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Yan-Qiu Yang
Fujian Academy of Agricultural Sciences
Su-Fang Deng
Fujian Academy of Agricultural Sciences
You-Quan Yang
Fujian Academy of Agricultural Sciences
Zhao-Yang Ying
zhaoyang_ying@163.com

Fujian Academy of Agricultural Sciences

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Comparative Metagenomic Analysis of Phyllospheric Microbial Diversity in *Azolla imbricata* and *Azolla pinnata*

Yan-Qiu Yang[1,2]*, Su-Fang Deng[1,2], You-Quan Yang[1,2], Zhao-Yang Ying[1,2]*

1. Institute of Resources, Environment and Soil Fertilizer, Fujian Academy of Agricultural Sciences, Fuzhou, China. 2. National Azolla Germplasm Resource Nursery (Fuzhou), China.

*Correspondence: yangyanqiu518@163.com; zhaoyang_ying@163.com

Abstract

Background: The aquatic fern *Azolla* grows rapidly, produces nutrient-rich biomass, and adapts well ecologically due to symbiotic interactions with phyllospheric microorganisms. *Azolla imbricata* (Aim) and *Azolla pinnata* (Api) are valuable for biofertilization and phytoremediation, with their ecological differences potentially influenced by species-specific microbial partnerships. This study examines their phyllospheric microbiomes under controlled conditions to explore adaptive strategies.

Method and Results: High-throughput metagenomic sequencing identified 10,857 bacteria, 986 eukaryotes. Proteobacteria dominated, particularly Burkholderiales, with higher abundance in Aim. Api exhibited greater Cyanobacteria abundance, especially Nostocaceae (genus *Trichormus*). Streptophyta constituted over 90% of fungal communities in both species. LEfSe analysis highlighted unique taxa in each species, including enriched Magnoliopsida and Bacillariophyta in Aim, while Api had more Marchantia and Streptophyta. KEGG pathway analysis identified ribosome, oxidative phosphorylation, photosynthesis, quorum sensing, and two-component systems among the most enriched pathways. Resistance gene analysis showed Aim had higher levels of cmlA9, qacEdelta1, tetD, and ANT3-Ii-AAC6-IId_fusion_protein, while Api had more AAC6-IIa.

Conclusions: Aim's enrichment in Proteobacteria may enhance resilience, while Api's nitrogen-fixing Cyanobacteria suggest distinct nitrogen management. Functional and resistance gene differences highlight ecological adaptability, providing insights for optimizing Azolla's agricultural potential.

Keywords: *Azolla*, phyllosphere, microorganisms, metagenome.

Background

Azolla, a genus of aquatic ferns belonging to the family Salviniaceae, encompasses approximately

seven species classified into two subgenera: *Rhizosperama*, which includes *A. pinnata*, *A. nilotica*, and *A. imbricata*, and *Euazolla*, which encompasses *A. filiculoides*, *A. mexicana*, *A. caroliniana*, *A. microphylla* and *A. rubra* [1,2]. *Azolla* is notable for its rapid biomass accumulation, achieving doubling within 2 to 5 days, facilitated by its symbiotic association with *Nostoc azollae*, a cyanobacterium that resides within specialized leaf cavities, thereby enabling *Azolla* to effectively fix atmospheric nitrogen [3,4]. This unique trait positions *Azolla* as a valuable organic fertilizer and nutrient source for rice cultivation, making it a crutical component of sustainable agriculture [5-7]. In addition to its agricultural significance, *Azolla* provides various ecological benefits, serving as a carbon sink and serving in phytoremediation [8-10]. Certain species of *Azolla* have also demonstrated potential medicinal applications, exhibiting antibacterial and antioxidant properties that inhibit pathogens such as *E. coli* and *S. aureus*, as well as providing hepatoprotective effects in animal models [11-14].

Azolla species thrive in freshwater environments, including ponds, lakes, and rivers, where dynamic conditions influence their growth and development [15]. The variability in *Azolla*'s germplasm resources across species leads to distinct physiological and biochemical traits, potentially affecting interactions with the associated microbiome of its fronds [16]. As interest in *Azolla*'s genetic resources increases, there is growing recognition of the role of species-specific microbial associations in influencing ecological functions, resilience, and nutrient cycling in different environments. The plant microbiome is essential for maintaining plant health and environmental adaptability, with endophytic communities in the phyllosphere playing key roles in nutrient acquisition, stress resilience, and overall plant fitness [17-20]. Recent studies have revealed the presence of complex bacterial communities in *Azolla*'s leaf cavities. These communities exhibit variability across species, suggesting that microbial associations are influenced by the plant's genetic diversity. Although the details of these interactions remain incompletely understood, they are critical for maximizing *Azolla*'s potential in agricultural and environmental applications.

