**RESEARCH ARTICLE**

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# Biocontrol: Endophytic bacteria could be crucial to fight soft rot disease in the rare medicinal herb, *Anoectochilus roxburghii*

**Bingcong Xing**[1,2] | **Ying Zheng**[1,2] | **Man Zhang**[2] | **Xinting Liu**[1,2] | **Lihong Li**[1,2] | **Chenhao Mou**[1,2] | **Qichao Wu**[1,2] | **Haipeng Guo**[3] | **Qingsong Shao**[1,2]

[1]State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou, China

[2]Zhejiang Provincial Key Laboratory of Resources Protection and Innovation of Traditional Chinese Medicine, Zhejiang A&F University, Hangzhou, China

[3]State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, School of Marine Sciences, Ningbo University, Ningbo, China

**Correspondence**
Qingsong Shao, State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou 311300, China.
Email: sqszjfc@126.com

Haipeng Guo, State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, School of Marine Sciences, Ningbo University, Ningbo 315211, China.
Email: guohaipeng@nbu.edu.cn

**Abstract**

Microbial destabilization induced by pathogen infection has severely affected plant quality and output, such as *Anoectochilus roxburghii*, an economically important herb. Soft rot is the main disease that occurs during *A. roxburghii* culturing. However, the key members of pathogens and their interplay with non-detrimental microorganisms in diseased plants remain largely unsolved. Here, by utilizing a molecular ecological network approach, the interactions within bacterial communities in endophytic compartments and the surrounding soils during soft rot infection were investigated. Significant differences in bacterial diversity and community composition between healthy and diseased plants were observed, indicating that the endophytic communities were strongly influenced by pathogen invasion. Endophytic stem communities of the diseased plants were primarily derived from roots and the root endophytes were largely derived from rhizosphere soils, which depicts a possible pathogen migration image from soils to roots and finally the stems. Furthermore, interactions among microbial members indicated that pathogen invasion might be aided by positively correlated native microbial members, such as *Enterobacter* and *Microbacterium*, who may assist in colonization and multiplication through a mutualistic relationship in roots during the pathogen infection process. Our findings will help open new avenues for developing more accurate strategies for biological control of *A. roxburghii* bacterial soft rot disease.

# INTRODUCTION

A balanced microbiome is important for humans, plants, and environmental health, while diseases are often associated with microbial community dysbiosis (Hooks & O'Malley, 2017). Plants grown in natural soils are colonized by a phylogenetically structured microbial consortium. And plant pathogens are diverse and ubiquitous in the environment. They are major causes of loss in vegetable, crop, and medicinal plant production (Lazcano et al., 2021). Destabilization caused by pathogen infection affects microbiome richness, evenness, and network complexity (Bello et al., 2018; Wang et al., 2021). Understanding the pathogen infection is a key challenge for medicinal plant cultivation which can exert significant impacts on the output and quality of herbal medicine (Huang et al., 2018; Tian et al., 2020). Biotic interactions, such as the network between pathogens and non-detrimental resident microbes, have severe impacts on the infection process via resource utilization and competition (Wei et al., 2015). Therefore, deciphering the microbiota variation of the pathogen-infected plants provides a crucial basis for estimating the severity and process of disease.

It has been well documented that root zone soils (bulk soils, RZS) are the main reservoir for microorganisms colonizing the rhizosphere. The first step of infection is the colonization of the plant rhizosphere, where the plant drives the migration of microorganisms by depositing the root-specific exudates that regulate bacterial gene expression and physiology at the soil-root interface, which means that the pathogens must outcompete other microbial taxa for successful colonization (De Coninck et al., 2015; Rico-Jiménez et al., 2022). Therefore, rhizosphere microbiota can function as the first line of defence against pathogen invasion (Bakker et al., 2018). Studies have also uncovered that endophytic microbes that colonize interior plant compartments without inducing disease may also contribute to host resistance against pathogens (Hu et al., 2020). Endophytes suppress pathogen infection via the induction of host resistance genes, competition, and the production of bioactive compounds (Wang et al., 2021). Surprisingly, the harmless endophytic microbes were found to assist pathogen invasion via a wide variety of mechanisms including cell–cell signalling (Gupta et al., 2021), metabolic interactions (Sun et al., 2021), evasion of the immune response (Ma et al., 2021), and a resident-to-pathogen switch (Venturi & Silva, 2012). These entirely opposite roles of endophytes call for serious consideration of pathogen–microbe interactions in the host for disease severity and control.

Bacterial soft rot caused by *Pantoea* species is a devastating disease of plants with large-scale crop losses worldwide, such as blackleg of potatoes, foot rot of rice, and bleeding canker of pears (Walterson & Stavrinides, 2015), and extensive efforts have been made to prevent and control this disease (Shin et al., 2019).

*Anoectochilus roxburghii* (Wall.) Lindl. (Orchidaceae), a perennial herb grows under evergreen broad-leaved forests or bamboo forests with relatively high humidity (Ye et al., 2017). It is widely used as a treatment booster and medicine because of its various beneficial properties, including the curative effects of heat dissipation and cooling of blood, elimination of dampness, detoxification, and immunity enhancement (Zeng et al., 2020). Its major chemical constituents are polysaccharides, flavonoids, glycosides, and Kinsenoside (Qi et al., 2018), which can be used for clinical treatment of hyperuricemia, type 2 diabetes, and chronic hepatitis B (Guo et al., 2019; Smoak et al., 2021). With a low reproduction rate and frequent forest management, the distribution ranges of *A. roxburghii* are sharply declining and artificial cultivation has been applied to improve its production (Li et al., 2019). However, bacterial soft rot occurs frequently during *A. roxburghii* cultivation (Shao et al., 2014). The outbreak of soft rot disease has led to a maximum of 70%–80% reduction in *A. roxburghii* yield, which severely hindered the development of the *A. roxburghii* medicinal industry and failed to keep up with the customers' increasing demand (Shao et al., 2014). However, the pathogen microbiota and their dynamics in the host plants remain largely uninvestigated.

