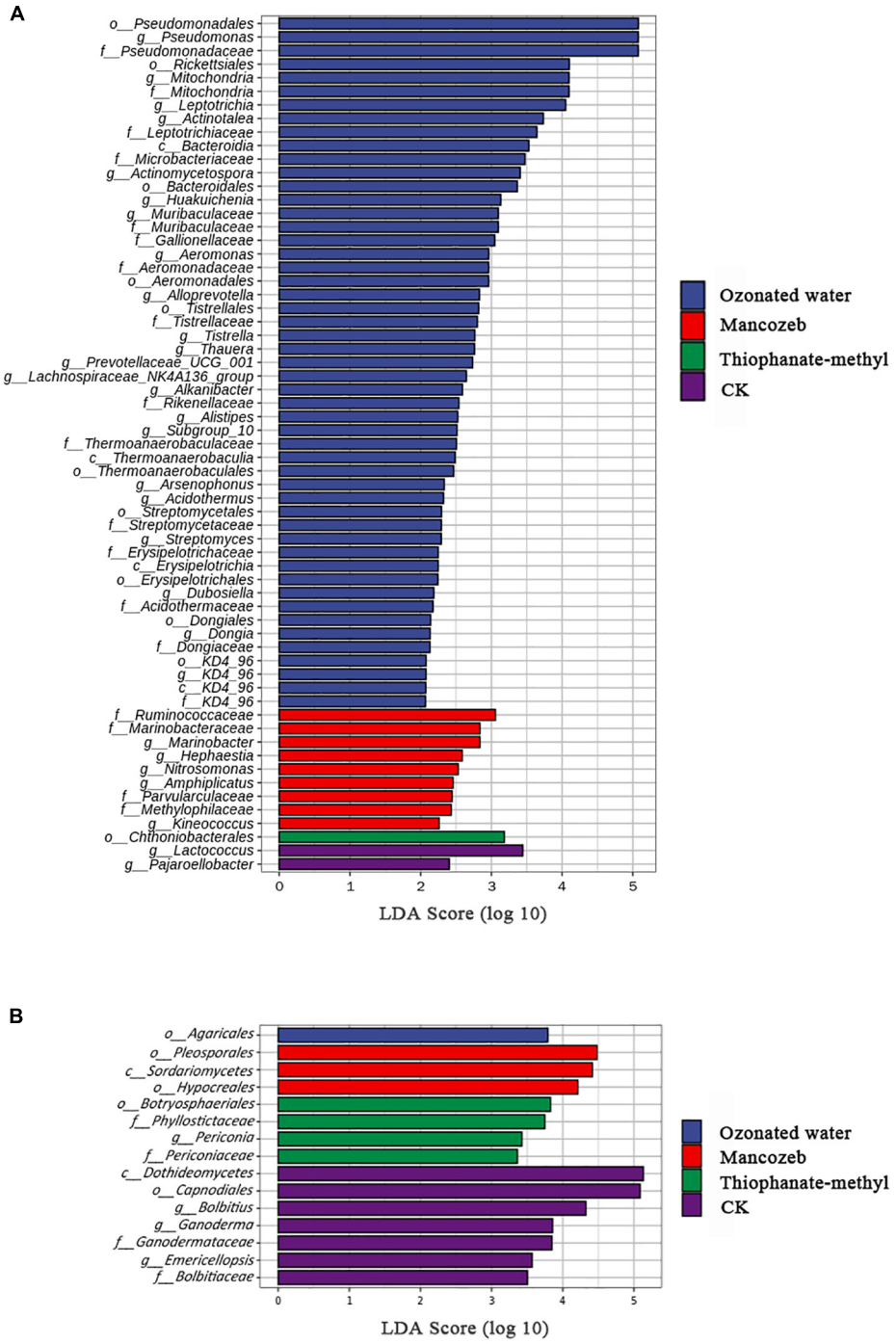

FIGURE 4
Linear discriminant analysis effect size (LEfSe) analysis of phyllosphere microorganisms of different treatments: **(A)** bacteria and **(B)** fungi.

fungi are the main phyllosphere microorganisms of strawberry plants, and most diseases of strawberry plants are directly related to fungi, with the yield and quality of strawberries greatly reduced when the disease is severe (Amil-Ruiz et al., 2011; Holmes et al., 2020). *Fusarium oxysporum* can cause strawberry wilt, which causes the plant to collapse and die (Koike and Gordon, 2015). The main pathogens of strawberry anthracnose are the following three species: *Colletotrichum fragariae*, *C. gloeosporioides*, and *C. acutatum*, which can lead to plant death in severe cases (Brooks, 1931; Arroyo et al., 2007;

Widiastuti et al., 2013). Powdery mildew is one of the main facility diseases of strawberry, mainly caused by *Podosphaera aphanis* (Feng et al., 2020). After spraying leaves with ozonated water, the richness of bacteria and fungi in the strawberry phyllosphere significantly increased, and the diversity of fungi was reduced, but the effect on bacterial diversity was not significant. Spraying mancozeb increased the bacterial richness and fungal diversity of strawberry leaves and reduced fungal richness and bacterial diversity, but the effect was not significant. The application of thiophanate-methyl had no significant effect on the bacterial and fungal richness and diversity of the strawberry phyllosphere. As a broad-spectrum green fungicide, ozonated water has a good bactericidal effect, but it also has a negative impact on beneficial bacteria. After spraying ozonated water, the richness of bacteria and fungi in the strawberry phyllosphere increased, which may be because, after ozonated water depopulated the phyllosphere, it was repopulated from the surrounding soil and air. Because the collected samples were in the same environment, the microorganisms recolonizing the leaves were similar, and the points causing ozonated water in the NMDS analysis were more concentrated compared to the other treatment groups. As a protective fungicide, mancozeb can form a protective layer on the surface of leaves and fruits, preventing pathogen invasion and reducing the activity of various enzymes required for the normal physiological metabolism of pathogens (Jabeen et al., 2011). Thiophanate-methyl is a systemic broad-spectrum fungicide that can kill microorganisms on the leaf surface (Papadopoulou-Mourkidou, 1991). This prevents the immediate repopulation of the phyllosphere from air and soil microbes, causing a decrease in the richness and diversity of phyllosphere microorganisms.

Effects of different treatments on the composition of strawberry phyllosphere microbial community

The present study found that *Pseudomonas*, *Halomonas*, *Nesterenkonia*, *Pantoea*, and *Ochrobactrum* were the dominant bacterial genera on strawberry leaves. *Moesziomyces*, *Aspergillus*, and *Dirkmeia* were found to be the dominant fungal genera. This is inconsistent with Olimi et al. (2022), but it may be caused by factors such as cultivation conditions and strawberry variety. At the fungal phylum level, ozonated water treatment decreased the relative abundance of Ascomycota and increased the relative abundance of Basidiomycota. The most common diseases of strawberry plants are fungal diseases (such as anthracnose, powdery mildew, and root rot), and the remaining pathogenic fungi belong to *Ascomycota* (Zhang and Zhong, 2018; Weber and Hahn, 2019; Yang et al., 2019). Therefore, the application of ozonated water sprays can effectively reduce pathogenic fungi populations in strawberry leaves, thereby reducing the incidence of plant diseases. Mancozeb and thiophanate-methyl are both fungicides and have no disinfecting effect on bacteria; thus, compared with water, the difference in bacterial composition is not obvious. Since mancozeb is a protective fungicide, the relative abundance of Ascomycota on the leaf surface is not clearly different from that of the CK group, while thiophanate-methyl is a broad-spectrum fungicide that can directly reduce fungal populations.

Some bacteria of the genus *Pseudomonas*, such as *P. fluorescens*, can symbiotically combat plant diseases (Sun et al., 2008; Bai et al., 2014). They can be used as important potassium-solubilizing and scale-solving bacteria in soil to improve soil nutrient utilization (Bai et al., 2014). In the post-harvest preservation of strawberries, apples, and grapes, these bacteria can also effectively reduce the incidence of gray mold and acid rot (Sun et al., 2008; Ilhan and Karabulut, 2013). *Actinomycetospora* has antibacterial activity and has an inhibitory effect on fungi (Phongsopitanun et al., 2020). After applying ozonated water, *Pseudomonas* and *Actinomyces* were significantly enriched in the strawberry phyllosphere, indicating that spraying ozonated water enriched beneficial bacteria and increased the resistance of strawberry plants to several agricultural pathogens.

Aspergillus and *Penicillium* are common fungi in the phyllosphere, and fungi from these two genera are often associated with diseases (Wang et al., 2008; Gupta and Rathore, 2013). *Aspergillus* causes mildew in tobacco and is the main fungus responsible for medicinal material decomposition during storage (Guo and Pang, 2018; Chen et al., 2019). *Penicillium* infects the surface of strawberries and produces mycotoxins, causing food-safety hazards (Chao et al., 2017). After applying ozonated water, the relative abundance of *Aspergillus* and *Penicillium* decreased, indicating that spraying ozonated water can kill harmful fungi in strawberry leaves and can effectively prevent pathogens from being transferred from leaves to fruit, thereby extending the shelf life of strawberries. The number of enriched fungi in the CK group was significantly higher than those in the other groups, indicating that ozonated water, mancozeb, and thiophanate-methyl could effectively kill strawberry phyllosphere fungi, with ozonated water showing the most promise.

Effects of different treatments on strawberry pathogens

Xanthomonas fragariae is the causal agent of strawberry bacterial angular leaf spot disease, which produces irregular spots on the leaves after infection and causes blackening and wilting of the eyes of the main stem shoots in severe cases (Kim et al., 2016). *Pseudomonas* and *Rhizobium* were found to inhibit the growth of *X. fragariae* (Henry et al., 2016). The highest relative abundance of *Pseudomonas* was found in the ozonated water treatment group, and the relative abundance

of *Xanthomonas* decreased after the application of ozonated water, indicating that ozonated water has the potential to control strawberry bacterial angular leaf spot disease. *Pseudocercospora fragarina* is the causal agent of gray spot disease in strawberry, which occurs mainly in the spring. The relative abundance of *Pseudocercospora* decreased after the application of ozonated water and the chemical mancozeb, which are used in production for the control of gray spots and may explain the low relative abundance of *Pseudocercospora*. In this study, the relative abundance of *Colletotrichum* was found to be higher in all four treatment groups compared to the other strawberry pathogens. The experimental area was located in Jinhua, Zhejiang Province, China, with high precipitation in October and average daytime temperatures of around 24°C, a period of high strawberry anthracnose incidence. Among the three groups of fungicide treatments, the ozonated water treatment group had the lowest relative abundance of *Colletotrichum*, indicating the potential of ozonated water to prevent anthracnose. In contrast, the relative abundance of the control *Colletotrichum* was 0.000895, which may be caused by the decrease in the richness of some other microbes inhibiting *Colletotrichum*. Although the relative abundance of *Colletotrichum* was higher in the ozonated water treatment group and the pharmaceutical treatment group than in the CK group, overall, the relative abundance of *Colletotrichum* was not high in either group, and the strawberry's own defense mechanisms resisted the invasion of these pathogens, and anthracnose had not yet occurred. The pathogenic fungi of strawberry root rot are more complex, and *Pestalotiopsis* and *Colletotrichum* can cause strawberry root rot (Salinas et al., 2020; Baggio et al., 2021). *Botrytis cinerea* and *P. aphanis* are the causal agents of gray mold and powdery mildew, respectively, in strawberry (Kamaruzzaman et al., 2018; Feng et al., 2020). The study reported that *Sphaerotheca macularis* can also cause the occurrence of powdery mildew in strawberries (Mahmud et al., 2020). These two diseases occur primarily in the spring in Zhejiang Province. In this study, the sampling time was October, when a high incidence of gray mold and powdery mildew was not reached, so pathogenic fungi were not detected.

Conclusion and prospects

As a green fungicide, ozonated water can effectively reduce the incidence of crop diseases. Our research on the phyllosphere microbial community of strawberry plants under different treatments showed that spraying ozonated water, mancozeb, and thiophanate-methyl did not have a great influence on the diversity of the strawberry phyllosphere microbial community. Ozonated water can effectively reduce the relative abundance of pathogenic fungi (*Aspergillus* and *Penicillium*) in strawberry leaves and foster the growth of beneficial microorganisms, including *Pseudomonas* and *Actinomyces*. Compared with traditional pesticides, ozonated water has a more obvious bactericidal effect and does not pollute the environment. In the future, diseased strawberries can be used to explore the effect of ozonated water on phyllosphere microorganisms, and *in vitro* experiments can be conducted to further explore the effect of ozonated water on strawberry phyllosphere fungi and bacteria.

Data availability statement

The data presented in this study are deposited in the Genome Sequence Archive, (GSA) repository, accession number: CRA007542.

Author contributions

PS, XL, JS, and HJ designed the experiments. JW, YiW, CC, JZ, and YqW performed the experiments. PS, JW, and XL analyzed the data and compiled the figures. PS, JW, and JS wrote the manuscript. YqW, HJ, and JS edited the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deciphering the core seed endo-bacteriome of the highland barley in Tibet plateau

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Highland barley (*Hordeum vulgare* var. *nudum* (L.) Hook.f., qingke) has unique physical and chemical properties and good potential for industrial applications. As the only crop that can be grown at high altitudes of 4200–4500 m, qingke is well adapted to extreme habitats at high altitudes. In this study, we analysed the seed bacterial community of 58 genotypes of qingke grown in different regions of Tibet, including qingke landraces, modern cultivars, and winter barley varieties, and characterised endophytic bacterial communities in seeds from different sources and the core endo-bacteriome of qingke. This study aim to provide a reference for the application of seed endophytes as biological inoculants for sustainable agricultural production and for considering microbe-plant interactions in breeding strategies. A total of 174 qingke seed samples from five main agricultural regions in Tibet were collected and subjected to investigation of endophytic endo-bacteriome using high-throughput sequencing and bioinformatics approaches. The phyla of endophytic bacteria in qingke seeds from different sources were similar; however, the relative proportions of each phylum were different. Different environmental conditions, growth strategies, and modern breeding processes have significantly changed the community structure of endophytic bacteria in seeds, among which the growth strategy has a greater impact on the diversity of endophytic bacteria in seeds. Seeds from different sources have conserved beneficial core endo-bacteriome. The core endo-bacteriome of qingke seeds dominated by Enterobacteriaceae may maintain qingke growth by promoting plant growth and assisting plants in resisting pests and diseases. This study reveals the core endo-bacteriome of qingke seeds and provides a basis for exploiting the endophytic endo-bacteriome of qingke seeds.