This study aims to investigate the differences in phyllospheric microbial communities associated with two *Azolla* species, *A. imbricata* and *A. pinnata*, cultivated under identical conditions. Using metagenomic analysis, we intend to explore how genetic diversity influences microbial community composition and function. By analyzing DNA sequences from these microbial

communities, the study aims to identify microbial diversity and elucidate species-specific interactions, thereby providing insights into the ecological roles of these communities. The findings will contribute to understanding the potential applications of *Azolla* in sustainable agriculture and environmental remediation.

Results

Sequence data summary

High-quality sequencing data were obtained from all samples, as confirmed by a clean data rate exceeding 97%, determined through rigorous quality control analysis. The average Q20 and Q30 values were 97.8% and 93.59%, respectively, indicating excellent data quality. The average GC content was 52.46%, which falls within the expected range for the samples as detailed in **Table 1**. To assess the consistency among biological replicates, correlation coefficient analysis was performed, revealing that all samples clustered individually within their respective replicates, indicating a high level of correlation (**Figure 1A**). This finding was further supported by the results of the principal component analysis (PCA) (**Figure 1B**). Collectively, these results demonstrate that the quality of the sequencing data met the necessary criteria for subsequent analysis, thereby ensuring reliable outcomes.

Table 1 Sequencing Data Statistics Information in samples

Samples	Total_ Reads	Raw bases (GB)	Clean_bases(GB)	GC_Content	Q20	Q30
Aim_1	37,399,688	5.2196	5.1629	54.89%	97.70%	93.38%
Aim_2	44,589,542	6.223	6.1532	54.13%	97.70%	93.38%
Aim_3	41,515,748	5.794	5.7397	55.01%	97.93%	93.95%
Api_1	43,793,934	6.112	6.0604	48.68%	98.23%	94.48%
Api_2	33,784,944	4.7151	4.653	48.33%	97.60%	93.10%
Api_3	38,189,846	5.3299	5.2669	53.73%	97.64%	93.24%

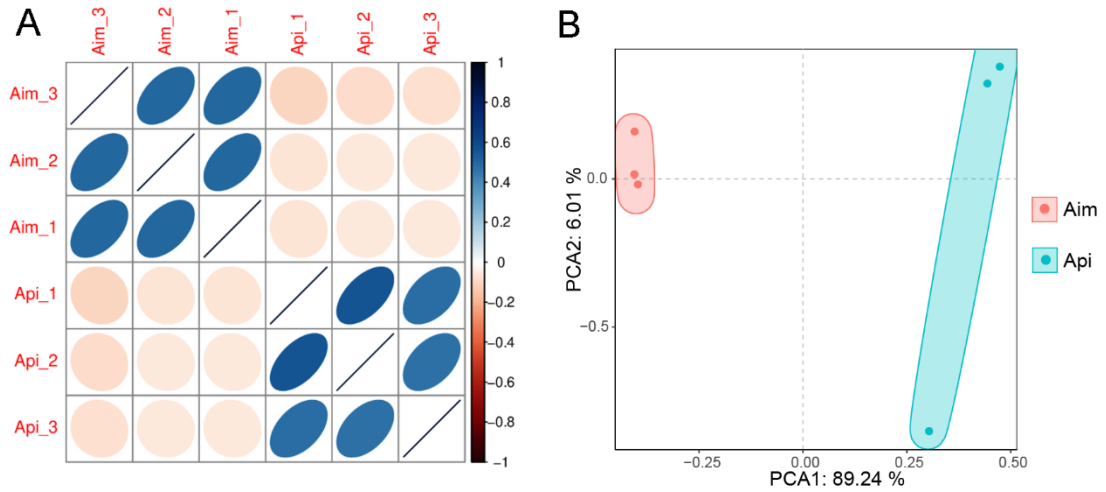

Figure 1 The analysis of *Sequence data for two species of Azolla*

(A) Biological correlations of the samples. The presence of significant correlations between two species is indicated by red ellipses for negative correlation and blue ellipses for positive correlations. (B) PCA of the samples. "Aim" represents *Azolla imbricata,* and "Api" represents *Azolla pinnata*.