Microbial diseases occur as a result of multifarious host–pathogen interactions which could be explored by the newly developed co-occurrence network approaches (Deng et al., 2012; Põlme et al., 2018). Within network structure, the configuration and distribution of links among plant soils and endophytic microbiota can provide strong predictions on the succession of pathogen infection (Deng et al., 2021; He et al., 2021; Wei et al., 2015). Network analysis could also identify keystone microbial members or other microorganisms that may function in the defence against pathogen invasion (Hu et al., 2020; Zamkovaya et al., 2021). Therefore, interactions both within the soil and endophytic communities and between the resident communities and invading pathogens are likely to be important for plant health and fitness (Michalska-Smith et al., 2021).

Here, we found that many *A. roxburghii* plants in the greenhouse were infected with stem rot when they were transplanted for 1 month in the soil. And other plants around it get sick when one plant gets sick. To investigate the mechanism of this disease occurrence and spreads, we studied the bacterial diversity, community composition, and dynamics in RZS, rhizosphere soils (RS), roots, and stems from healthy and soft rot infected *A. roxburghii* plants by high throughput sequencing. We then tracked the source of microbial migration from soils to endophytic communities during pathogen invasion. Furthermore, the interactions between pathogens and other host-associated microbiota were investigated through network analysis.

# EXPERIMENTAL PROCEDURES

## Sample collection and processing

The field experiment was conducted in 2019. All *A. roxburghii* were planted in Jinhua Academy of Agricultural Sciences, Zhejiang, China (29.08N, 119.65E). On September 2, 2019, after tissue was cultured for 4 months, and domesticated in the greenhouse for 2 months, the *A. roxburghii* plants were transplanted into soils. The culture conditions were 14 h of light and 10 h of darkness under 25 ± 2°C. The soft rot disease was observed and stems of *A. roxburghii* began to decay about 1 month after being transplanted. A total of 46 samples were collected on October 15, 2019. Four soil-root system compartments, including the RZS, RS, ES, and ER from both healthy and severely diseased plants, were sampled. Each sample type has six replicates except RZS, which has four replicates. Briefly, each sample of *A. roxburghii* was taken intact from the greenhouse using an ethanol-sterilized shovel. Soils loosely attached to the plant roots were collected by gently shaking, which was designated as RZS. The roots and stems were separated, using a sterile pair of scissors, and the roots were transferred to a new 50 ml Falcon tube filled with 30 ml sterile phosphate-buffered saline solution. Soils from the roots of each plant were collected by centrifugation at 8000 *g* for 10 min following vigorous vortexing and designated as RS. The roots and stems were surface-sterilized for 2 min in 75% ethanol, 10 min in 2.5% sodium hypochlorite, and then five times washed with sterile water, respectively. Then the sterilized tissues were stored at −80°C until used for DNA extraction. Sterility was assessed by placing 100 μl of the last washing water on Luria-Bertani agar (LB) plates for 3–7 days cultivated at a 28°C incubator. The LB plate with no microbial growth shows that disinfection is thorough. Endophytes in stems and roots are designated as ES and ER, respectively.

## DNA extraction and amplicon sequencing

The total DNA of both soil, root, and stem samples was extracted using the MP Biomedical FastDNA™ Spin Kit (MP Biomedical) according to the manufacturer's instructions. DNA concentration of each sample was quantified by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and stored at −80°C until downstream analysis. Negative controls were performed to confirm the absence of contamination in the used kit and solution. Approximately 10 ng DNA was used as the template to amplify the V3-V4 region of the 16S rRNA gene, using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′)

through polymerase chain reaction (PCR). The triplicate PCR products were pooled and purified using a PCR miniBEST DNA Fragment Purification Kit (Takara Biotech). The purified PCR products were amplified again to add the adapters and barcodes to form the libraries, which were pooled at equimolar concentrations after purification and quantification. The samples were sequenced using an Illumina MiSeq platform (Illumina) by Novogene Co., Ltd.

## Sequence data preprocessing and bioinformatics approaches

The 16S rRNA sequences obtained from the MiSeq platform were used for microbial ecology analysis. The bioinformatics processing was referred to Hong et al. (2021) and Zheng (Zheng & Gong, 2019; Zheng & Lin, 2020) using QIIME (Caporaso et al., 2010), USEARCH (Edgar, 2010), and in-house scripts. Briefly, after the generation of high-quality reads, unique reads were clustered to form OTUs at the 99% sequence similarity by the *pick_open_reference_otus.py* script. Singletons and OTUs assigned as chloroplasts, mitochondria, unassigned, and unclassified sequences were removed from the data before downstream analysis. A large OTU table was created with 46 samples in column and 12,400 OTUs in the row. To correct for the unequal sequencing depth, the OTU table was rarefied at the lowest sequences (3500) per sample to calculate alpha-diversity estimates and Bray–Curtis dissimilarity between samples.

## Statistical analysis

Statistical analyses were mainly performed in R and STAMP (Parks et al., 2014). Normal distribution of the data was checked with the Shapiro–Wilk test and homogeneity of variances was analysed using the Levene test in R with the "car" package (Fox & Weisberg, 2019). Student's *t*-test was conducted to test the statistical significance of differences between healthy and diseased plants. PD was estimated according to Faith's approach via the Picante package in R. PCoA based on Bray–Curtis distance matrix was used to examine the difference in microbial community structures. The analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA), based on the Bray–Curtis dissimilarity, were analysed using the Past 3.0. Functional prediction and metabolic characteristics of representative OTUs were carried out using PICRUSt (Langille et al., 2013). Significantly differed catalogues between different sample types were estimated in STAMP with Welch's *t*-test corrected by Bonferroni (Parks et al., 2014).