KEYWORDS

highland barley, seed, endophytes, core endo-bacteriome, qingke

Introduction

Endophytes are a class of non-pathogenic microorganisms that complete part or all their life cycles in plant tissues and are closely related to their host plants (Wilson, 1995; Farrar et al., 2014; Pitzschke, 2016). The existence of some endophytes can promote the growth of host plants and enhance the ability of host plants to utilise environmental nutrients, help plants resist biotic and abiotic stresses, and affect the competitiveness of host plants (Hardoim et al., 2012; Vandenkoornhuyse et al., 2015; Cope-Selby et al., 2017; Khalaf and Raizada, 2018; Tyc et al., 2020). Although complex endophyte communities are distributed in all plant organs, seed-distributed endophytes are of particular interest; in contrast, non-systemically horizontally transmitted endophytes, which systematically propagate through host plant seeds, and sexually vertically transmitted endophytes appear to have a higher probability of mutualism (Ewald, 1987; Rudgers et al., 2009; Saari et al., 2010; Gundel et al., 2011; Truyens et al., 2015).

Seeds are one of the most important stages in plant life. It is the beginning of a new life cycle of plants and the key to expanding the distribution range of plant populations and adapting to new environments (Truyens et al., 2015). Microorganisms in mature seeds are the endpoint of plant endophyte community assembly in seeds and the starting point of seedling endophyte community assembly, which may be an important source of endophytes in seedlings and adult plants (Khalaf and Raizada, 2018; Khalaf and Raizada, 2016). Compared to many soil-borne microorganisms, the endophyte community of seeds can better adapt to the life of plant tissues and has the advantage of colonisation (Truyens et al., 2015; Cope-Selby et al., 2017; Mukherjee et al., 2020). A suitable endophyte community play a positive role in the process of seed germination and seedling establishment, which can regulate the growth and development of seedlings and enhance the disease resistance (Pitzschke, 2016; Cope-Selby et al., 2017; Verma et al., 2017; Verma and White, 2018; Matsumoto et al., 2021). The development of biological inoculants with seed endophytes is considered a feasible method for improving agricultural productivity and developing sustainable agriculture; however, our research on seed endophytes is still limited (Mukherjee et al., 2021), particularly in plants grown in extreme environments (Compant et al., 2016).

The Qinghai-Tibet Plateau, known as the "Roof of the World" and the "Third Pole", is the highest plateau in the world, with an average altitude of over 4,000 m and a changeable and extreme climate (Yao et al., 2012). The highland barley (*Hordeum vulgare* var. *nudum* (L.) Hook.f., qingke) is called "Qingke" in Chinese and "nas" in Tibetan. As one of the earliest crops domesticated by humans, qingke is an important crop for the production of food and medicines, beverages, and energy and has good industrial application potential (Palumbo et al., 2017; Zeng et al., 2018; Guo et al., 2020; Obadi et al., 2021; Yin et al., 2022). Qingke is the only crop that can be grown at high altitudes of 4200-4500 m. Its good frost resistance makes it a staple food for Tibetans at least 3500-4000 years ago, the main crop varieties accounting for 43% of the total area of crops planted on the Qinghai-Tibet Plateau (Liu et al., 2013; d'Alpoim Guedes et al., 2015; Wang et al., 2017; Zeng et al., 2018). Because of the unique climatic conditions and geographical environment of the Qinghai-Tibet Plateau, compared with other barley genotypes, qingke had a founder effect event that lasted for 2,500 years before approximately 4,500-2,000 years, which makes qingke an untapped resource of unique seed endophytes (Zeng et al., 2018).

In this study, we employed a high-throughput sequencing approach to investigate the seed endophytic bacterial community in 58 qingke genotypes collected from five major agricultural regions in the Tibetan Plateau, including qingke landraces, modern cultivars, and winter barley. By analysing the diversity and composition of endophytic bacteria in these qingke seeds, the potential impact of seed core endo-bacteriome on the growth of qingke was inferred, which provides a basis for harnessing bacterial seed endophytes for sustainable barley production.

Materials and methods

Seeds sampling

The qingke landrace varieties were provided by the Tibet Academy of Agriculture and Animal Husbandry Sciences, and the modern cultivars and winter barley varieties were collected from Shigatse and Qamdo, Tibet, respectively. No pesticides were applied to the seed collection area. For comparison, we divided 58 qingke genotypes seeds from different sources into Lhasa landraces (Lh), Lhoka landraces (Lk), Nyingchi landraces (Ny), Qamdo landraces (Qa), Shigatse landraces (Sh), modern cultivars (MC), and winter barley (WB) 7 categories, and each seed contained three biological replicates for a total of 174 samples. One gram of barley seeds (composed18-22 seeds) was used for each sample.

Total community DNA extraction

The seed samples were soaked in 75% alcohol for 5 min and then rinsed with sterile deionised water for 1 min. Surface cleaned seed samples are ground using a sterile mortar after liquid nitrogen freezing. Total DNA was extracted using a Plant DNA Extraction Mini Kit B (Mabio Co., Guangzhou, China) according to the manufacturer's instructions under sterile conditions, and the purity and concentration of the total DNA were detected using a Thermo NanoDrop One spectrophotometre (Thermo Fisher Technology Co., USA).

16S rRNA gene amplification

According to the Earth Microbiome Project protocol (Thompson et al., 2017), universal bacterial primer set (pair) 515f (5′ GTGYCAGCMGCCGCGGTAA), 806r (5′ GGACTACHVGGTTWTAAT), and TaKaRa Premix Taq® Version 2.0 (TaKaRa Biotechnology Co., Dalian, China) were used for each PCR amplification of the total DNA of the samples, and all PCR reactions were performed in triplicate. The PCR products were sent to Magigene Co., Ltd. (Guangzhou, China) to sequence the constructed amplicon library using the Illumina Nova PE250 platform after initial quality control and adapter ligation.

Bioinformatics analysis

Clean paired-end reads were obtained after the primer sequences were removed using Cutadapt. Usearch V10 was used to filter unmatched tags to obtain the original spliced sequence. Using Fastp V0.14.1 to trim the original spliced sequence data with sliding window quality and obtain valid spliced fragments, the UPARSE software was then used to cluster operable taxonomic units (OTUs) with 97% similarity. Representative sequences of each OTU were aligned in the SILVA V132 database using Usearch V10 to obtain species annotation information. After the annotation information was obtained, OTUs that could not be annotated to the kingdom and the OTUs annotated to chloroplasts, mitochondria, and archaea and their tags were deleted. Principal coordinate analysis (PCoA), ANOSIM analysis, and alpha diversity index were calculated using the vegan package of R V4.1.2, based on the flattened OTU table, and the Wilcoxon rank sum test and Kruskal-Wallis test were calculated using R V4.1.2. Correlation network analysis was performed using the Hmisc package of R V4.1.2, and Gephi was used for image drawing. Finally, we used LEfSe analysis to identify endophytic bacterial taxa with significantly different abundances in the seeds of different types.

Result

Diversity of endophytic bacteria in qingke seeds

From the α diversity index (Figure 1A), in terms of the average Chao1 index, Nyingchi landraces were the highest, followed by Shigatse landraces, Qamdo landraces, modern cultivars, and winter barley is relatively low, the difference analysis showed that the Chao1 index of Nyingchi landrace was significantly higher than that of winter barley; in terms of the mean value of the Shannon diversity index, Nyingchi landrace was the highest, followed by modern cultivars, Shigatse landrace and Qamdo landrace were also higher, and Lhasa landrace was relatively lower, difference analysis showed that the Shannon diversity indices of Nyingchi and Shigatse landraces were significantly higher than those of winter barley; in terms of the average Simpson diversity index, Nyingchi landraces were the highest, followed by modern cultivars, Qamdo landraces and Shigatse landraces were also higher, and Lhasa landraces were relatively low. The uniformity of endophytic bacteria in qingke seeds from different types showed that the distribution of endophytic bacteria in Nyingchi landrace was the most uniform, the distribution of endophytic bacteria in modern cultivars, Qamdo landrace, and Shigatse landrace was also relatively uniform, and the distribution of endophytic bacteria in Lhasa landrace was the most uneven.

Community structure of endophytic bacteria in qingke seeds from different sources

The β-diversity of qingke seed endophytes was assessed by PCoA analysis based on species-level Bray-Curtis distance, and the differences in the community structure of seed endophytes from different sources were calculated using ANOSIM (Figure 1B). The results showed significant differences in the community structure of endophytic bacteria in seeds from different sources (R=0.219, P=0.001). Among the qingke landraces, the endophytic bacterial community structures in seeds from Nyingchi and Qamdo were relatively similar, those from Lhoka and Shigatse were similar, and modern cultivars were similar to those of winter barley.

The composition of endophytic bacterial phyla in qingke seeds from different sources was similar. As shown in Figure 2 and Figure 3A, Proteobacteria is the most important bacterial phylum (64.6%), in Lhasa landrace (71.7%), Lhoka landrace (72.6%), Nyingchi landrace (37.8%), Qamdo landrace (56.7%), Shigatse landrace (66.9%), modern cultivars (57.0%) and winter barley (69.5%) accounted for a higher proportion. Firmicutes (12.4%, 9.4%, 12.4%, 14.6%, 8.8%, 8.6%, and 9.7%), Bacteroidetes (3.8%, 7.3%, 18.4%, 10.2%, 9.5%, 15.6%, and 5.6%), the two accounted for 10.1% and 9.5% of the total OTU respectively. Actinobacteria (6.9%, 5.4%, 12.4%, 6.2%, 5.7%, 5.7%, and 9.5%), and Fusobacteria (3.4%, 1.3%, 7.4%, 4.3%, 1.8%, 4.9%, and 3.7%) accounted for a relatively small proportion, the two accounted for 6.5% and 2.9% of the total OTU number, respectively, and other categories accounted for less than 2% of the seeds from different sources. Among the 27 phyla, bacteria from 11 phyla were shared by all types of qingke (Figure 3C). Among the seeds from different types, Shigatse landrace had the most endophytic bacteria phyla, including 23 phyla, and landraces from Lhoka and Qamdo also had more endophytic bacterial phyla, including 18 phyla, which originated

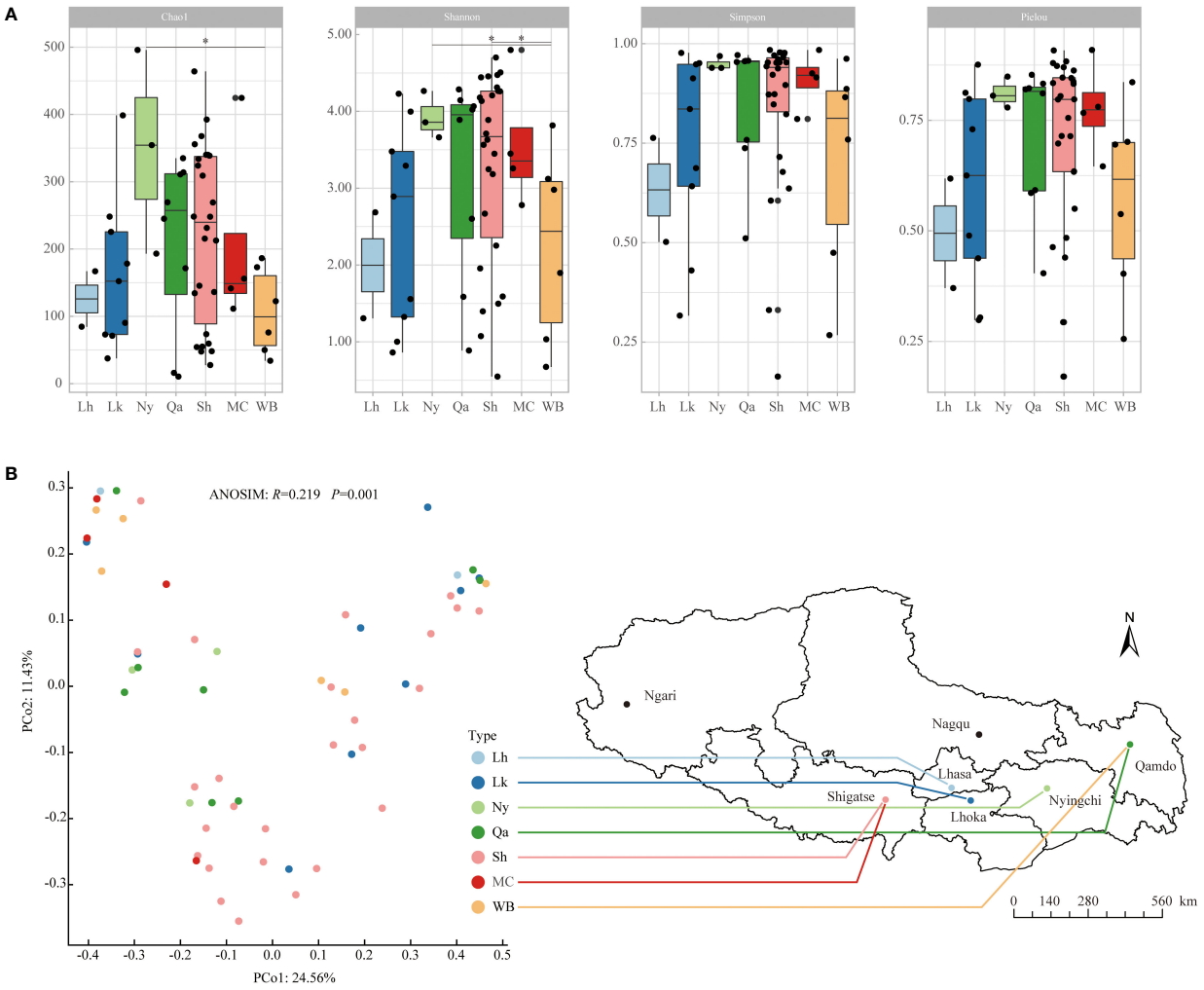

FIGURE 1
Alpha diversity and beta diversity of endophytic bacterial communities in different types of qingke seeds. **(A)** Alpha diversity. The "*" represent significant difference among different types of qingke seeds at 0.05 level. **(B)** Beta diversity was evaluated by PCoA ranking based on Bray-Curtis distance. Different colored dots represent different types of qingke seeds. Using ANOSIM analysis to assess the significance of differences in endophytic bacterial community structure in different types of qingke seeds. Lh, Lhasa landrace; Lk, Shannan landrace; Ny, Nyingchi landrace; Qa, Qamdo landrace; Sh, Shigatse landrace; MC, modern cultivars; WB, winter barley.

from the Lhasa landrace, and there were relatively few endophytic bacterial phyla, including 12 phyla.