Taxonomic Composition of the Microbial Communities

The taxonomic composition of the microbial communities was evaluated by aligning sequences with the NCBI-NT database. The annotation results indicated the identification of 10,857 operational taxonomic units (OTUs) as bacteria, 986 as eukaryotes. Cyanobacteria and Proteobacteria emerged as the most abundant phyla in both Api and Aim samples, collectively representing over 70% of the total microbial population as illustrated in **Figure 2A**. Notably, Proteobacteria was the dominant phylum in both samples, accounting for 42.39% of the total in Api and 51.35% in Aim. At the order level, the most prevalent orders within Proteobacteria were Burkholderiales (21.54% in Api and 27.98% in Aim), Hyphomicrobiales (2.72% in Api and 5.01% in Aim), and Cellvibrionales (0.62% in Api and 1.28% in Aim), as shown in **Figure 2B**. Cyanobacteria was the second most abundant phylum, constituting 42.19% of the total in Api and 22.40% in Aim. Within Cyanobacteria, the dominant orders were Nostocales (43.92% in Api and 22.73% in Aim) and Pseudanabaenales (0.75% in Api and 0.86% in Aim, as depicted in **Figure 2B**. At the genus level, a total of 2,271 genera were identified across all samples, with *Trichormus*, *Calothrix*, *Methyloversatilis*, and *Haliscomenobacter* being the predominant genera. The relative abundance of *Trichormus* reached 27.49% in Api and 12.06% in Aim, while the relative abundance of the other genera remained below 10%, as shown in **Figure 2C**. The relative abundance of *Haliscomenobacter* demonstrated significant variation with values of 1.25% in the

Api group and 4.06% in the Aim group. Furthermore, a Linear Discriminant Analysis Effect Size (LEfSe) was performed on the bacterial data at the phylum level. The results revealed distinct biomarker profiles between the two groups. The Aim group exhibited a higher proportion of the phylum Proteobacteria, specifically within the order Burkholderiales and class Betaproteobacteria, among others (as illustrated in **Figure 2D and 2E**). In contrast, the Api group showed an increased prevalence of the phylum Cyanobacteria, particularly within the order Nostocales, family Nostocaceae, and genus *Trichormus*, among others (as depicted in **Figures 2D and 2E**).

We identified numerous OTUs annotated as eukaryotes. Notably, the phylum Streptophyta accounted for over 90% of the relative abundance of all identified fungi, as illustrated in **Figure 3A**. Within the phylum Streptophyta, we observed genera such as *Vitis*, *Glycine*, *Selaginella*, *Cajanus*, and *Marchantia*, along side several other fungi with lower abundance, as depicted in **Figure 3B**. Further analysis utilizing LEfSe indicated that the class Magnoliopsida, phylum Bacillariophyta, and class Bacillariophyceae were more prominently represented in the Aim group, as shown in **Figures 3C and 3D**. In contrast, the phylum Streptophyta, the species *M. polymorphic*, and the genus *Marchantia* were more prevalent in the Api group, as demonstrated in **Figures 3C and 3D**.