## SourceTracker and network analysis

SourceTracker analysis was applied to estimate the sources of bacteria in the root, rhizosphere, roots, and steam samples, as described by Knights et al. (2011). Groups of closely connected OTUs were identified by GCNA with a soft thresholding power of 9 (Langfelder & Horvath, 2008) and an "unsigned" network model. Hierarchical clustering was performed based on the topological overlap matrix. Negative and positive co-occurrence relationships are based on strength of correlation at $r \leq -0.9$, $r \geq 0.9$, and $p \leq 0.05$. All $p$-values were adjusted for multiple testing using the Benjamini and Hochberg FDR controlling procedure (Benjamini et al., 2006). The constructed networks of stem and root endophytic communities in healthy and diseased samples and the sub-network of specific interactions between the pathogen and other microbial members were visualized by Cytoscape 3.3.0 (Morris et al., 2015).

To elucidate microbial interactions in soil and endophytic communities during soft rot disease invasion, we constructed phylogenetic MENs via a Random Matrix Theory-based approach in the molecular ecological network analysis pipeline (MENA, http://ieg2.ou.edu/MENA/) (Deng et al., 2012; Zhou et al., 2010). Threshold values ranging from 0.01 to 0.99 with 0.01 intervals were applied to the Spearman rank correlation matrix (Deng et al., 2012). The optimal threshold value was estimated when the nearest-neighbour spacing distribution followed the Poisson distribution well, which was associated with characteristic nonrandom properties in a complex system. Furthermore, the appropriate identical threshold value (0.76) was selected to generate networks for comparing the different networks under the same conditions. The empirical networks of soil and endophytic communities were analysed by the above methods, and the random networks were generated by rewiring the positions of all links of MENs with the same numbers of nodes and links in corresponding empirical networks. A final network concerning the *Pantoea* and the top 18 most abundant genera in healthy samples and diseased samples were visualized by Cytoscape (Morris et al., 2015).

## RESULTS

### Bacterial community diversity and composition of healthy and diseased samples

The soft rot disease was observed about 1 month after the *A. roxburghii* plants were transplanted into soils from a tissue culture medium. Then four soil-root system compartments, including the RZS, RS, stems, and roots from both healthy and severely diseased *A. roxburghii* plants, were sampled. Six independent replicates were taken except RZS, which has four replicates. After DNA extraction and amplification, a total of 3,489,006 high-quality reads (healthy samples: 1,800,011 and diseased samples: 1,688,995) were obtained from 46 samples through high-throughput sequencing analysis. After removing the potential chloroplast, mitochondrial, and eukaryotic sequences, 12,400 operational taxonomic units (OTUs) (healthy samples: 9760 and diseased samples: 8458) were obtained.

The bacterial alpha-diversity indices were significant difference between healthy and diseased samples ($p < 0.05$) (Figure 1). Specifically, the bacterial diversities of soils (RZS, RS) were higher than the endophytes in stems (ES) and endophytes in roots (ER) according to the Chao1 index and observed species evenness (Figure 1A). The difference in phylogenetic diversity (PD) in RZS was non-significant between healthy plants and diseased plants, but it was remarkably higher in ER and ES of healthy plants (Figure 1A). A significant difference in Shannon diversity was detected between healthy and diseased plants wherever the bacteria come from, which indicated more bacterial species in the healthy plants than diseased ones (Figure 1A). No clear separation of microbial communities between healthy and diseased samples was detected according to the principal coordinate analysis (PCoA) (Figure 1B). However, the ER community composition in healthy plants was remarkably different compared to the other sample types along the PCoA2 axis. And ES in diseased plants showed different bacterial communities from the others along the PCoA1 axis.