The genus composition of qingke seeds from different sources showed (Figure 3B), Enterobacteriaceae in Lhasa landraces (36.0%), Lhoka landraces (26.9%), Nyingchi landraces (8.2%), Qamdo landraces (18.6%), Shigatse landraces (18.8), and winter barley (17.2) accounted for a higher proportion; *Acinetobacter* accounted for a higher proportion of Lhasa landraces (24.6%) and modern cultivars (21.4%); *Pseudomonas* accounted for Lhoka landraces (21.0%), Shigatse landraces (16.6%) and winter barley (24.1%) accounted for a higher proportion; *Prevotella* accounted for a higher proportion of Nyingchi landraces (11.5%) and modern cultivars (8.9%); *Paenibacillus* accounted for Qamdo landraces (7.7%), Lhasa landrace (7.7%) and Lhoka landrace (3.9%) accounted for a higher proportion. Among the seeds from all sources, Shigatse landraces had the most genera (579), Lhasa landraces had the least genera (82), and 38 genera (5.2%) were shared by different types of qingke seeds (Figure 3D).

Correlation network of endophytic bacteria in qingke seeds

Symbiotic bacterial network analysis can reveal the relationships among bacterial genera. To avoid complex

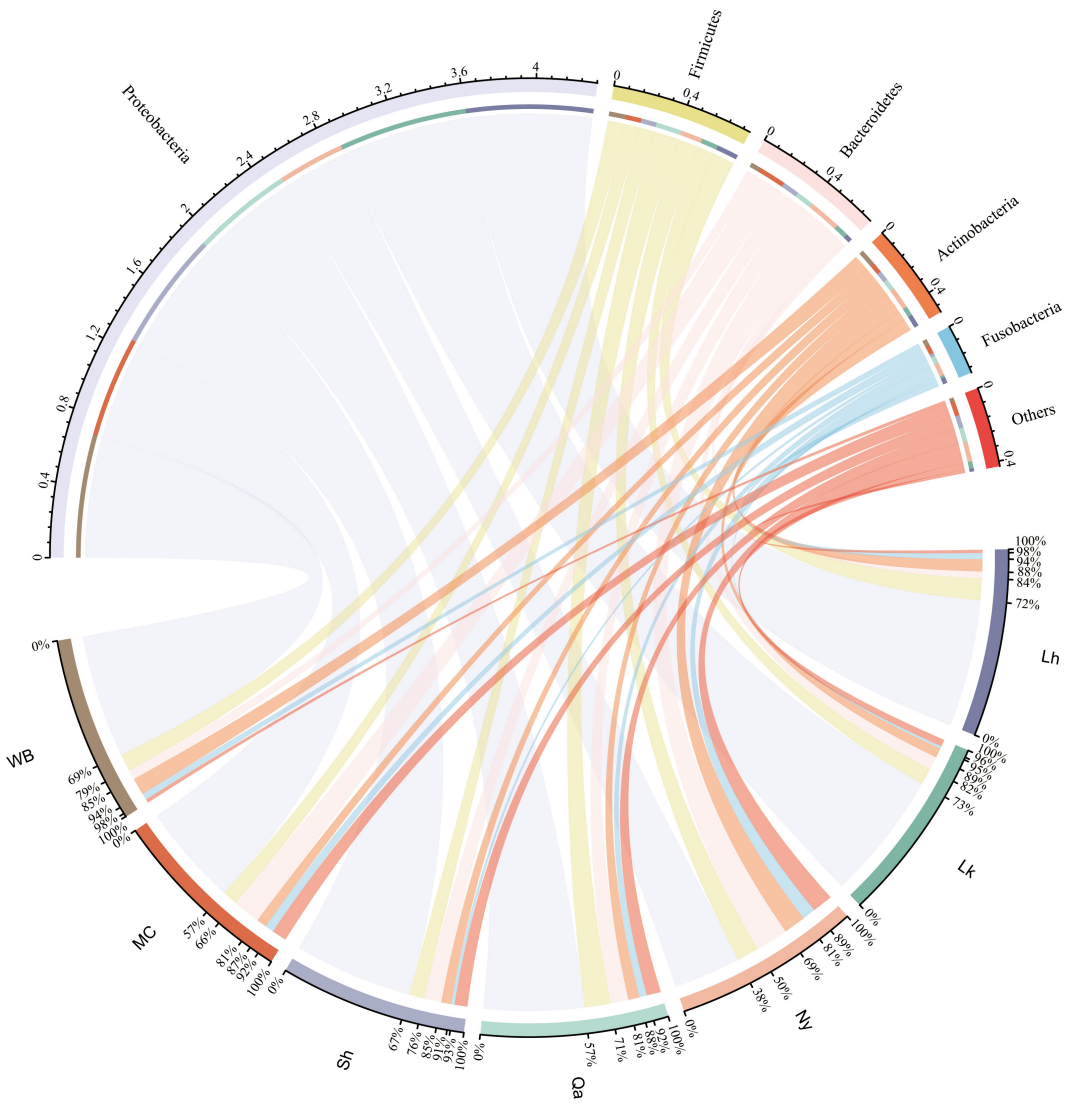

FIGURE 2
Composition of endophytic bacteria in different types of qingke seeds. Only the top 5 most abundant phyla are displayed, and the less abundant phyla are summarized as "Others".

images, the number of nodes is limited to 200. The network has a total of 1233 edges, of which 1193 edges are positively correlated and 40 are negatively correlated, and the average degree of the network is 12.3. As shown in Figure 4, the qingke endophytic bacterial genera can be divided into two groups. The *hgcI_clade* in the left-hand association group was closely related to other bacterial genera in the group, and its degree of connection was the highest among all nodes (2.1%). This was followed by o_Acidobacteriales (2.0%), *Escherichia-Shigella* (1.9%), *ADurb.Bin063-1* (1.8%), *CandidatusSolibacter* (1.7%) and *Pontibacter* (1.7%). In the right association group, *Prevotella* and *Burkholderia-Caballeronia-Paraburkholderia* were closely related to other bacterial genera, and the degree of connection was 1.4%. This was followed by *Streptococcus* (1.3%), *Fusobacterium* (1.2%), and f_Pasteurellaceae (1.2%).

Differences in abundance of endophytic bacteria in qingke seeds from different types

Significant differences in the endophytic bacterial communities or species in different types of qingke seeds were investigated using LEfSe. Statistical analysis from the phylum to species level was performed in the cladogram, and LEfSe confirmed an LDA score of 3.3 or higher (Figures 5A, B), and

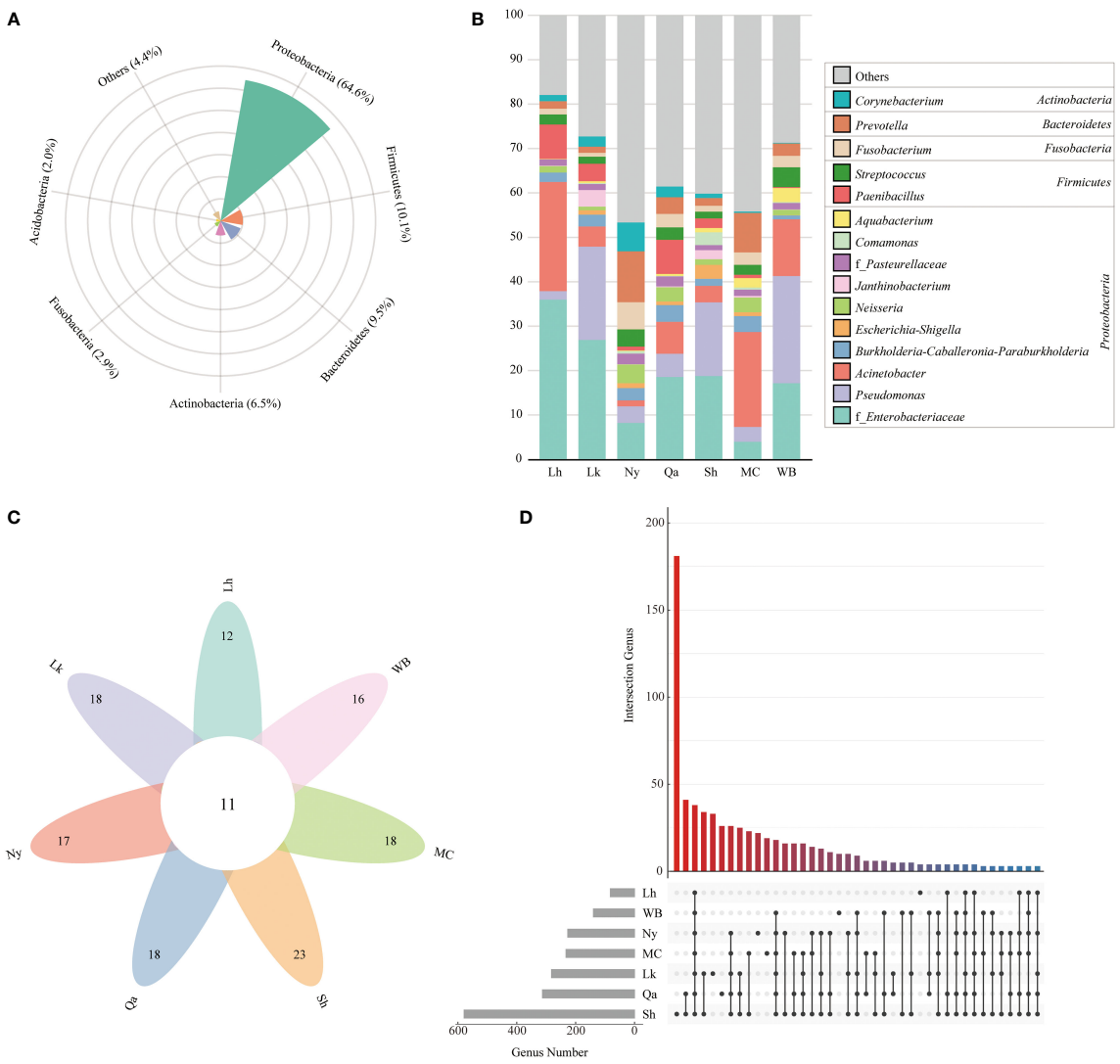

FIGURE 3
Similarity of endophytic bacterial composition of different types of qingke seeds. **(A)** The composition of endophytic bacteria in qingke seeds. The phyla with abundances below 2% are summarized as "Others". **(B)** The genus composition of different types of qingke seeds. Taxa with abundances below 1% are summarized as "Others" **(C)** Venn diagram of the seed phylum level of different types of qingke. **(D)** The genus level upset of different types of qingke seed genus.

LEfSe analysis showed that there were 68 biomarkers. There were 10 taxa in Lhasa landraces, the taxa that contributed the most to Lhasa landraces were Paenibacillaceae (at the family level), *Paenibacillus* (at the genus level), and *Paenibacillus*_sp (at the species level). There are 9 taxa of Lhoka landraces, among which the taxa that contributes the most to Lhoka landraces is Actinobacteria (at the class level); there are 13 taxa of Nyingchi landraces, of which Bacteroidetes (at the phylum level) and Bacterodia (at the class level) are the taxa that contribute the most to Nyingchi landraces; there are 5 taxa of the landraces in Qamdo, of which the taxa that contributes the most to the landraces in Qamdo is the uncultured_bacterium (at the genus level) of the order Leptotrichaceae; There are 18 taxa of landraces in Shigatse, among which Betaproteobacteriales (at the order level) contributes the most to the landraces of Shigatse; there are 6 taxa of modern cultivars, of which the taxa that contributes the most to the modern cultivars is *Acinetobacter* (at the genus level); there are 7 taxa of winter barley, of which the taxa that contributes the most to winter barley is Pseudomonadales (at the order level).

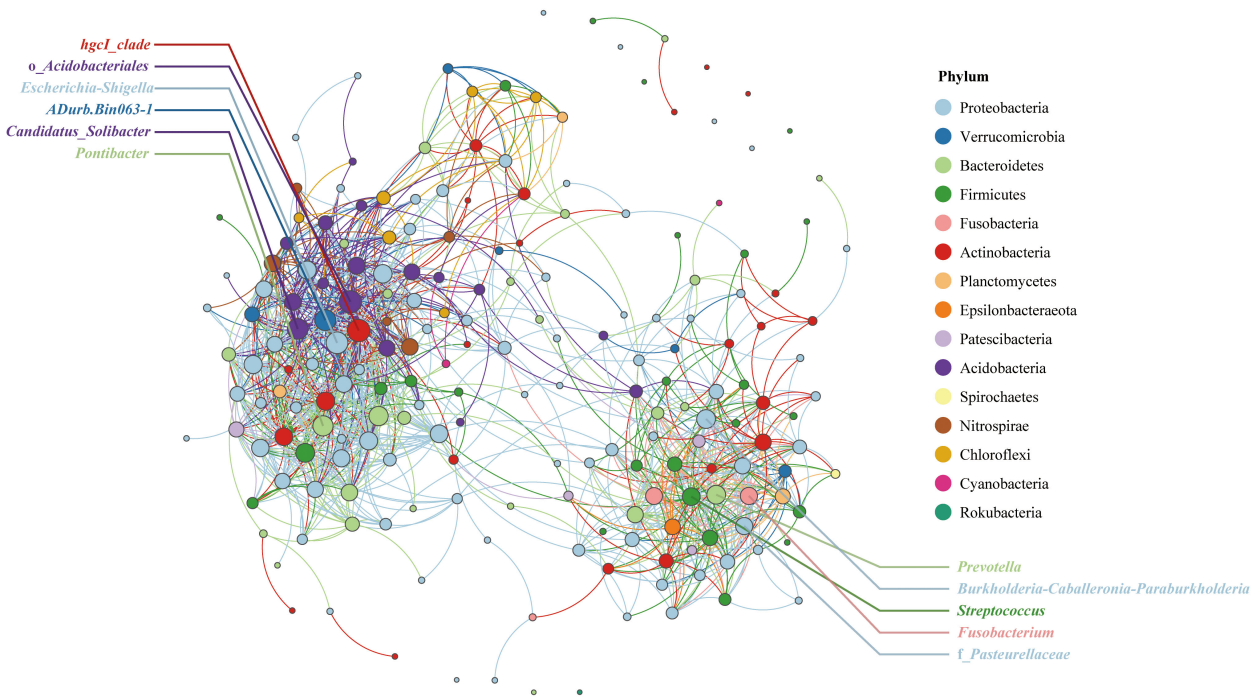

FIGURE 4
Correlation network analysis at the genus level of endophytic bacteria in qingke seeds. The node colors represent different bacterial phyla, and the node size is proportional to the degree.