After assigning to different taxonomic levels using the SILVA classifier, 99.97% of OTUs were classified at the phylum level, 91.30% were classified at the genus level, however, only 55.18% of sequences were identified at the species level. Therefore, the downstream analyses were mainly conducted at the genus and OTU levels. Generally, the most abundant phyla were Proteobacteria (47.62%), followed by Chloroflexi (16.17%), Actinobacteria (13.64%), and Acidobacteria (7.39%). The majority of detected Proteobacteria in diseased plants derived from endophytes, otherwise, most Proteobacteria members in healthy plants were from soils. All OTUs were classified into 735 genera (diseased plants: 547 and healthy plants: 656) belonging to 324 families (diseased plants: 286 and healthy plants: 316). The top 20 dominant genera of soil and endophytic samples were shown in Figure 1C. The average relative abundance of genus lower than 0.83% and genus classified as "metagenome" were combined as "Others." Apart from the unclassified taxon and "Others," *Pantoea* exhibited extraordinarily high relative abundance in diseased plants (especially the DES: 21.17%), followed by *Rhizobium*, *Ktedonobacter*, and *Enterobacter*. However, the relative abundance of *Rhizobium* (short for *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*) was much higher in diseased endophytes (diseased endophytes roots, DER and diseased endophytes stems,
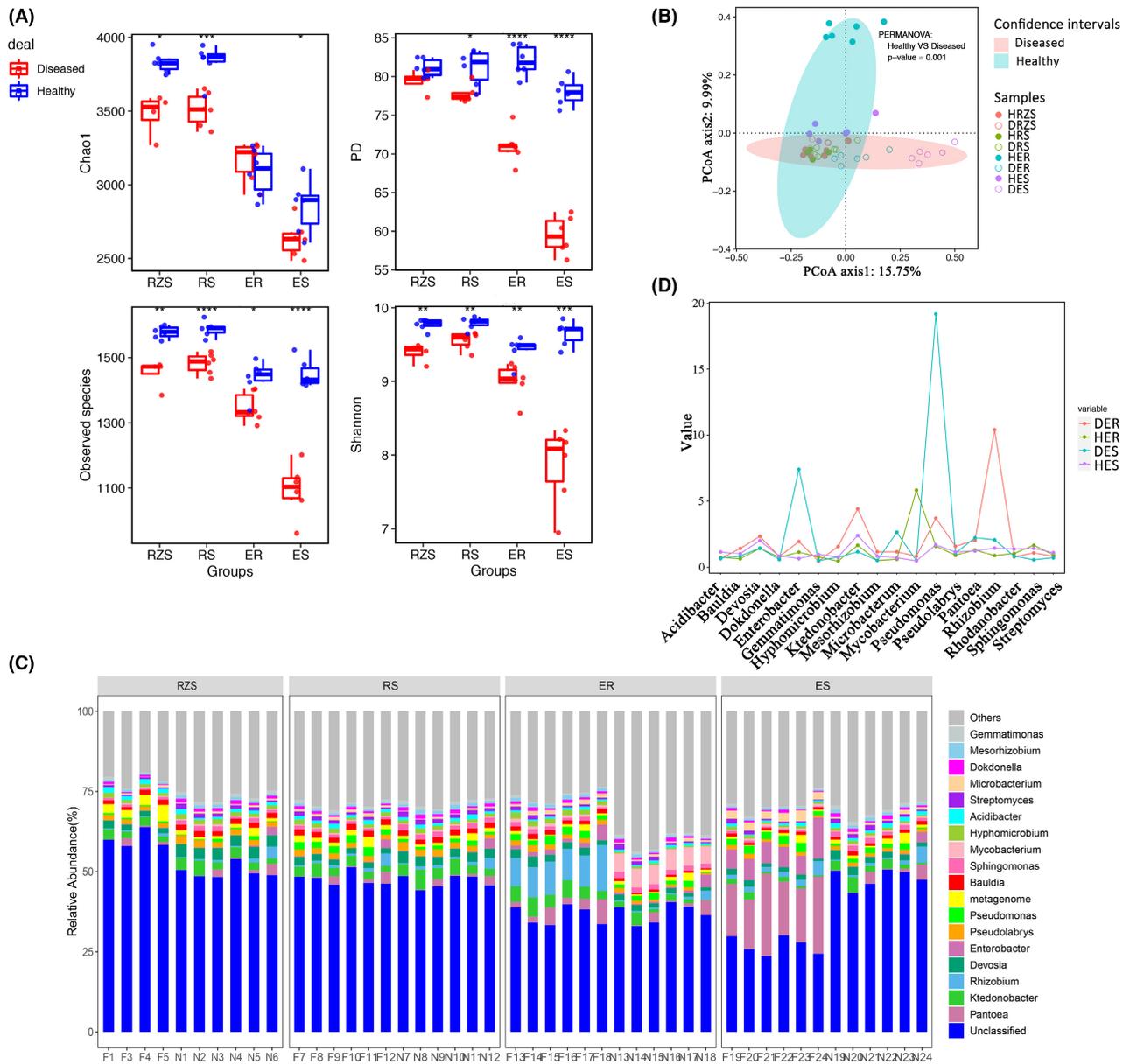
**FIGURE 1** The alpha diversity and bacterial community structure in healthy and soft rot disease-infected plants. (A) Comparative analysis of the alpha diversity of 16S rRNA sequences in RZS (root zone soils), RS (rhizosphere soils), ER (endophytes in roots), and ES (endophytes in stems) samples between healthy and diseased samples. Different asterisks indicated a significant difference at *$p < 0.05$ and **$p < 0.01$ based on the one-way ANOVA analysis; (B) Principal coordinate analysis (PCoA) in healthy and diseased samples based on the Bray–Curtis dissimilarity. (C) Bacterial community composition variation among different sample types at the genus level. Significant differences in the bacterial community between healthy and diseased samples were evaluated by ANOSIM and PERMANOVA; (D) Bacterial community differences of the top 18 dominant genera between root and stem endophytes.

DES) than in soils (diseased root zone soils, DRZS and diseased rhizosphere soils, DRS), which was the same trend in *Enterobacter*. For healthy plants, the most abundant genus was *Ktedonobacter*, followed by *Devosia, Mycobacterium*, and *Pseudolabrys*. The relative abundance of *Mycobacterium* was higher in endophytic compartments than in soils, while the other three predominant genera exhibited the opposite trend, that was, higher abundance in soils than in endophytic roots and stems.

According to LEfSe, more genera were detected in healthy plants than diseased ones (120 for healthy plants and 41 for diseased plants), which could be treated as potential biomarkers. For example, *Mycobacterium* was supposed to be a biomarker for healthy endophytes roots (HER), and *Microbacterium* for healthy endophytes stems (HES) which may be beneficial to healthy plants. While, *Acidibacter* (DRZS), *Gemmatimonas* (DRS), *Pantoea* (DES), *Pseudomonas* (DES), and *Rhizobium* (DER) were biomarkers for diseased samples.

To get further insight into the community difference between root and stem endophytes, the top 18

dominant genera were compared (relative abundance >0.83%; Figure 1D). It is worth noting that the DES were dominated by *Pantoea* and *Enterobacter*, while, the DER were occupied by species from *Rhizobium* and *Ktedonobacter*, which is largely consistent with the abovementioned results. Therefore, *Pantoea* was highly likely the predominant pathogens of *A. roxburghii* soft rot disease with the collaboration with *Enterobacter*.

## SourceTracker analysis of bacterial community from soil to endophytic compartments

The differences in the potential sources of endophytic bacteria between diseased and healthy plants were detected according to the source-tracking analysis (Figure 2A). In healthy samples, the majority of RS bacteria were derived from the RZS (68.3%). For roots, 29.3% of endophytes were from RS and 28% from RZS. There were three main sources of ES, most of them from RS (30%), 24.7% from RZS, and 13.5% from

roots indicating that frequent microbial transfer may exist from the exterior to the interior compartments of healthy plants. In diseased samples, the RZS (62%) were the main sources of the RS bacteria, which supported 48.5% of the ER bacteria, and the ES were primarily from ERs (55%), indicating that there is microbial flow from soils to the interior of diseased plants which may be responsible for the pathogenic invasion.

## Molecular ecological network analysis on soil and endophytic communities

Since different microbial participate in diverse biological processes, weighted gene co-expression network analysis (WGCNA) and dynamic hierarchical clustering approaches were used to define modules of shared OTUs between root and stem endophytes from both healthy and diseased samples (Figure 2B). We first defined the module eigengene (ME), a single value that represents the highest percentage of variance in the relative abundance of OTUs for all
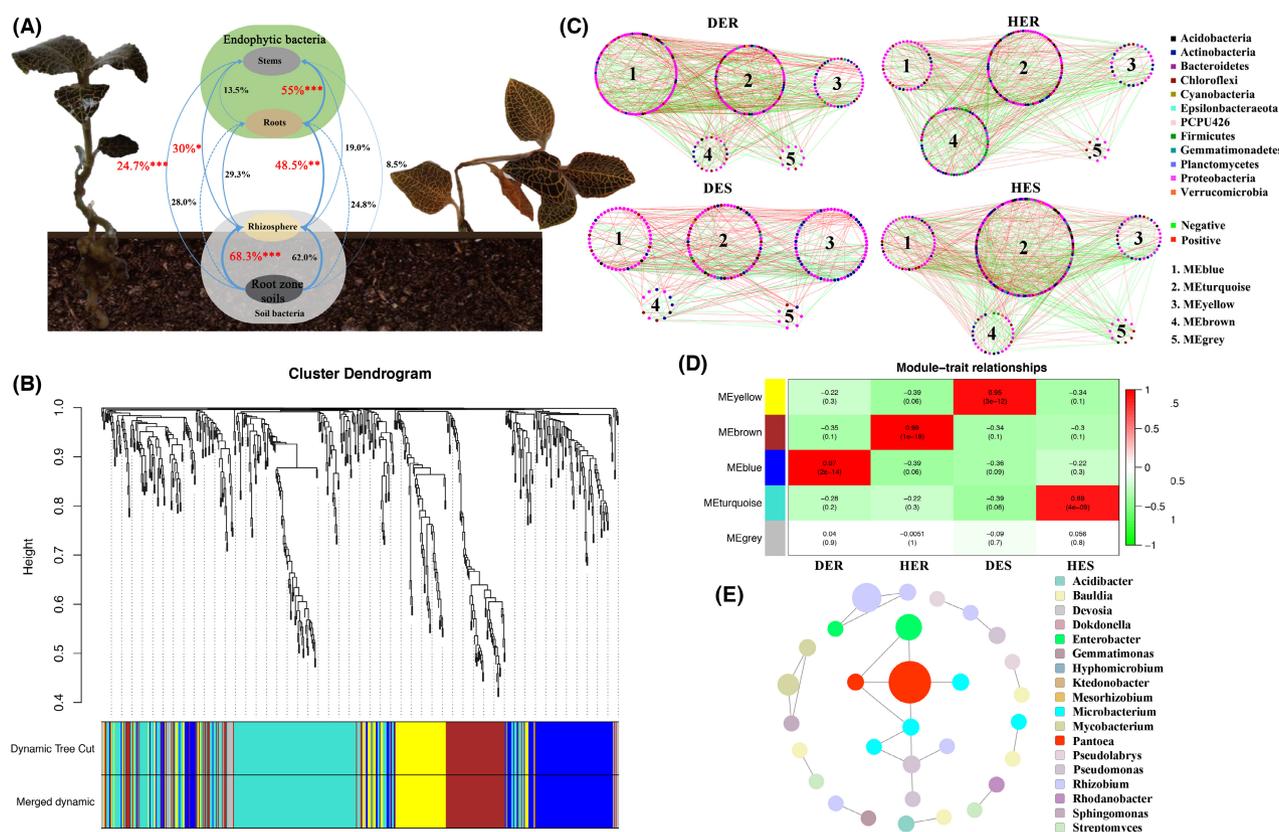


**FIGURE 2** Sample-specific OTU modules and network visualization of the interaction architecture in bacterial communities of healthy and diseased *A. roxburghii*. (A) SourceTracker analysis results of healthy (left) and diseased (right) samples. (B) Hierarchical cluster tree showing modules of bacterial OTUs, with a total of five modules identified by WGCNA dendrograms. The height (*y*-axis) indicated the level of correlation. Colours represent the five different OTU modules. (C) The constructed networks of stem and root endophytic communities in healthy and diseased samples. (D) Heatmap of correlations between modules and different samples with numerical Pearson's correlation coefficient (top) and corresponding *p* values (bottom). (E) Network of interactions between the pathogen and other species in soft rot disease infected roots. Each node was identified at the genus level. The size of nodes represented the relative abundance of each genus. DER, diseased endophytes roots; DES, diseased endophytes stems; HER, heathy endophytes roots; HES, heathy endophytes stems.

modules in a stage. Thus, the bacterial profiles of module traits among different compartments can be summarized as the bacterial profile of MEs. Five modules consisting of 576 OTUs were identified (Figure 2B, Table S1). Apart from Proteobacteria, which took an overwhelming majority in the composition of each module, different modules have variations in bacteria composition. The OTU-enriched module 2 "MEturquoise" (221/576) were abundant in Actinobacteria and Chloroflexi. Actinobacteria was also the dominant phylum in module 3 "MEyellow." However, Firmicutes in the "MEbrown" module was more predominant than Actinobacteria and Chloroflexi apart from Proteobacteria (Figure 2C, Table 1).

Pearson's correlation coefficient values ($r$) between MEs and different compartments were then used to determine the relationship between a module and a plant compartment (Figure 2D). Collectively, four significantly "compartment-specific" modules were identified ($r > 0.8$ and $p < 0.001$, Figure 2D). Module 1 was positively correlated with ER of diseased plants, module 2 with ES of healthy plants, module 3 with ES of diseased plants, and module 4 with ER of healthy plants. In addition, 224 significantly different OTUs were detected among different modules via ANOVA. Noticeably, OTU3524 (*Pantoea*, relative abundance: $16.89 \pm 3.82$) was extremely enriched in the DES followed by OTU128 (*Enterobacter*, relative abundance: $6.91 \pm 2.35$), both of which belonged to module 3. The healthy roots-enriched endophyte OTU2885 (*Mycobacterium*, relative abundance: $4.34 \pm 0.38$), presented in module 4, may play role in resistance to pathogen infection.