Discussion

Beneficial endophytes in seeds can promote seedling growth, increase the nutrient supply to host plants, and improve their resistance to biotic and abiotic stresses (Gond et al., 2015; Berg and Raaijmakers, 2018). At present, the possibility of targeted modification of seed microbiota to create optimal plant microbiota combinations to improve plant performance has been demonstrated (Mitter et al., 2017). The climatic conditions of the Qinghai-Tibet Plateau are harsh. As one of the most important crops on the Qinghai-Tibet Plateau, qingke has become a staple food for Tibetans because of its adaptation to extreme environments at high altitudes. Research on the endophytic bacteria in qingke seeds will provide a reference for the development and application of seed endophytes as biological inoculants in sustainable agriculture. In this study, we used high-throughput sequencing technology to study endophytic bacteria in the seeds of qingke landraces, winter barley, and modern cultivars widely planted in Tibet in five major agricultural regions. The average altitude of the five regions generally increased from east to west. Shigatse has the highest altitude, with an average altitude of more than 4000 m, followed by Lhoka (3700 m), Lhasa (3658 m), and Qamdo (3500 m), Nyingchi City has the lowest average altitude (3100 m). Alpha diversity showed that the abundance, diversity, and uniformity of endophytic bacteria in qingke seeds were the highest among the Nyingchi landraces, with the lowest altitude in the planting area and higher in the Qamdo area, where the planting area was relatively low. The climatic conditions in plateau areas are affected by altitude, compared with higher altitudes, areas with lower altitudes tend to have more suitable hydrothermal conditions. The more suitable hydrothermal conditions were possible reasons for the high diversity of endophytic bacteria in the seeds of qingke in the above two regions. It is worth noting that the abundance, diversity, and uniformity of endophytic bacteria in the landraces of Shigatse at the highest altitude in the planting area were higher than those of the Lhoka and Lhasa landraces, showing that the landraces of Shigatse have better adaptability to the high-altitude climate. As the main producing area of qingke in Tibet, Shigatse has the largest number of qingke genotypes, the rich endophytic bacterial community of the landrace in Shigatse may play a role in the high-altitude adaptation of qingke. It is generally considered that microbial communities with higher diversity are more stable, similar to the findings of Abdullaeva et al. (2021), we found that the modern breeding process increased the diversity of endophytic bacteria in the seeds of qingke. Compared with the landraces, except Nyingchi, the diversity of endophytic bacteria in the seeds of the modern cultivars was higher. In addition, we found that the diversity of

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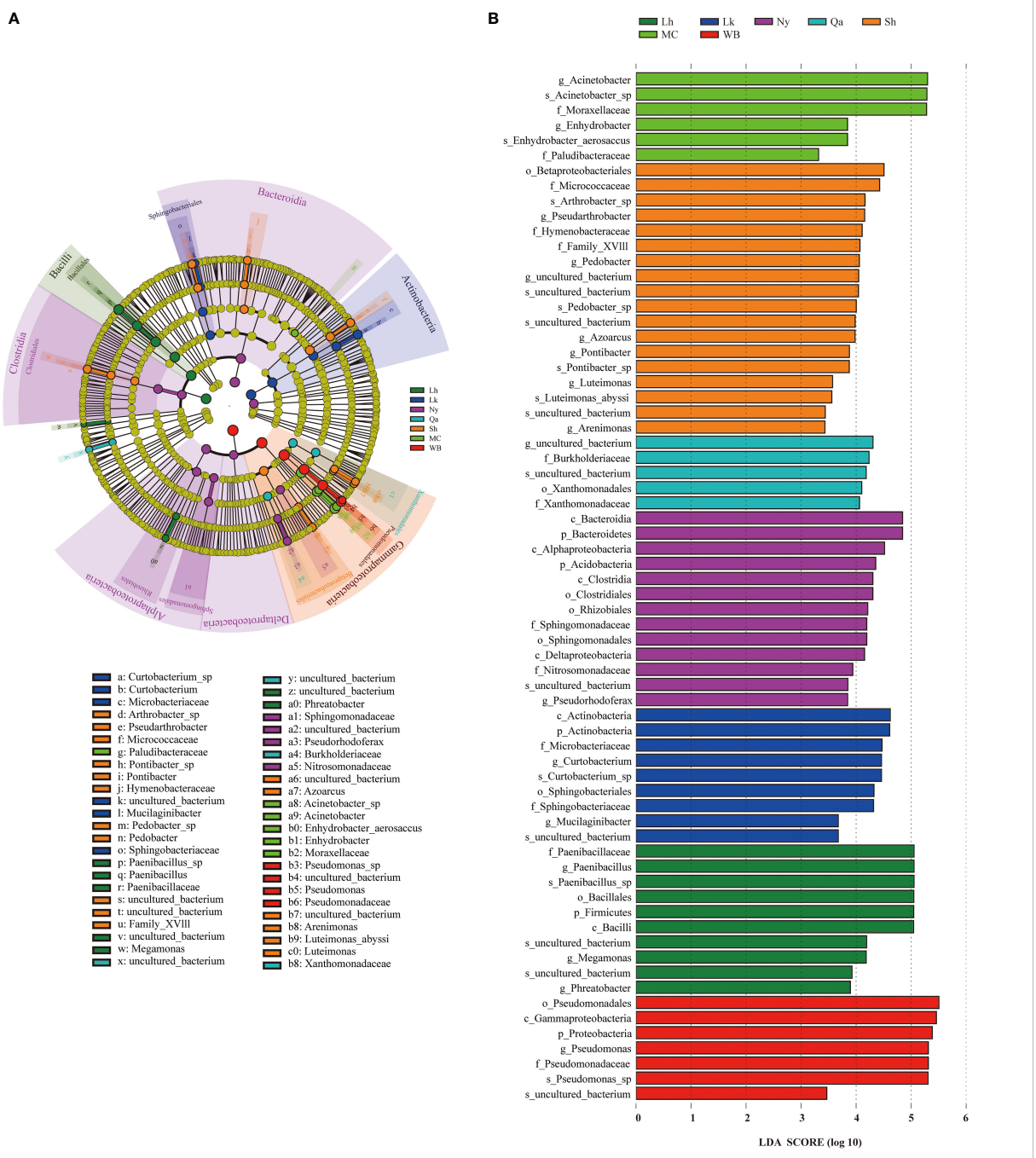

FIGURE 5
Using the Linear discriminant analysis Effect Size (LEfSe) method, the abundance of qingke seeds of different types of different bacterial taxa was revealed. **(A)** Clade diagram representation of the phylogenetic relationships of taxa with differences in abundance among qingke seeds from different sources. Nodes from inside to outside represent phyla, classes, orders, families, genera and species. **(B)** LEfSe analysis of endophytic bacteria in qingke seeds from different sources ($P < 0.05$, log LDA score threshold ≥ 3.3).

endophytic bacteria in the seeds of winter barley varieties was low. These results show that the seed endophyte community of modern cultivars has higher stability, modern breeding processes and different seed growth strategies have certain effects on the diversity of qingke seed endophytes, and plant growth strategy has a greater impact on seed endophytes.

The results of the PCoA and ANOSIM analyses showed significant differences in the endophytic bacterial community

structure of qingke seeds from different sources. Owing to the founder effect event in the qingke population, our findings indicate that local planting for more than 2,000 years has significantly changed the endophytic bacterial community structure of qingke in different regions of Tibet. Among all landraces, the endophytic bacterial community structure of the Nyingchi and Qamdo landraces was relatively similar, and the endophytic bacterial community structures of the Lhoka and Shigatse landraces were relatively similar. The similar geographical location and altitude are possible reasons for their similar endophytic bacterial community structure.

Previous studies have shown that seed endophytes mainly belong to the phyla Proteobacteria and Firmicutes. Yang et al. (2017) also found that Proteobacteria and Firmicutes were the main endophytic phyla in the seeds of German barley cultivars. Rahman et al. (2018) studied barley seed endophytes in different geographical locations and harvest years in Germany and found that the relative abundance of Proteobacteria was the highest (94.8%), followed by Actinobacteria (3.4%), Firmicutes (1.2%), and Bacteroidetes (0.39%). Bziuk et al. (2021) studied the endophytes of seven different genotypes of barley seeds and found that Proteobacteria, Actinobacteria, and Firmicutes were the main endophytes in barley seeds. In our study, different types of qingke seeds had similar endophytic bacterial phyla composition: Proteobacteria (64.6%), Firmicutes (10.1%), Bacteroidetes (9.5%), and Actinobacteria (6.5%) were the main phyla of qingke endophytes. Proteobacteria, and Firmicutes, exhibiting biological control and plant growth-promoting traits in various plants and are the most important bacterial phyla in barley seeds (Rahman et al., 2018). In contrast to previous studies, Bacteroidetes and Firmicutes were relatively more abundant in our study. In studies of the rhizosphere microbiome of various plants, including barley, Bacteroidetes were found to be more abundant in the rhizosphere of various wild plant relatives than in domesticated species. In addition, wild relatives of domesticated barley have a higher abundance of Firmicutes (Pérez-Jaramillo et al., 2018). Studies have shown that endophytes originating from seeds can enter the soil and develop into the rhizosphere microbial community, suggesting that seed endophytes can shape the rhizosphere microbiota (Cope-Selby et al., 2017). For the connection between the seed endophytic microbiome and the rhizosphere microbiome, although this study did not examine the endophytic microbial community of the wild relatives of qingke, we suppose that compared with the previously studied barley genotypes, the endophytic microbiome of qingke seeds closer to wild barley populations, Bacteroidetes' strong ability to degrade complex biopolymers, and organophosphorus mineralisation may help qingke obtain nutrients from the environment more easily (Lidbury et al., 2021).

In bacterial genus composition, although there are differences in endophytic bacteria in qingke seeds from different sources, at least 38 bacterial genera were conserved in the whole qingke population. In previous studies, we used to refer to the conserved microbiota as the core microbiota of the host plant, the vertically transmitted core microbiota in seeds is usually closely related to the healthy development and normal functional expression of the host plant (Johnston-Monje and Raizada, 2011). Consistent with the results of previous studies, we found that the core endo-bacteriome of Enterobacteriaceae accounted for a higher proportion of qingke seeds. Enterobacteriaceae are a group of gram-negative bacteria with strong degradation ability for organic compounds, which dominate in many plant endophytic microflora and are the endophytic core microflora of many plant seeds; members of this family are related to plant health and/or disease (Rossmann et al., 2012; Erlacher et al., 2015; Cernava et al., 2019; Zhang et al., 2020; Bziuk et al., 2021). In other relatively high-abundance core endo-bacteriome, *Acinetobacter* is a class of gram-negative bacteria, and some strains of this genus can grow hormones, solubilise phosphate, and produce siderophores, which can promote plant growth (Sachdev et al., 2010; Rokhbakhsh-Zamin et al., 2011; Adewoyin and Okoh, 2018). In this study, *Acinetobacter* accounted for a relatively high proportion of Lhasa landraces and modern cultivars and was the most abundant bacterial genus in the seeds of modern cultivars. Modern breeding activities may have changed the community structure of the seed endophytes to a certain extent (Johnston-Monje and Raizada, 2011). The plant growth-promoting effect of *Acinetobacter* strains is one of the possible reasons for the higher yield of modern cultivars. *Pseudomonas* is a class of Gram-negative bacteria with diverse species, different species of this genus exhibit diverse metabolic characteristics in different ecological niches, many species are closely related to plants and have excellent plant colonization ability, antibiotic production ability and Mineral phosphate solubilizing ability plays an important role in promoting plant growth and assisting plants in resisting pathogenic microorganisms (Shen et al., 2013; Oteino et al., 2015; Strunnikova et al., 2015; Girard et al., 2020). The genus *Pseudomonas* occupies a high proportion of Lhoka landraces, Shigatse landraces, and winter barley, and the enrichment of bacteria in this genus can reduce the diseases caused by pathogenic fungi in the soil during seed germination. Although most *Prevotella* species are derived from mammals, they are also found in paddy soils and in rice roots (Ueki et al., 2007). As a growth-promoting bacterium, *Prevotella* is the most important genus in Nyingchi landraces, which may be related to its higher optimum growth temperature of *Prevotella* (Arafat et al., 2020). Among all the regions involved in this study, Nyingchi had the lowest altitude and highest annual average temperature. Members of the genus *Paenibacillus* are biochemically and morphologically diverse, and many species of this genus are considered to promote plant growth (Xie et al., 2016). In this study, the *Paenibacillus* genus accounted for a higher proportion in the Qamdo, Lhasa, and Lhoka landraces,

and the climate conditions and altitude of the above three regions were similar, and the relative abundance of *Paenibacillus* in seeds may be affected by climate and altitude. Podolich et al. (2015) suggested that plants have a core microbiome that responds dynamically to environmental factors, and our study verifies this conclusion.

Symbiotic bacterial network analysis divided the endophytic bacterial communities of Tibetan qingke seeds into two groups. The *hgcI_clade* is widely distributed in freshwater ecosystems worldwide; however, its role as a plant endophyte has not been investigated (Zhou et al., 2020). In another association group, *Prevotella* is a widely studied plant growth-promoting bacteria. Although *Burkholderia-Caballeronia-Paraburkholderia* is closely related to a variety of plants, its role in plants is unclear. Given that *Burkholderia*, *Caballeronia*, and *Paraburkholderia* have been reported to increase plant nutrient absorption, promote plant growth, and degrade complex organic matter (Madhaiyan et al., 2021), the effect of *Burkholderia-Caballeronia-Paraburkholderia* on qingke growth deserves further study.

In previous studies, it was found that the endophytic microbiota of plant seeds is affected by the plant growth environment, the endophytic microbiota of seeds produced under field conditions showed more activities related to the inhibition of plant pathogens, and the endophytic microbiota of seeds produced in the environment showed more activities related to promoting nutrient absorption (Bergna et al., 2018). Using LEfSe to analyse the endophytic bacterial community of qingke seeds from the phylum to the species level, it was found that the landraces in Shigatsehad the most different groups of endophytic bacteria, and the landraces in Qamdo showed the least difference. By analyzing the main differential endophytic bacterial groups of seeds from different types, it can be found that the main differential endophytic bacterial groups of Lhoka landrace and winter barley seeds can promote plant growth and resist pests and diseases (Palaniyandi et al., 2013). The main differential endophytic bacterial groups of Lhasa landrace seeds can promote plant growth (Arafat et al., 2020). The main differential endophytic bacterial groups in the seeds of Nyingchi landraces have the ability to degrade complex biopolymers (Clocchiatti et al., 2021). The main differential endophytic bacterial groups in the seeds of modern cultivars have the ability to promote plant growth and inorganic salt acquisition (Adewoyin and Okoh, 2018). The main differential endophytic bacterial groups of the seeds of Shigatse landraces is a common and important part of the plant microbiome, this bacterial family member interacts with arbuscular mycorrhizal fungi (AMF) and cooperates with AMF to participate in the adaptation process of plants to stress tolerance, increasing the nutrient acquisition and growth rate of plants (Emmett et al., 2021). The main differential endophytic bacterial taxa of the Qamdo landraces are members of a bacterial family (Leptotrichaceae) containing pathogenic bacteria, however, several studies have shown that some members of this family also have biological control traits (Lory, 2014; Das and Dhal, 2022). Although there were differences in the endophytic bacterial taxa of qingke seeds from different sources owing to climatic conditions, sowing strategies, and breeding processes, most of the major differential bacterial taxa had plant growth-promoting and biological control traits. It is worth noting that the main differential endophytic bacterial taxa of Shigatse landraces can interact with AMF, and AMF and seed endophytic microbiota have been shown to affect biotic or abiotic soil properties directly or indirectly, thereby affecting plant growth and communities (Idbella et al., 2021). The interaction between the qingke seed endophytic bacterial community and the soil microbiota may play a positive role in the establishment of qingke seedlings under harsh climatic conditions.