To gain a deeper insight into the interactions among endophytic microorganisms, the networks of five identified modules were visualized by Cytoscape and significantly different network structures were found (Figure 2C). A higher positive/negative edge ratio was detected in DES1.62 than in other sample types (Table 2). In diseased plants, ER had a more complex and highly connected bacterial community than those of ES. Microbes in module 1 contributed most to the root endophyte community, followed by microbes in module 2, implying that microbes in module 1 are likely responsible for the soft rot of *A. roxburghii*. In addition, modules 2 and 3 predominantly account for the majority of microbial contributions to stem endophytes, indicating that the invasion by soft rot pathogens may associate with the interactions with other microbial members in plants. For healthy plants, the topological properties of networks were remarkably different compared to diseased plants, exhibiting that module 2 accounts for the majority of microbial contributions to both root and stem endophytes, suggesting that microbes from module 2 may be capable of resistance to soft rot pathogens and benefit to the well-being of host plants.

## Network of interactions among potential pathogenic Pantoea and other microbial members in diseased plants

To further reveal which endophytic microorganisms may be important in aiding or inhibiting soft rot

**TABLE 1** The composition of bacterial phylum in different modules

| Phylum | Module 1 (MEblue) | Module 2 (MEturquoise) | Module 3 (MEyellow) | Module 4 (MEbrown) | Module 5 (MEgrey) |
|---|---|---|---|---|---|
| Proteobacteria | 96 | 95 | 48 | 43 | 14 |
| Actinobacteria | 8 | 40 | 22 | 16 | 2 |
| Chloroflexi | 9 | 35 | 4 | 15 | 5 |
| Acidobacteria | 9 | 20 | 3 | 4 | 2 |
| Gemmatimonadetes | 2 | 8 | | 4 | |
| Verrucomicrobia | 4 | 6 | | 3 | 1 |
| Bacteroidetes | 1 | 5 | 2 | 6 | |
| Firmicutes | | 5 | 2 | 19 | |
| Planctomycetes | | 4 | 2 | | |
| Entotheonellaeota | | 1 | | | |
| Patescibacteria | | 1 | | | |
| Unassigned | | 1 | | | |
| Cyanobacteria | 2 | | | | |
| Epsilonbacteraeota | | | 1 | 1 | |
| FCPU426 | | | | | |
| Latescibacteria | | | 1 | 1 | |
| WPS-2 | | | 1 | 1 | |
| WS2 | 1 | | | | |

**TABLE 2** The significantly different OTUs between endophytic roots and stems from both healthy and infected samples with their relative abundance >1%

| Module | HER | HES | DER | DES |
| --- | --- | --- | --- | --- |
| MEblue | 98 | 149 | 280 | 96 |
| MEbrown | 162 | 72 | 42 | 32 |
| MEgrey | 11 | 30 | 35 | 18 |
| MEturquoise | 234 | 505 | 227 | 125 |
| MEyellow | 53 | 88 | 98 | 104 |
| Positive/negative | 281/277 | 458/386 | 361/321 | 232/143 |

outbreaks, subnetworks for the interactions among pathogenic *Pantoea* and other microbial members were analysed. The network of interactions in diseased plants revealed that potential pathogenic bacteria *Pantoea* were positively correlated with *Enterobacter* and *Microbacterium* (Figure 2E). *Enterobacter* was positively correlated with *Rhizobium* and *Microbacterium* was positively interacted with *Pseudomonas* and *Bauldia*. No negative interactions were detected between *Pantoea* and the other top abundant genera. These bacteria that correlated positively with potential pathogenic *Pantoea* members may play important roles in assisting bacterial soft rot infection.

## Function prediction and metabolic characteristics

To explore the potential ecological/biological function divisions of microbial communities between healthy and diseased plants, the metabolism pathways were predicted. Significant differences in metabolic characteristics were found with regard to where the microbe inhabited (interior plant organs or exterior soils). Generally, greater function divisions were detected in endophytes than in soil microbes between healthy and diseased plants (Welch's $t$-test corrected by Bonferroni, $p < 0.05$) (Figure 3). For example, no metabolic differentiation was detected when comparing root-soil microbes between diseased and healthy plants, and only 14 significantly different pathways were found in RZS, most of them were enriched in healthy plants. Amino sugar and nucleotide sugar metabolism, and galactose metabolism were exclusively abundant in diseased plants. However, for endophytes, 28 and 23 differential pathways were found in ES and ER, respectively. Soft rot infected plants of ES had higher relative abundance in cell motility, cellular processes, and signalling, membrane transport, amino acid metabolism, carbohydrate metabolism than the healthy plants, while, the healthy ones were abundant in energy metabolism (especially the photosynthesis), glycan biosynthesis, lipid metabolism, and

metabolism of cofactors and vitamins. Similarly, more abundant pathways (17/23) were detected in DER, and energy metabolism, metabolism of cofactors and vitamins were particularly enriched in healthy endophyte roots.

## DISCUSSION

*Pantoea ananatis*, which could symbiosis with plants, is better known as a phytopathogen affecting the yield of many economically important plants that causes blight and dieback of *Eucalyptus* (Arriel et al., 2014), maize leaf spot disease (Krawczyk et al., 2021) and brown stalk rot (Weller-Stuart et al., 2014), leaf blight and bulb rot of onion (Weller-Stuart et al., 2014), palea browning and stem necrosis of rice (Azizi et al., 2020), and fruit rot of netted melon (Özdemir, 2021). However, there are also many plant-associated microbes that can perform a wide range of life-beneficial functions, including plant nutrient acquisition, immune development, and plant tolerance of multiple stresses (Hu et al., 2022). The plant-microbe balance keeps plant's normal growth. And higher microbial diversity increases community invasion resistance due to interactive effects on community stability (Gao et al., 2021; Ravi et al., 2022). Our results showed that the microbial diversity of soils, roots, and stems were higher in healthy samples than in diseased samples according to both Shannon diversity and PD indices (Figure 1). It could be explained by the fact that the dominance of pathogens depressed the resident bacteria growth during the onset of soft rot disease.

Through the species classification, we found that OTUs assigned to potential pathogenic *Pantoea* were rarely observed in all healthy samples (HRZS, HRS, HES, and HER), but showed fairly high abundance in the diseased samples (DRZS, DRS, DES, and DER), consistent with field observations of plant soft rot. Correspondingly, the relative abundances of several bacteria were clearly altered after bacterial soft rot infection. There was a decline in the relative abundances of *Ktedonobacter*, *Devosia*, *Enterobacter*, *Pseudolabrys*, *Sphingomonas*, *Mycobacterium*, *Streptomyces*, *Dokdonella*, *Mesorhizobium*, *Gemmatimonas*, and *Rhodanobacter* in DRZS and DRS. These compositional changes could be a consequence of pathogen invasion. *Streptomyces* was report could induction of microbial autophagy by secrets rapamycin. The effect of rapamycin on TOR can promote the degradation of the histone acetyltransferase Gcn5, thereby reducing the acetylation level of Atg8 and promoting autophagy (Wang et al., 2021). In the endophytic compartments, the relative abundances of *Enterobacter*, *Microbacterium*, *Pseudomonas*, and *Rhizobium* showed significant increases in diseased samples compared to the healthy ones. It is worth noting that the abundance of *Enterobacter* exhibits a contrasting trend
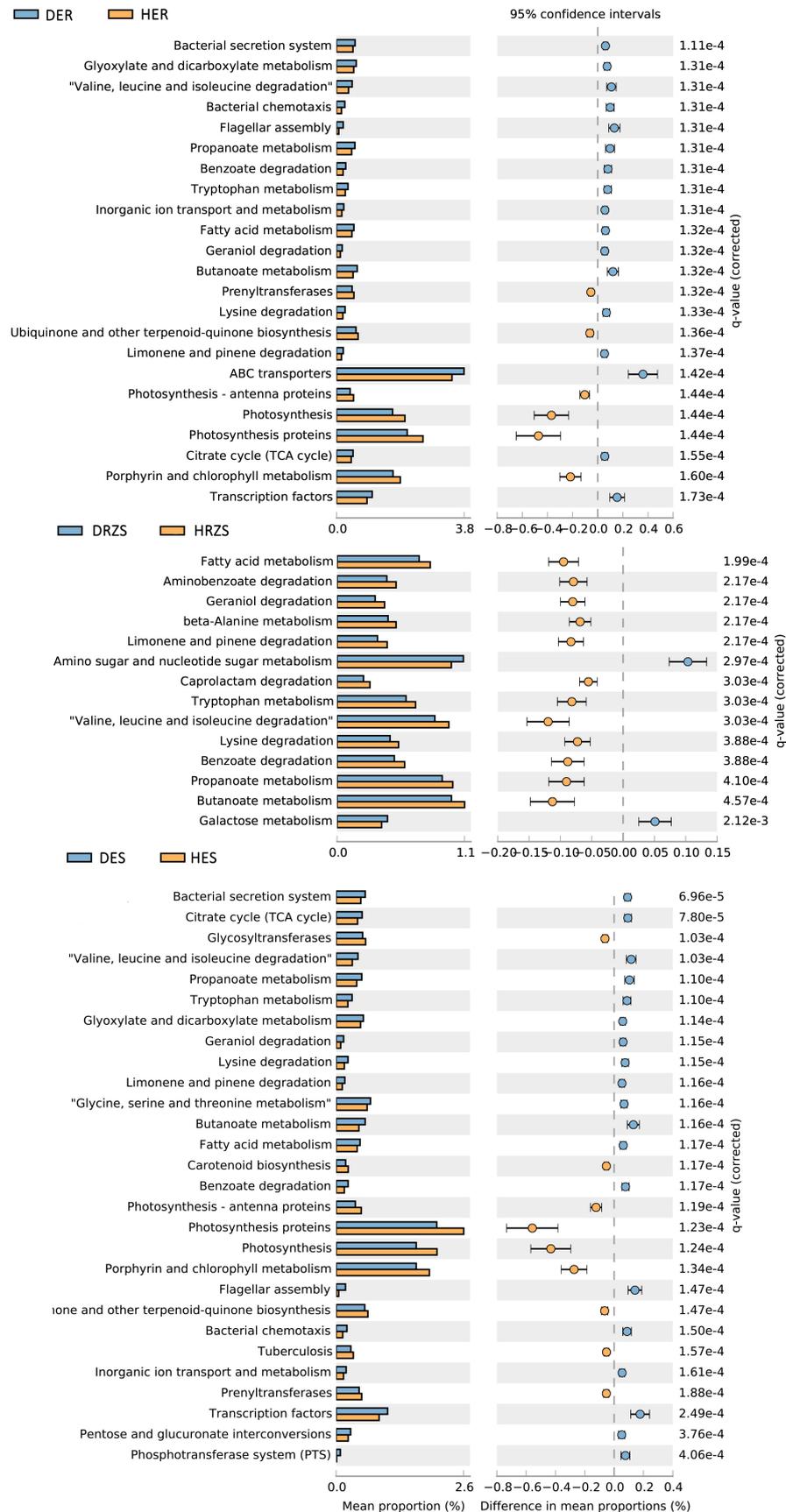
**FIGURE 3** Significantly different pathways between healthy and diseased plants at KEGG functional category 2 with their statistical significance estimated in STAMP. DER, diseased endophytes roots; DES, diseased endophytes stems; DRZS, diseased root zone soils; HER, heathy endophytes roots; HES, heathy endophytes stems; HRZS, healthy root zone soils.

in soils and endophytic samples. Actually, some microbes have dual attributes. Genera like *Pseudomonas*, *Streptomyces*, *Bacillus*, *Paenibacillus*, *Enterobacter*, *Pantoea*, *Burkholderia*, and *Paraburkholderia* have been reported for their roles in pathogen suppression (Ma et al., 2021; Sun et al., 2021). *Enterobacter* is the most promising candidates against *Gaeumannomyces graminis* (Compant et al., 2019). However, soft-rot *Enterobacter* were broad host-range pathogens that cause wilt, rot, and blackleg diseases on a wide range of plants (Charkowski et al., 2011). Some species of *Rhizobium* are capable to fix nitrogen when in symbiosis with leguminous plants, but some were pathogenic bacteria to plants (Ji et al., 2010). The relatively high abundances of *Rhizobium*, *Ktedonobacter*, and *Enterobacter*, suggest they may be involved in the process of pathogen invasion and have mutualistic relationships with pathogenic members of *Pantoea* (Elsas et al., 2012), or they are opportunists, which take advantage of potential ecological niches opened by pathogen invasion (Lundberg et al., 2012).