Conclusion

In this study, high-throughput sequencing was used to study the endophytic bacteria of qingke seeds. In the analysis of different qingke landraces, modern cultivars, and winter barley, we found that: the modern breeding process has increased the endogenous bacterial diversity of qingke seeds, the growth strategy of winter barley overwintering reduced the diversity of endophytic bacteria, and there were significant differences in the bacterial community structure of different types of qingke seeds. In addition, we found that the core endo-bacteriome, dominated by Enterobacteriaceae, *Acinetobacter*, *Pseudomonas*, *Prevotella*, and *Paenibacillus*, is conserved in different types of qingke seeds and that the core endo-bacteriome may maintain qingke growth by promoting plant growth or assisting plants to resist pests and diseases. In the analysis of the association network of endophytic bacteria, a variety of key taxa of qingke endophytic bacteria, including *hgcI_clade*, *Prevotella*, and *Burkholderia-Caballeronia-Paraburkholderia*, were found. Further research on key taxa will help us better understand the endophytic bacterial communities of qingke seeds. This study reveals the community structure of the core endo-bacteriome of qingke seeds with multiple host-friendly traits and provides a feasible strategy for the application of qingke seed endophytes as bioinoculants for sustainable agricultural production of qingke and other high-altitude crops.

Data availability statement

The data is presented in the study are both in the NCBI's Sequence Read Archive (SRA) repository, accession number PRJNA874323.

Author contributions

XG, JD and ZH designed the study. YW, ZH, XG and JD performed the research. XG, JD, YW and ZH wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Citrus Huanglongbing correlated with incidence of *Diaphorina citri* carrying *Candidatus* Liberibacter asiaticus and citrus phyllosphere microbiome

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In China, citrus Huanglongbing (HLB) disease is caused by the *Candidatus* Liberibacter asiaticus bacterium, which is carried by the Asian citrus psyllid *Diaphorina citri* Kuwayama. It was hypothesized that the epidemic of the HLB may related with the rate of bacterium presence in the insect vector and bacterium content in plant tissues, as well as the phyllosphere microbe communities changes. This study systematically analyzed the presence or absence of *Ca.* L. asiaticus in citrus tree leaves and in the insect vector *D. citri* over a 6-year period using real-time PCR. In addition, changes in the number of bacteria carried by *D. citri* over 12 months were quantified, as well as the relationship between the proportion of *D. citri* carrying *Ca.* L. asiaticus and the proportion of plants infected with *Ca.* L. asiaticus were analyzed. Results showed that the proportion of *D. citri* carrying bacteria was stable and relatively low from January to September. The bacteria in citrus leaves relatively low in spring and summer, then peaked in December. The proportion of *D. citri* carrying bacteria gradually declined from 2014 to 2019. The proportion of *D. citri* carrying *Ca.* L. asiaticus showed a significant positive correlation with the proportion of diseased citrus. The phyllosphere bacterial and fungal communities on the healthy citrus leaf were significantly different with the disease leaf in April and December. Pathogenic invasions change the citrus phyllosphere microbial community structure. It could be summarized that

citrus Huanglongbing correlated with incidence of *Diaphorina citri* carrying *Candidatus* Liberibacter asiaticus and citrus phyllosphere microbiome.

KEYWORDS

citrus huanglongbing, *Candidatus* Liberibacter asiaticus, *Diaphorina* citri, phyllosphere microbiome, pathogen detection and monitoring

Highlights

(1) We monitored citrus Huanglongbing (HLB) for 6 years to obtain enough samples and accumulate long-term continuous data to analyze the spread and prevalence of Huanglongbing in Zhejiang Province, China.

(2) The number of pathogenic bacteria in citrus leaves and the rate of insect vector *Diaphorina citri* carrying bacterium was lowest in spring and highest in December.

(3) Phyllosphere microorganisms of citrus are correlated with HLB.

Introduction

Citrus Huanglongbing (HLB) disease has been reported over 50 countries around the world, which causes serious damage to the citrus industry (Faghihi et al., 2009; Gottwald, 2010; Lopes et al., 2010). HLB is caused by a group of bacteria called *Candidatus* Liberibacter that inhabit in the phloem of citrus trees. Three species of HLB-causing bacterium have been reported: *Candidatus* Liberibacter africanus, *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus. The bacteria can infect different tissues of host plant once invading, and then affect plant growth and development, such as causing metabolism disorders, leaves yellowing, fruits deformity, and roots rot (Pustika et al., 2008; Koh et al., 2012; Etxeberria et al., 2009; Johnson et al., 2014). Infected citrus trees will be significantly shortened profitable lifetimes and lower yields (Gottwald, 2010).

In plant pathological systems, many parasites infect plants and increase their prevalence by host vectors. HLB is transmitted by insect vectors feeding on the phloem of citrus foliage. The HLB causing by africanus species is transmitted by the African citrus psyllid *Trioza erytreae*. Meanwhile, the HLB causing by the asiaticus and americanus species is transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Grafton-Cardwell et al., 2013). Studies have shown that HLB disease in China is caused by *Ca.* L. asiaticus, which is associated with the vector *D. citri* (Hall et al., 2013). Suitable growth area of the bacteria and the insect hosts has been expanded with the rising winter temperatures in recent decades due to global warming (Wang et al., 2020). Bacteria are acquired by *D. citri* when they feed on the infected plant, after that, bacteria will proliferate in *D. citri* and maintain throughout the life history of the adult psyllid (Aubert and Quilici, 1984; Tabachnick, 2015; Luo et al., 2016). Citrus HLB is optimal and limitted by temperature conditions (Narouei-Khandan et al., 2015). However, there is still no long-term quantitative monitoring of bacterial content levels in different hosts, and lack of understanding of this aspect.

The population dynamics of insects are closely related to the growth rhythm, desirable food intake and nutritional quality of host plants (Wallner, 1987). The phenological characteristics of host plants will affect the growth of insects, leading to genetic variation among insect individuals and genetic differentiation among insect populations (Knolhoff and Heckel, 2014). Although tremendous progress has been made in understanding the ecological and evolutionary underpinnings of the Liberibacter disease pyramid, little is known about the quantitative relationship between these factors in the pyramid.

The phyllosphere (aboveground part of terrestrial plants) is an important niche of the plant, inhabited by diverse microbes which are collectively called the phyllosphere microbiome (Vorholt, 2012). The phyllosphere microorganisms could influence host plant by affecting nutrient acquisition, promoting host stress tolerance, altering plant hormones, and mediating plant pathogen interactions (Stone et al., 2018). The phyllosphere microbiomes were found to differ between infected and uninfected citrus leaves by melanose pathogen, and part of the phyllosphere microbiome shift could positively affect plant performance against pathogen invasion (Li et al., 2022).

In this study, we hypothesizes that the epidemic of the HLB may related with the rate of bacterium presence in the insect vectors and bacterium content in plant tissues, as well as the phyllosphere microbe communities. *Citrus unshiu* orchards in different regions of Zhejiang and Hunan Province were systematically analyzed to determine the level of threat from HLB disease (Figure 1). The number of *D. citri* carrying *Ca.* L. asiaticus over time was quantified. The phyllosphere microbiomes of healthy citrus leaves and HLB diseased leaves

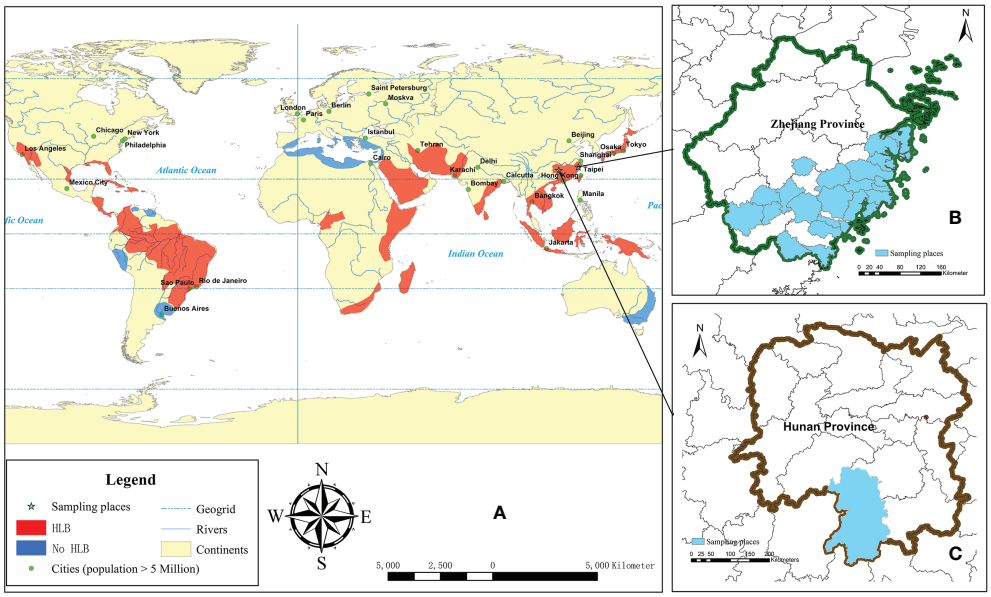

FIGURE 1
Illustration of the major citrus growing areas (orange and blue), HLB happened areas (orange) adapted from Dala-Paula et al. (2018) and Liu et al. (2012) **(A)**, and the sampling areas in Zhejiang **(B)** and Hunan **(C)** province.

were investigated in April and December to understand the correlation among phyllosphere microbial community structure, HLB disease and seasonal effects. The aim of this study is to determine the influence of the bacteria carrier rate of *D. citri* in citrus orchards at different growth stages, in order to provide a theoretical basis for future exploring the ecological regulation and comprehensive control of *D. citri* and *Ca.* L. asiaticus.

Materials and methods

Orchards location and sample collection

For monitoring the *D. citri* carring *Ca.* L. asiaticus over a six-year period from 2014 to 2019, random sites from locations in different counties in the south of Zhejiang Province were chosen for sample collecting (Figure 1, Table 1).

All the *C. unshiu* trees monitored were over 12 years old and at the high yield stage. In each site, 60 psyllids were collected from one *C. unshiu* tree and stored into an individual 1.5-ml tubes, then frozen at –20°C for the future tests. DNA was extracted from 30 psyllids randomly selected from the 60 psyllids (the other 30 psyllids were stored for backup) and processed to detect and quantify *Ca.* L. asiaticus.

For monitoring the rate of *D. citri* and *C. unshiu* leaves carring *Ca.* L. asiaticus changes during the year, An orchards in Yueqing (Zhejiang Province), in which the disease incidence rate were over 90.0%, were sampled by using five-point sampling of the orchards, once a month for 12 months from May 2015 to April 2016. Five leaf samples were also collected from the same point where the insects collected to determine the bacterial content in the midvein. Approximately 45–70 insects were collected around the 20th day of each month. Thirty insects of each month were processed to determine the presence of the bacterium.

To determine the rate of *D. citri* carring *Ca.* L. asiaticus in orchard with different disease incidence, psyllids were also collected by using five-point sampling of the orchards in the early August from 22 orchards in Huangyan, Yueqing, Wenling and Yuhuan (Zhejiang Province) in 2019 (Table 2).

Ten healthy and HLB leaves were collected by using five-point sampling within an area of 600 m^2 for phyllosphere microbiome study at each sampling point in April (Spring) and December (Autumn), respectively, in Yongzhou (Hunan Province) in 2018. Ten leaf samples of each sampling point were mixed put into a sterile bag and refrigerated at -80°C. Every sterile bag contained 10 leaves which were cut into tiny pieces and the samples mixed into 4 treatments which are healthy Apirl (SK), diseased Apirl (SB), healthy December (AB) and diseased December (AK), before subsequent processing. The phyllosphere microorganisms were collected following the procedures of Xie et al. (2015). The microbes were filtered by a 0.22 μm filter microfiltration membrane using the air pump filtration, and then the samples were stored at -80°C for subsequent DNA extraction.

TABLE 1 Proportion of *Diaphorina citri* carrying *Candidatus Liberibacter* asiaticus bacteria in orchards in southern counties in Zhejiang Province from 2014 to 2019.

Years	locations and No.of Samples took	No.of Samples detected with HLB
2014	Cangnan (10), Ruian (10), Ouhai (10), Yongjia (10), Yueqing (10), Longquan (10), Yunhe (10), Qingtian(20), Songyang(10), Liandu (10), Yuhuan (10),Wenling(10), Luqiao (10), Jiaojiang (10), Linhai (10), Shanmeng (10), Xianju (10), Ninghai (10), Xiangshan (10), Yongkang (20)	Cangnan (6), Ruian (7), Ouhai (3), Yongjia (3), Yueqing (6), Longquan (4), Yunhe (5), Qingtian (4), Songyang (9), Liandu (8), Yuhuan (10), Wenling (4), Luqiao (4), Jiaojiang (4), Linhai (1), Shanmeng (3), Xianju (0), Ninghai (3), Xiangshan (1), Yongkang (4)
2015	Wenling (30), Liandu (30), Ninghai (12), Huangyan (10)	Wenling (13), Liandu (11), Ninghai (0), Huangyan (3)
2016	Yuhuang (20), Wenling (20), Luqiao (30), Jiaojiang (30), Linhai (10), Shanmeng (14)	Yuhuang (10), Wenling (10), Luqiao (10), Jiaojiang (10), Linhai (10), Shanmeng (10),
2017	Yongjia (24), Yueqing (60), Wenling (30), Liandu (30)	Yongjia (3), Yueqing (18), Wenling (10), Liandu(10)
2018	Ruian (30), Longwan (40), Yueqing (40), Wenling (10), Linhai (10)	Ruian (6) Longwan (10), Yueqing (10), Wenling (0), Linhai (8)
2019	Taishun (30), Wenling (20), Ruian (15) Longwan (63), Yueqing (61), Cangnan (97), Huangyan (17), Liandu (13), Qingtian (31)	Taishun (3), Wenling (15), Ruian (4) Longwan (34), Yueqing (7), Cangnan (23), Huangyan (1), Liandu (0), Qingtian (3)

Detection and quantification of *Ca.* L. asiaticus

The insects or leaves were crushed with a glass rod, and DNA was extracted using the CTAB extraction method. 400μL prechilled buffer (100 mmol/L Tris-HCl, 10 mmol/L EDTA, 700 mmol/L NaCl, pH 8.0) was added into the crushed powder, then added 500μL 65°C preheated buffer (2% CTAB, 50mmol/L Tris-HCl, 10 mmol/L EDTA, 800 mmol/L NaCl, pH 8.0). Samples were mixed and incubated at 65°C for 120 min, during which mixing the samples by inverting the tubes gently per 20 min. After that, add 450μL chloroform and mix by inverting the tubes and centrifuge 2 minutes at 12000r, the aqueous phase (above the white interface layer) to a clean microtube (then discard the rest), add 1 μL RNase (DNase-free) and incubate for 30 min at 37°C. Add 0.6 mL of isopropanol (2/3 of the recovered volume). Gently invert the microtube to be sure mixing is complete. Leave to precipitate for overnight at room temperature to precipitated the DNA, spin 15 min at 12000r at 4°C to pellet the DNA, remove the supernatant carefully, then wash the pellet once or twice with cold EtOH, spin 15 min at max speed, 4°C, remove supernatant and dry the pellet by leaving tube open, resuspend pellet in sterile H2O, store at -20°C (Winnepenninckx et al., 1993). The bacterium was quantified using RT-qPCR, The primers pair: 5'-CAAGG AAAGA GCGTA GAA-3' and 5'-CCTCA AGATC GGGTA AAG-3' were used. The PCR is carried by 25μL system with 2μL DNA and 0.3 μmol/L primers and 1X PCR master mix, on the iCycler™(Bio rad, USA) marchin with the program of 94° C, 5 minute, 40 cycles of 95°C, 5 second, 59°C, 15 second, and 72° C, 45 second, ended with 72°C, 7 minute. A standard curve was prepared and calculated using T vectors with DNA fragments of the target bacterial gene (382 bp) insertion. Copy numbers of T vectors were diluted to obtain copy numbers of 10, 10^2, 10^3, 10^4, 10^5, 10^6 for a standard curve. The standard linear regression (Y=a +bX) of the log concentration of the target DNA copies(Y) versus the mean Ct value(X) were obtained (Li et al., 2008).

DNA extraction, PCR amplification and pyrosequencing for phyllosphere microbiome

The total DNA was extracted from phyllosphere samples according to the manufacturer's protocol using the MP FastDNA® SPIN Kit for soil (MP Biochemicals, Solon, OH, USA). The PCR amplification were performed following Luo et al. (2019). The bacterial and fungi forward and reverse primers with a unique 12 nt barcode were included as the modification in the study, respectively. The bacterial and fungal ITS regions were amplified as previously described by Kong et al. (2019). PCR products were purified with an E.Z.N.A. ® Gel Extraction Kit, pooled in equimolar amounts using Qubit (CA, USA). And mixed PCR products were sequenced (2×250 bp) on an Illumina MiSeq platform by ANNOROAD Gene Technology Co., Ltd. (Beijing, China) according to standard protocols.

Raw sequence data reads were processed with an in-house pipeline (http://mem.rcees.ac.cn:8080). In brief, a separate sample was generated according to a series of 12-bp barcodes and primers, and allowing for one mismatch. Paired-end reads (overlap > 30 bp) were combined by the FLASH program (Magoc & Salzberg, 2011). The combined sequences (Quality Score < 20) were filtered by Btrim program (Kong, 2011). Then the sequences with either an ambiguous base or the sequence length less than 200 bp were discarded. The UPARSE algorithms were used to detect and remove chimera

TABLE 2 To study the relationship between the proportion of diseased plants and the proportion of *D. citri* carrying the bacterium, 22 different orchards located in Huangyan, Yuhuan, Wenling,and Yueqing were investigated.

tag	Location	Samled time	Investigated trees	% of deseased trees	Tested insects	The number of tested insects carrying bacterium	% oftested insects carrying bacterium
1	Huangyan	Aug-05	300	0	200	13	6.5
2	Huangyan	Aug-05	300	0	200	7	3.5
3	Huangyan	Aug-02	300	23.1	200	28	14
4	Huangyan	Aug-02	300	12.3	200	11	5.5
5	Huangyan	Aug-02	300	11.67	200	13	6.5
6	Huangyan	Aug-05	300	0	200	7	3.5
7	Huangyan	Aug-04	300	7	200	26	13
8	Huangyan	Aug-04	300	12.9	200	26	13
9	Huangyan	Aug-04	300	13.1	200	20	10
10	Huangyan	Aug-04	300	7	200	27	13.5
11	Huangyan	Aug-04	300	12.9	200	28	14
12	Huangyan	Aug-04	300	13.1	200	20	10
13	Yueqing	Aug-04	300	49.2	200	34	17
14	Yueqing	Aug-04	300	67	200	60	30
15	Yueqing	Aug-04	300	91.7	200	85	42.5
16	Yueqing	Aug-04	300	98.5	200	134	67
17	Yueqing	Aug-04	300	98	200	134	67
18	Wenling	Aug-04	300	64.8	200	74	37
19	Wenling	Aug-04	300	76.9	200	32	16
20	Yuhuan	Aug-02	200	100	200	156	78
21	Yuhuan	Aug-02	200	3.2	200	6	3
22	Yuhuan	Aug-02	300	1.5	200	3	1.5

sequences (Edgar, 2013). Low abundance OTUs (≤ 1 count) were eliminated from the OTU table. The microbial representative sequences for each OTU were assigned to taxonomic groups using the RDP Classifier database (Silva database 132 version) and UNITE database (Version 12.01.2017) (Abarenkov et al., 2010). The data were resampled randomly with the lowest sequence number (17,590 for bacteria and 27,286 sequences for fungi). The resampled OTU table was used for the subsequent analysis. In this study, all the microbial raw sequences were deposited in the SRA database short-read archive PRJNA844183.

Statistical analysis

To plot the curve to fit the relationship between *D. citri* bacterial infection rate and the HLB incidence, the linear mixed model uses the lmer function performed in the "nlme" package, and all statistical analyses were performed in the R3.2.5 (R Core Team, 2016). The Chao1 and Inv_Simpson index were used to assess the difference of α diversity indices between healthy and diseased citrus phyllosphere samples. The weighted principal coordinate analysis (PCoA) based on UniFrac matrix and nonparametric permutational multivariate (PERMANOVA) based on Bray Curtis were used to assess the difference of microbial community structure between healthy and disease phyllosphere samples (Anderson, 2001; Caporaso et al., 2010).

Results

Quantitative detection of bacteria in the insect vector *D. citri*

In total, 1037 insect samples were collected from different sites in the south of Zhejiang Province over a six year-period from 2014 to 2019. Of these samples, the presence of the bacterium was detected in 319 *D. citri* samples using RT-qPCR (Table 1). Copy numbers of the bacterium gene per nanogram of DNA ranged from approximately 10^4 to 10^9 (Figure 2), however, 10^4–10^6 copy numbers of the bacterium gene were detected in the majority of samples. Approximately 10^4 copy numbers of the bacterium gene per nanogram of DNA were found in 47 samples, 10^5 in 81 samples, and 10^6 in 98 samples. The copy number of the bacterium gene per nanogram of DNA was 85.6% in 10^5–10^6. However, only eight samples were found with more than 10^9 copy numbers of the bacterium gene per nanogram of DNA. These samples were collected from orchards located in the counties of Jiaojiang (2), Wenling (1), and Yuhuang (3) in 2014 and Wenling (2) in 2015.

Although *D. citri* carrying bacteria were found in orchards in all counties, some *D. citri* samples lacked bacteria (Table 1).

Proportion of *D. citri* carrying the *Ca.* L. asiaticus bacterium over a six-year period

Among the 1037 samples, the proportion of *D. citri* carrying *Ca.* L. asiaticus decreased and finally stabilized over time. In 2014, *D. citri* were found at 220 sites and, *D. citri* were found to be carrying the bacterium in 92 sites accounting for 41.8% of these sites. In 2015, *D. citri* were found at 82 sites and *D. citri* were found to be carrying the bacterium in 27 sites accounting for 32.9% these sites. By 2016, 2017, 2018, and 2019, *D. citri* were found at 114, 144, 130, and 347 sites but *D. citri* were only found to be carrying the bacterium at 30.7% (35), 28.5% (41), 26.2% (34) and 25.9% (90) of these sites, respectively (Figure 3, Table 1).

Changes in the proportion of *D. citri* carrying the bacterium throughout the year

Analysis of *D. citri* collected from a diseased orchards in Yueqing revealed that more than 30% of *D. citri* were carrying *Ca.* L. asiaticus throughout the year (Figure 4B). The proportion of *D. citri* carrying *Ca.* L. asiaticus gradually decreased from January to March and was stable and relatively low from May–September, then gradually increased and peaked in December (Figure 4B). The content of *Ca.* L. asiaticus in the citrus leaves was greatest in December. The content of *Ca.* L. asiaticus in the citrus leaves was relatively low in the fall and spring (close to 30% in May, June, September, and March). In spring and summer (February, April, May, June, and August), the bacterial content of citrus leaves was lowest, but reaching a high peak in December (Figure 4A).

Relationship between the proportion of diseased plants and the proportion of *D. citri* carrying the bacterium

In total, 6600 *Citrus. unshiu* plants were investigated in 22 different orchards located in Huangyan, Yuhuan, Wenling, and Yueqing (Table 2). Based on investigations of 300 trees at each location, the proportion of diseased trees ranged from 0–98.5% (Table 2). Two hundred insects were collected at each location and were tested by RT-qPCR to determine the presence or absence of the bacterium. The proportion of diseased trees and the proportion of *D. citri* carrying the bacterium showed a significant positive correlation, and the number of plants showing

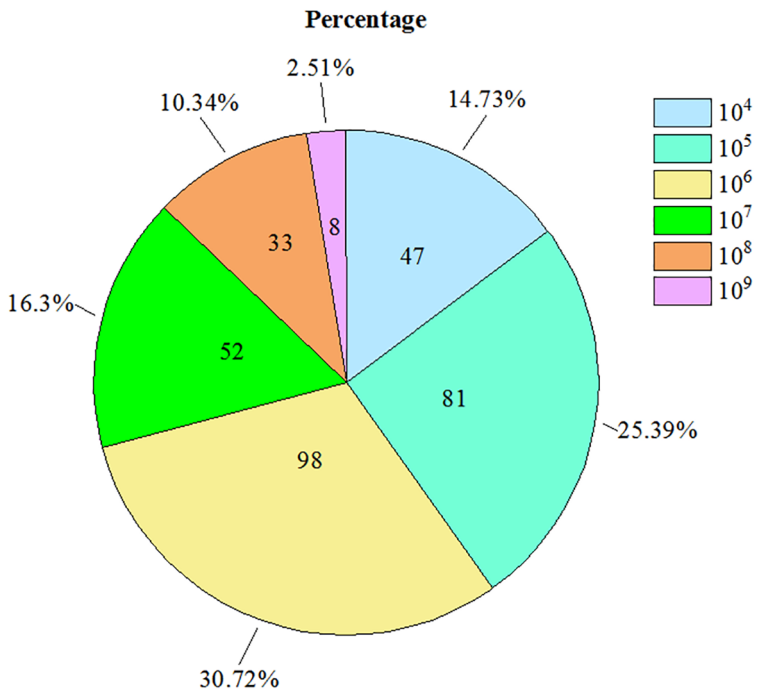

FIGURE 2
The detected amount of *Candidatus* Liberibacter asiaticus bacterium DNA by qPCR.

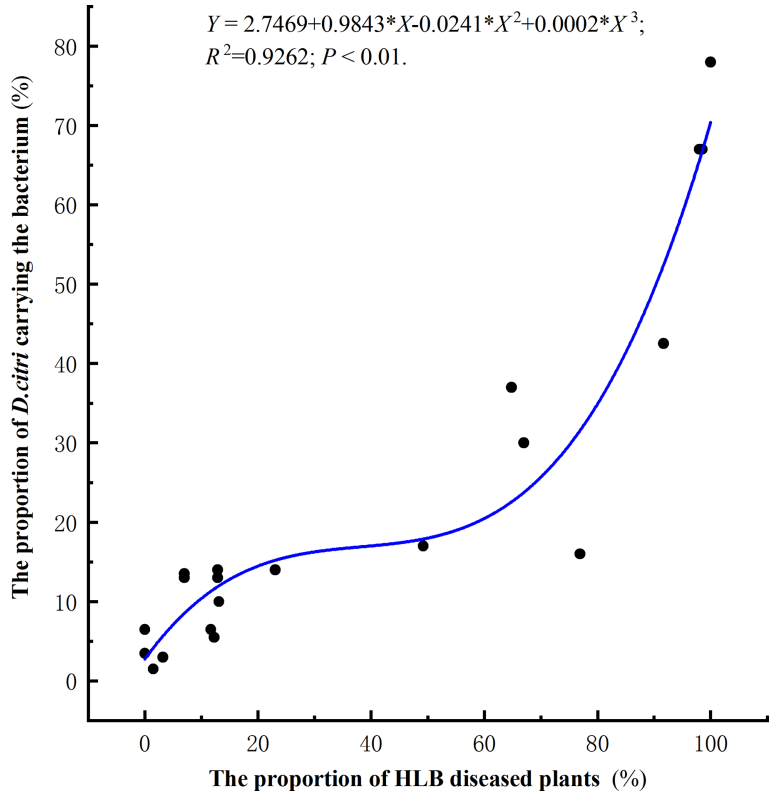

FIGURE 3
Proportion of *Diaphorina citri* carrying the pathogen *Candidatus* Liberibacter asiaticus between 2014 and 2019.

symptoms of HLB increased as the proportion of *D. citri* carrying the bacterium increased (R^2 = 0.93, P<0.01, Figure 5).

The phyllosphere microbiomes differ between healthy and HLB citrus in April and December

A total of 1626061 and 913399 high-quality sequences for bacteria and fungi were obtained after quality control of the original data using high-throughput sequencing technology. There were 17,590-184,257 for bacterial sequences and 27286-80816 for fungal sequences of each sample. Inv_Simpson index and Chao1 were used to evaluate α diversity of phyllosphere microorganisms with different treatments. The bacterial α diversity and the fungal α diversity in healthy leaves were significantly higher than that in diseased leaves in spring. But in autumn, there was no significant difference in α diversity of microbial communities between healthy and diseased citrus leaves (Figure 6).

PERMANOVA and pCoA based on bray_cuits distance matrix were used to analyze phyllosphere microbial differences among different leaf group samples. The pCoA results showed that bacterial and fungal sampled in the phyllosphere healthy and diseased citrus leaves at the same time, could be completely separated (SBB and SKB, ABB and AKB). The phyllosphere bacterial and fungal samples in healthy and diseased citrus leaves at two time points were compared respectively, and there were also significant differences between SBB and ABB, and SKB and AKB groups (Figure 7). The results of dissimilarity analysis also showed that there was a significant difference in the phyllosphere microorganisms between healthy and diseased citrus leaves sampled in the same time and between healthy and diseased citrus leaves in different seasons (Table S1).