As the differences in microbial diversity and community composition between healthy and diseased plants have been observed, a further understanding of where the pathogens come from and how they interact with each other will provide a "road map" to disentangle the pathogen invasion process which contributes to disease control. Many studies have investigated that microorganisms can enter roots through the root tip and root hair or enter shoots via stomata and enter the seed during seed germination (Hugouvieux-Cotte-Pattat et al., 2014; Compant et al., 2021; Synek et al., 2021). Previous studies showed that soft rot pathogens invaded *A. roxburghii* via the roots and wounds, and then aggressively spread to the aerial compartments throughout the vascular system (Peeters et al., 2013; Walterson & Stavrinides, 2015). In line with this view, our SourceTracker analysis revealed that the bacteria communities in the stems were mainly derived from roots (55%), and the root endophytes largely derived from RS (48.5%), which come from RZS (62%). This is supported by the evidence that the RZS was the main source of microbial species richness in the plant rhizosphere (Jansson & Hofmockel, 2020), and plant endophytes were largely originated from soils and then transferred upwardly to the above-ground tissues via the vascular system (Figure 2A). Therefore, the soft rot pathogen invasion investigated in this study may begin in the RZS, break into the plant roots, colonize the stems, and finally induce soft rot disease.

Recent studies have shown that harmless resident bacteria can be important to the incoming pathogens and in the outcome of the disease (Venturi & Silva, 2012). From the perspective of resource utilization and competition, plants and pathogens can have direct co-evolutionary relationships (Friesen, 2020; Singh et al., 2018). To further investigate how the pathogens interact with each other and their impacts on neutral or beneficial microbes after infection. We performed network analyses on root and stem bacterial community interactomes of diseased and healthy plants, and revealed their topological features (Figure 2C,D). Five modules were uncovered with each one matched to a specific sample type. For example, the soft rot disease-infected samples were positively correlated with modules 1 and 3, while the healthy plants were correlated with modules 2 and 4, indicating that microbes in modules 1 and 3 were more likely to be pathogens or pathogen helpers, and microbes in modules 2 and 4 may contribute to the host plant's resistance to pathogen invasion. Crucially, highly connected and modular microbiota could prime the plant immune system for accelerated activation of defence against the pathogen (Burdett et al., 2019; Tzipilevich & Benfey, 2021). In this sense, by changing community structure, invasive pathogenic microbes generate positive feedback that enhances both their own competitiveness and subsequent interactions with their "resident" partners.

In addition, we found that pathogenic *Pantoea* was positively associated with *Enterobacter* and *Microbacterium* (Figure 2E). It has been reported that the highly connected and anomalously correlated nodes were either targets or helpers of diverse pathogens (Ahmed et al., 2018). Such as, microbes that positively interact with *Ralstonia* were the preferred helpers for pathogen attack in tobacco bacterial wilt disease (Wei et al., 2015). Similarly, the positively correlated *Enterobacter* and *Microbacterium* were also possible the preferred helpers during soft rot pathogen invasion. Certain species from *Enterobacter* are phytopathogens, causing bacterial palea browning of rice (Cao et al., 2020), sprouting decay, and seedling stunting of upland cotton (Nagrale et al., 2020), as well as affecting other plants including onion bulb (Weller-Stuart et al., 2014), edible ginger (Liu et al., 2020), mulberry (Zhu et al., 2010), and papaya fruit (Keith et al., 2008). Another important finding was that an olive tree pathogen *Pseudomonas savastanoi* collaborated with a harmless resident endophyte and induced a significantly bigger tumour via interspecies quorum sensing cell–cell signalling (Hosni et al., 2011). The ecological functioning changes, like cellular processes and signalling, membrane transport, amino acid metabolism, carbohydrate metabolism were more abundant in diseased plants than in healthy ones. Taken together, we infer that the infection by pathogenic *Pantoea* members may be highly associated with positive interactions between them and non-detrimental bacteria, including *Enterobacter* and *Microbacterium*, and that these non-detrimental bacteria benefit from promoting pathogens, which might lead to the assemblage of additional bacterial genera into plant roots and stems from RZS, eventually causing the outbreak of *A. roxburghii* soft rot disease. These discoveries will

open new avenues for developing more accurate strategies for *A. roxburghii* bacterial soft rot disease control. Further work is needed to confirm these findings.

## CONCLUSIONS

Here we showed that the onset of *A. roxburghii* bacterial soft rot disease may begin in the RZS, migrated into the plant roots, transferred to the stems, and finally induce soft rot disease (Figure 2A). The pathogenic *Pantoea* invasion is associated with a drastic reduction in microbial diversity, abundance, and community composition, especially with the microbes in diseased plant stems. By changing community structure, invasive pathogenic microbes generate positive feedback that enhances both their own competitiveness and subsequent interactions with resident endophytic partners. These non-detrimental bacteria benefit from promoting pathogens, which might lead to the assemblage of additional bacterial genera into plant roots and stems from RZS, eventually causing the outbreak of *A. roxburghii* soft rot disease. These results will provide more accurate guidelines for soft rot disease control.

## AUTHOR CONTRIBUTIONS
BX and YZ analysed the data and wrote the manuscript. BX revised the manuscript. MZ, MC, and WQ conducted the experiments, and XL and LL analysed the data. HG and QS conceived the experiments and revised the manuscript. All authors read and approved the final manuscript.

## CONFLICT OF INTEREST
The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT
The raw 16S rRNA gene amplicon sequences supporting this study have been deposited in the NCBI Sequence Read Archive (SRA) database under the BioProject accession number PRJNA772090.

## ORCID
*Haipeng Guo* https://orcid.org/0000-0002-0368-9507
*Qingsong Shao* https://orcid.org/0000-0003-0171-5190

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