The changes in bacteria and fungi OTUs by HLB pathogen invasion

In order to further study the phyllosphere microbial population structure of healthy and diseased leaf samples, the OTUs that were common and unique among these groups were analyzed and were plotted as a Venn plot (Figure 8). A total of 702 bacterial OTUs and 896 fungal OTUs were obtained. According to the results of Venn diagram, 124 (bacteria) and

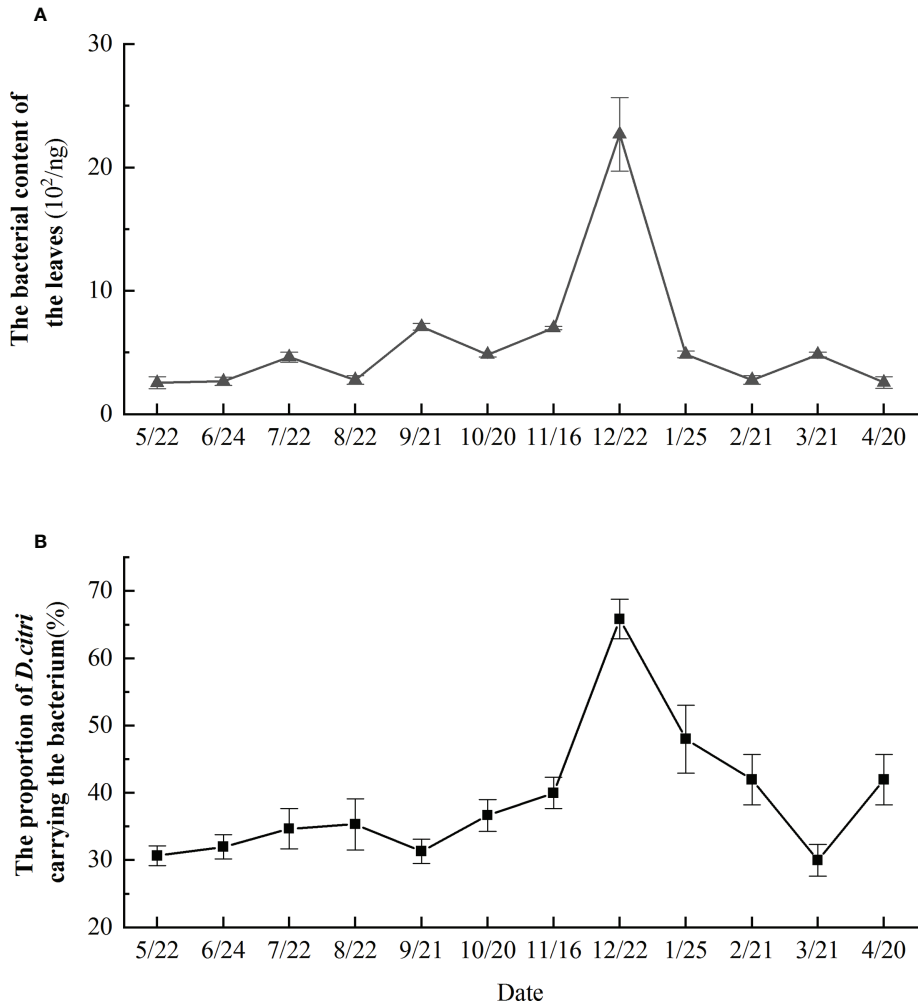

FIGURE 4
Proportion of. Monthly changes in *Candidatus* Liberibacter asiaticus bacterial content in citrus leaves over the course of a year **(A)**, and monthly changes in proportion of *Diaphorina citri* carrying the *Candidatus* Liberibacter asiaticus bacterium over the course of a year **(B)**.

109 (fungi) common OTUs were found in the phyllosphere samples of different treatments (Figure 8A). There were 269 common bacterial OTUs found in ABB group and AKB group, while 67 and 93 unique OTUs were found respectively in the two groups. And 206 common bacterial OTUs were found in SBB group and SKB group, while 65 and 193 unique OTUs were found in the two groups respectively. Besides, there were 163 and 515 common fungal OTUs found between ABF and AKF, SBF and SKF group (Figure 8B). 27 and 85 unique OTUs were found in ABF and AKF group, while 176 and 78 unique OTUs were found in SBB and SKB group, respectively.

Discussion

Citrus HLB has become the most important quarantine disease in citrus-producing areas all over the world (Abdullah et al., 2009; Martinez et al., 2009; Bové, 2014; Puttamuk et al., 2014; da Graça et al., 2015). Once infected, the quite few of options to prevent the disease from spreading further, one of which is to cut off symptomatic branches. Since the development of the citrus industry and the scale of the expansion of citrus in world trade in recent years, clarifying the mechanism of the spread of *Ca.* L. asiaticus to find an effective control method for HLB is urgently required.

Ca. L. asiaticus has a long incubation period, therefore, infected citrus plants do not show symptoms of HLB during the early stages. In order to distinguish diseased plants from healthy plants, the RT-qPCR method is usually used to detect whether *Ca.* L. asiaticus bacteria are present (Fujikawa et al., 2013). In this study, *Ca.* L. asiaticus and the insect vector *D. citri* were detected from almost all southern citrus-producing regions of Zhejiang. This confirmed previous reports that HLB is an aggressive disease in citrus plantations and that it is widely distributed and spreading rapidly in Zhejiang Province (Zhou, 2020).

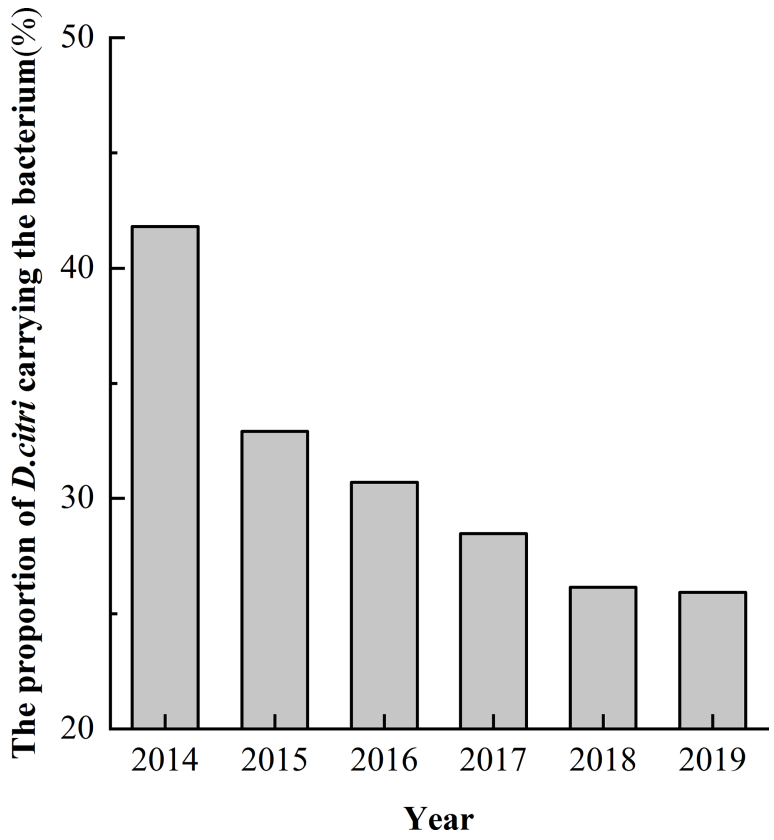

FIGURE 5
Correlation between the proportion of citrus plants with symptoms of HLB and the proportion of *Diaphorina citri* carrying the bacterium *Candidatus* Liberibacter asiaticus.

The dynamic of insect population is closely related to the growth rhythm and desirable food intake of insects, as well as the nutritional quality of host plants (Wallner, 1987). In addition, the phenological characteristics of host plants will also affect the growth of insects, leading to genetic variation among insect individuals and genetic differentiation among insect populations (Knolhoff and Heckel, 2014). Although tremendous progress has been made in understanding the ecological and evolutionary underpinnings of the Liberibacter disease pyramid, little is known about the quantitative relationship between these factors in the pyramid. By dynamically monitoring the bacterial content in the midvein of diseased leaves over the course of a year, we observed that values in December were more than 100 times higher than they were from March to May. This may be that citrus trees are cold-tolerant and evergreen, therefore, still lush in the fall, which significantly increasing the life span of *D. citri* (George et al., 2020). In addition, whether host plants can develop shoots in early spring and over the whole winter will affect the diapause of the insect population, which could exert a high selection pressure on the insect population to adapt to this extended host resource, and lead to species differentiation (Danks, 2013; Joyce et al., 2016). Furthermore, the chemoreceptors of insect are essential for the recognition and perception of plant secondary metabolites. Plant-eating insects can become conditioned to the host's secondary metabolites and chemical constituents, which leads to change in insect behavior and drive host-associated differentiation (Powell et al., 2006; Medina et al., 2014). Moreover, the main rainy season in this area is from March to May. This long period of rain scours the bacterial communities on citrus trees, decreasing the number of bacteria on the leaves. From the beginning of July, the quantity of bacteria on leaves gradually increases. *Ca.* L. asiaticus can stand with high temperatures (Hoffman et al., 2013; Lopes et al., 2013). Our analyses showed that *D. citri* carried more bacteria in January and December than at other times, indicating that *D. citri* also has a certain level of cold tolerance. The *D. citri* life span is generally from spring to winter, by which time bacteria have fully proliferated in its body and, hence, the December insects were found to carry the most bacteria. In addition, large numbers of *D.*

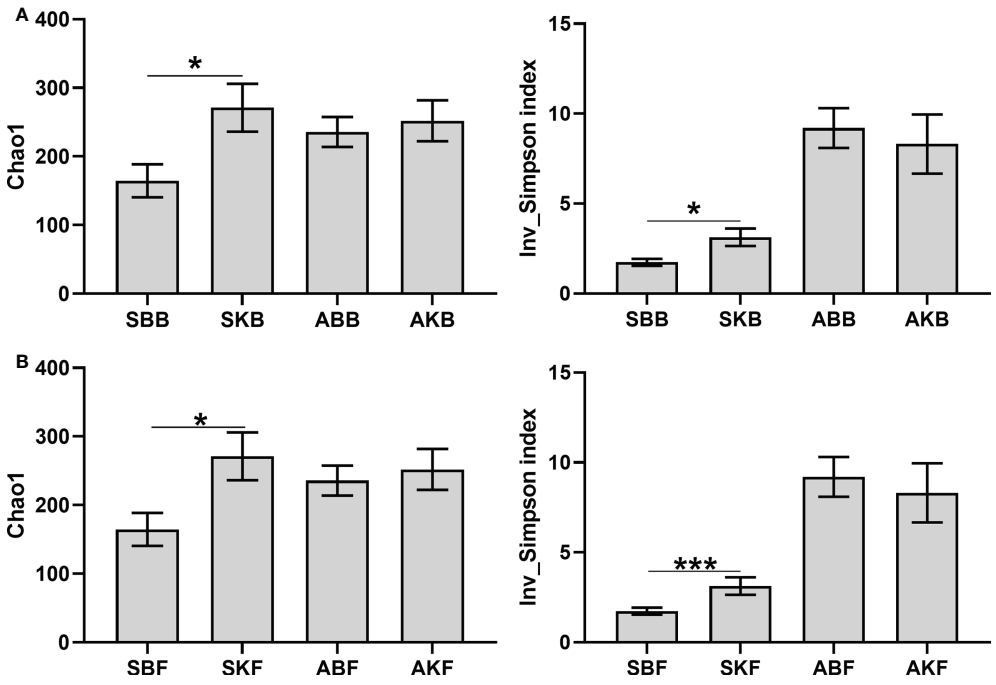

FIGURE 6
The α diversity indices of bacteria **(A)** and fungi **(B)** SBB, phyllosphere bacterial samples of spring disease leaves; SKB, phyllosphere bacterial samples of spring healthy leaves; ABB, phyllosphere bacterial samples of autumn disease leaves; AKB, phyllosphere bacterial samples of autumn healthy leaves; SBF, phyllosphere fungal samples of spring disease leaves; SKF, phyllosphere fungal samples of spring healthy leaves; ABF, phyllosphere fungal samples of autumn disease leaves; AKF, phyllosphere fungal samples of autumn healthy leaves. *P < 0.05, ***P < 0.001.

citri with bacterium were attracted to very few shoots in December. The abundance of *D. citri* on citrus shoots was highest in August and October, which is when their feeding and reproduction peaks. The rate of bacterial transmittance and population density also increased which may be related to the biotic factors that influence *D. citri* populations such as temperature and food. Although temperature is the main factor affecting the growth and development of insect, humidity has little effect on its survival and growth (Shang et al., 2013). *D. citri* carry a low level of bacteria in spring, which may be affected by climate and the bacterial content of the host tissue.

We found that the spread of HLB in Zhejiang Province was limited from 2014 to 2019. In 2014, *D. citri*-carrying bacteria were found at 41.8% of sites. By 2015, the level had dropped to 32.9% and by 2019 the level had dropped even further to 25.9%. This phenomenon may be related to the removal of diseased plants to control HLB. *D. citri* has a poor ability to fly long distances (Sakamaki, 2005). Long-range flight is made up of multiple short-range flights and relies on short-range wind diffusion. As a result, citrus HLB epidemics are dependent on sources of the pathogens and movement of the insect vectors. The removal of diseased plants therefore has a significant effect on controlling the spread of HLB (Abdullah et al., 2009; Bové, 2014).

Based on our observations, we showed a significant relationship between the plant infection rate and the proportion of *D. citri* carrying the bacterium (Figure 5). Our analysis showed that high levels of HLB disease in orchards correlate with high frequencies of *D. citri*-carrying bacteria. Coy and Stelinski (2015) found that infection levels of *Ca.* L. asiaticus in *D. citri* populations across Florida (USA) ranged from 37.5% to 100%, which was similar to the diverse infection levels found in different orchards in this study. Lee et al. (2015) have described a transmission mechanism that explains the high numbers of *Ca.* L. asiaticus-positive psyllids in retail environments based on an infection experiment. In retail environments, there are ample opportunities for each newly arriving the newly developed cluster of young leaves of healthy plant to be colonized by resident infected-psyllids, only after 15 days of being inoculated by *Ca.* L. asiaticus, the plants are infectious and transmit the pathogens to the next generation of psyllids. In this study of naturally infected orchards, we conclude that there is a relationship between the proportion of *D. citri* carrying the bacterium and the incidence of HLB disease. This correlation might due to that the physiological and biochemical characteristics of plants that have been infected with *Ca.* L. asiaticus may affect the behavior and performance of *D. citri* on susceptible plants. In addition,

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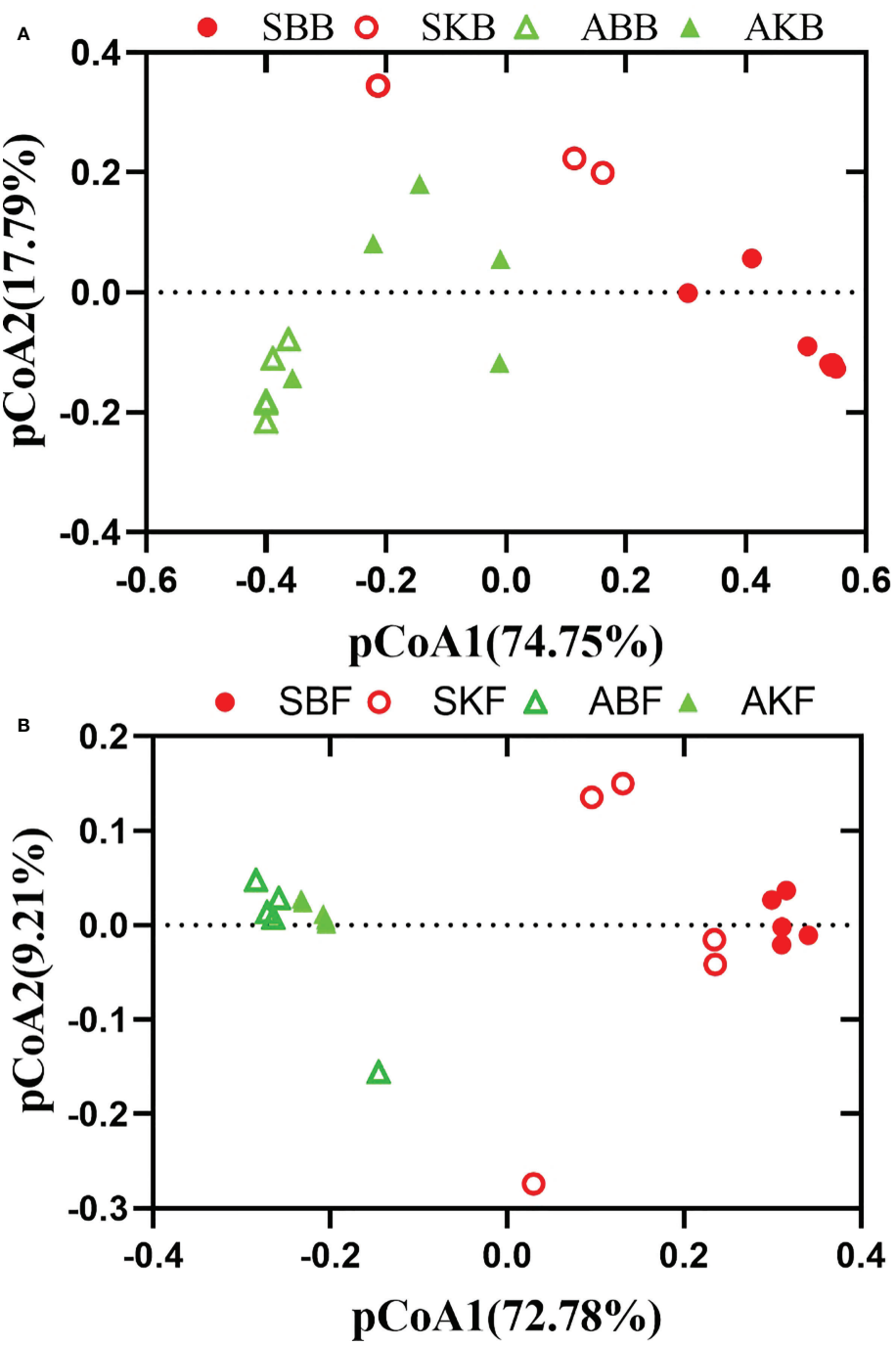

FIGURE 7
The pCoA of bacteria **(A)** and fungi **(B)** based on bray_cuits distance. SBB, phyllosphere bacterial samples of spring disease leaves; SKB, phyllosphere bacterial samples of spring healthy leaves; ABB, phyllosphere bacterial samples of autumn disease leaves; AKB, phyllosphere bacterial samples of autumn healthy leaves; SBF, phyllosphere fungal samples of spring disease leaves; SKF, phyllosphere fungal samples of spring healthy leaves; ABF, phyllosphere fungal samples of autumn disease leaves; AKF, phyllosphere fungal samples of autumn healthy leaves.

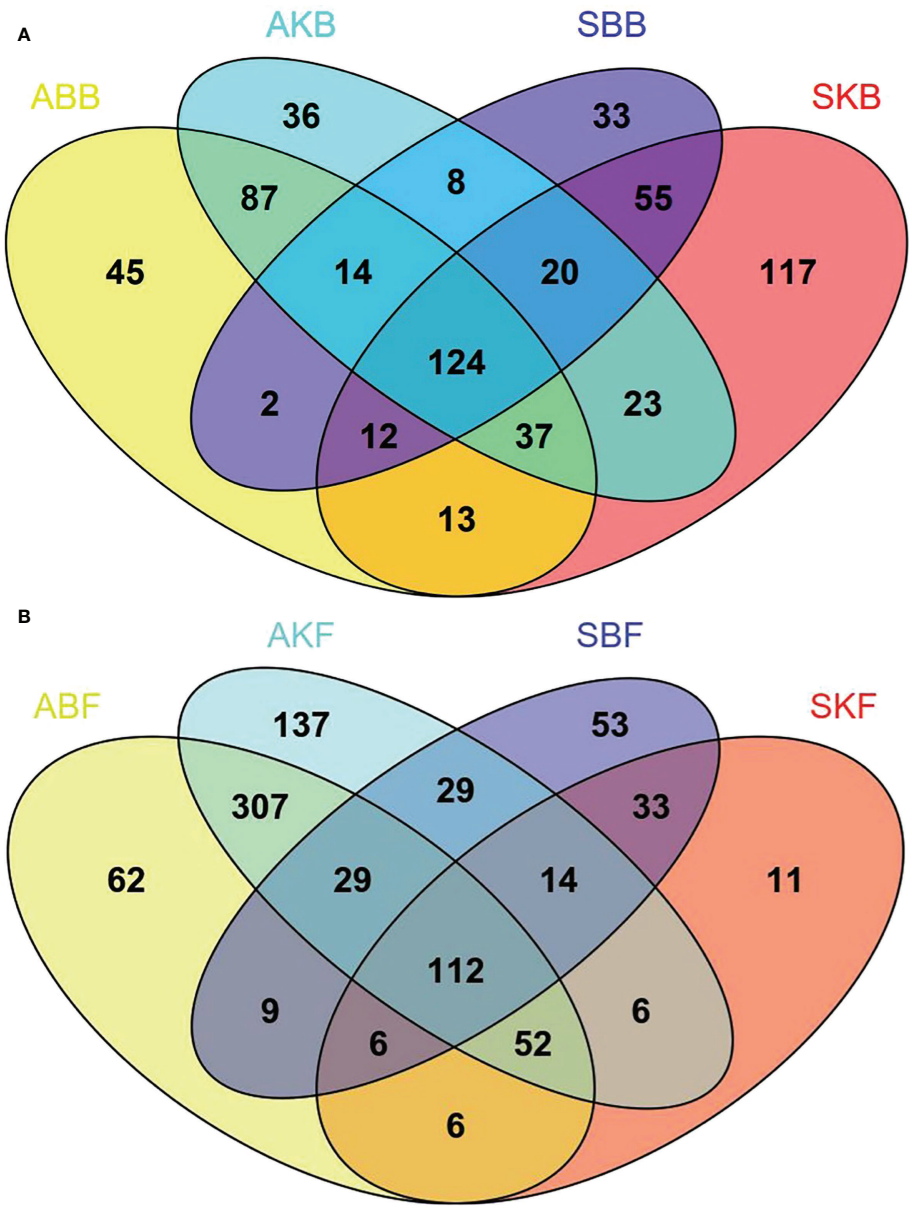

FIGURE 8
The analysis of united and shared OTU of bacteria **(A)** and fungi **(B)**. SBB, phyllosphere bacterial samples of spring disease leaves; SKB, phyllosphere bacterial samples of spring healthy leaves; ABB, phyllosphere bacterial samples of autumn disease leaves; AKB, phyllosphere bacterial samples of autumn healthy leaves; SBF, phyllosphere fungal samples of spring disease leaves; SKF, phyllosphere fungal samples of spring healthy leaves; ABF, phyllosphere fungal samples of autumn disease leaves; AKF, phyllosphere fungal samples of autumn healthy leaves.

plant defense responses induced by the bacterial infection may indirectly affect *D. citri* by increasing the attractiveness of infected plants. *Ca.* L. asiaticus can also indirectly affect the adaptability of *D. citri*, which also affects the epidemic of citrus HLB. Host plant changes promote the genetic variation of insect adaptability and affects the genetic diversity and genetic structure of the insect population (Feder, 1995; Medina et al., 2014). *D. citri* infected with *Ca.* L. asiaticus bacteria spend more time feeding and show higher levels of fecundity than bacteria-free *D. citri* (Duan et al., 2009; Cen et al., 2012). *Ca.* L. asiaticus bacteria obtain nutrients and energy for their own invasion and proliferation by regulating the metabolic activities of substances in *D. citri* (Duan et al., 2009). These studies also indicated it must be correlation between the *D. citri* carrying with *Ca.* L. asiaticus and HLB disease incidence.

Controlling *D. citri* is the premise and foundation for preventing citrus HLB (Manjunath et al., 2008). At present, disease control strategies mainly involve chemical, physical, or biological methods of control. *D. citri* has a strong reproductive ability. Although chemical control has an obvious control effect on these insects (Boina et al., 2011), it is impossible to eliminate the insects completely, and chemical control can lead to resistance and enhance the reproductive ability of the insects. In addition, chemical control methods cause serious pollution and chemical residues can accumulate in the environment. As a result, the development of accurate and efficient pesticides to control *D. citri* has become a focus of research (Qureshi and Stansly, 2010). In this study, the proportion of the insect population carrying the bacterium changed over the course of the year, peaking in winter. Even though the insect population may be small, the application of pesticides could still be important to reduce the potential risk of *Ca.* L. asiaticus infection. Hybridization of resistant varieties using molecular biology and molecular breeding is another strategy for HLB control. No HLB symptoms were found in transgenic plants after grafting them with HLB-infected plants (Dutt et al., 2008). However, in the main citrus-producing industrial belt, controlling *D. citri* has been the main focus, and diseased plants have been destroyed by digging out and removing diseased trees (Wang and Trivedi, 2013), as well as high-density planting and shoot control to reduce *D. citri* feeding, strengthening field and orchard cultivation and management, and enhancing plant disease resistance, which can control citrus HLB to a great extent. Our observation of a reduction in disease levels in Zhejiang Province of China indicate that the application of these methods has had a significant effect on reducing the HLB disease epidemic. The phyllosphere (aboveground part of terrestrial plants) is an important niche of the plant, inhabited by diverse microbes which are collectively called the phyllosphere microbiome (Vorholt, 2012). The phyllosphere microorganisms could influence host plant by affecting nutrient acquisition, promoting host stress tolerance, altering plant hormones, and mediating plant pathogen interactions (Stone et al., 2018). The phyllosphere microbiomes were found to differ between infected and uninfected citrus leaves by melanose pathogen, and part of the phyllosphere microbiome shift could positively affect plant performance against pathogen invasion (Li et al., 2022).

Our analysis showed that there were significant differences among different healthy citrus leaves and HLB diseased leaves in April and December (Figure 7). The finding was consistent with Li et al. (2022), which also reported the changes of microbe community of citrus phyllosphere due to the invasion of fungal pathogen *Diaporthe citri*. HLB has been found to cause decreased relative abundance and/or expression activity of rhizoplane-enriched taxonomic and functional properties, hence collectively resulting in impaired plant host-microbiome interactions (Zhang et al., 2017). Our data showed that the effect of HLB may also apply on phyllosphere. It indicated that, in the early stage of citrus HLB, diversity of phyllosphere microorganisms is not only affected by the disease, but also regulated by seasonal factors. In spring, phyllosphere microorganisms showed a reverse trend of changes at bacteria and fungi in response to pathogenic invasion. The Venn results indicated that the OTUs number of diseased phyllosphere bacteria taxa were lower than Apirl citrus, but higher in December leaves phyllosphere fungal samples in both diseased and healthy leaves (Figure 8). The number of bacteria decreases, and fungi increases in the presence of pathogenic bacteria, which indicates that microbial communities exhibit ubiquitous taxa with special functions selected during pathogenic bacteria invasion.

Conclusions

Through the long-term observation of citrus HLB, we conclude that there is a significant positive correlation between the level of citrus HLB in an orchard and proportion of *Ca.* L. asiaticus-carrying *D. citri*. The outbreak and spread of HLB in Zhejiang Province are declining. The number of bacteria in citrus leaves and the rate of *D. citri* carrying bacterium varies with seasonal factor. The phyllosphere bacterial and fungal communities were significantly different between healthy and disease phyllosphere in April and December. Pathogenic invasions change the citrus phyllosphere microbial community structure.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

YM, EW, ZZ, and YH planted the experiment. YM, EW, MZ, and ZZ carry on the experiment. YW, LD, H-eZ, TT, WX, LL, XF, and LY analysis data. LY, LL, and YH write the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.964193/full#supplementary-material

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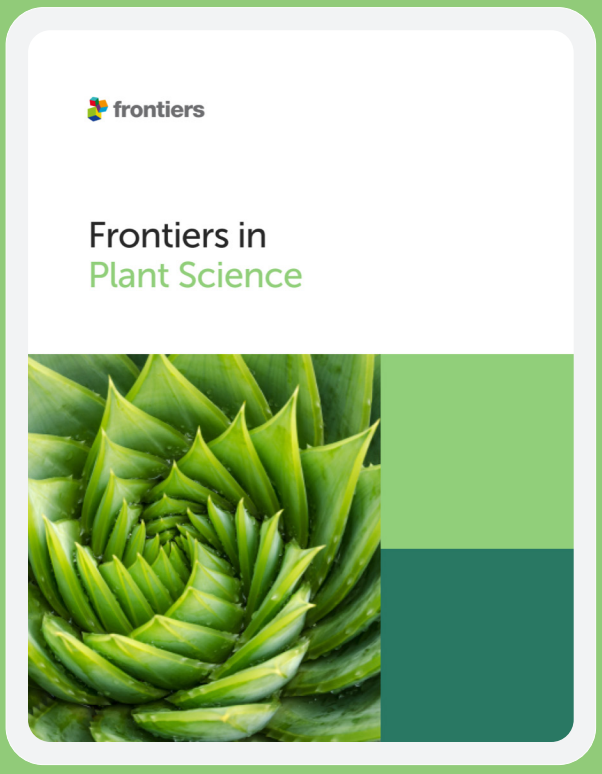

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