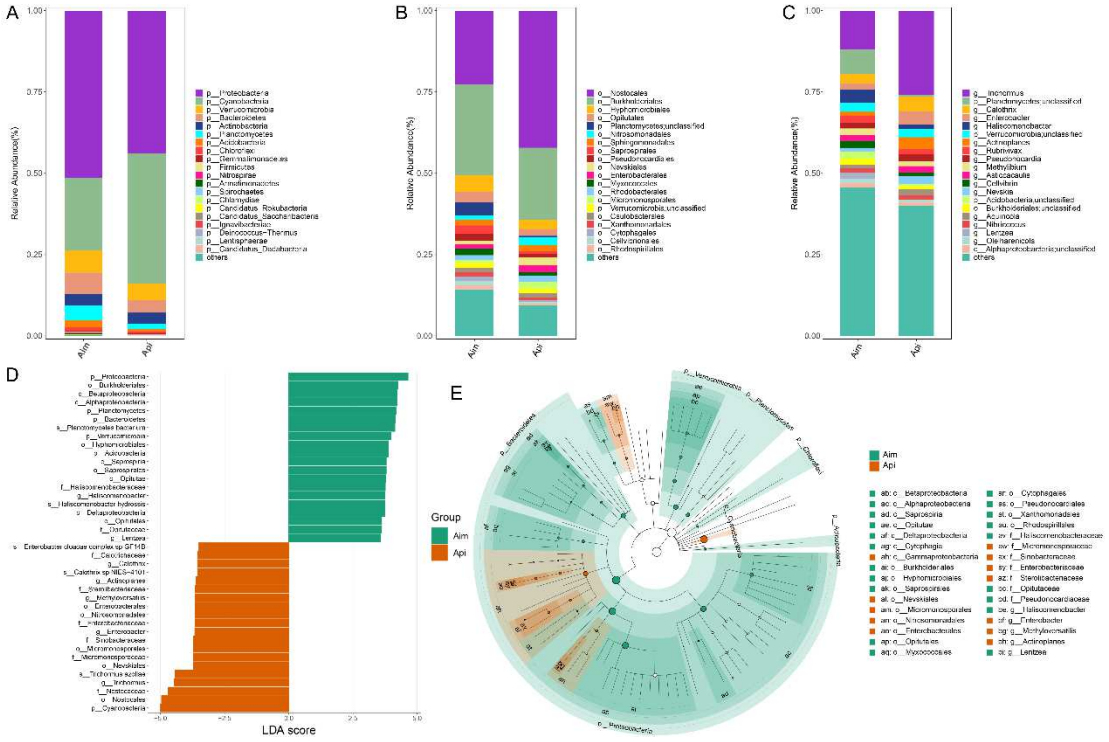

Figure 2 Taxonomic composition of bacteria communities

(A), (B) and (C) represent the relative abundance of bacteria at the phylum, order and genus level, respectively. (D) and (E) shows the graphical summaries of LEfSe (Linear discriminant analysis Effect Size) results. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

Pathogenic Microorganisms

Utilizing data from the Pathogenic Bacteria Database (https://globalrph.com/bacteria/), we identified 70 pathogenic bacteria in our analysis (Figure 4A). A significant proportion of these bacteria displayed a higher relative abundance in the Aim group. Furthermore, we performed LEfSe analysis, which revealed 13 bacteria that were significantly different between the two groups (Figure 4B). Notably, Actinomyces, Porphyromonas, Salmonella, Achromobacter, Stenotrophomonas maltophilia, Clostridium, Nocardia, and the Enterobacter cloacae complex were found to be more abundant in the Aim group compared to the Api group. In contrast, Enterobacter cloacae, Enterobacter, Pseudomonas aeruginosa, and Nocardia exhibited greater abundance in the Api group than in the Aim group.

KEGG Functional Analysis

The functional capabilities of the microbial community were evaluated through a metagenomic study employing KEGG functional analysis. According to our criteria, pathways representing more than 1% of the total pathways were classified as dominant. Consequently, we identified 20 predominant pathways across the two groups (Figure 5A). Among these, the five most enriched KEGG pathways included ribosome biogenesis, oxidative phosphorylation, photosynthesis, quorum sensing, and the two-component system. Additionally, we performed a detailed analysis to identify KEGG pathways that displayed significant differences between the two groups. The findings indicated that pathways such as Staphylococcus aureus infection, melanogenesis, chloroalkane and chloroalkene degradation, sphingolipid metabolism, biosynthesis of various secondary metabolites, Vibrio cholera infection, apoptosis, the RAS signaling pathway, stilbenoid, diarylheptanoid and gingerol biosynthesis, and butanoate metabolism ranked among the top ten significantly enriched KEGG pathways (Figure 5B).

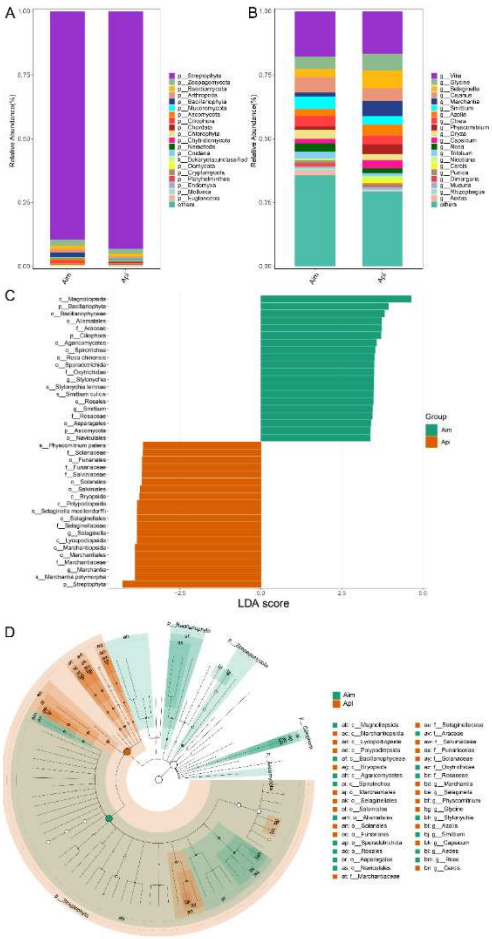

Figure 3 Taxonomic composition of fungi communities

(A) and (B) represent the relative abundance of fungi at the phylum and genus level, respectively. (C) and (D) display the graphical summary of LEfSe results. “Aim” represents *Azolla imbricata,* and “Api” represents *Azolla pinnata*.

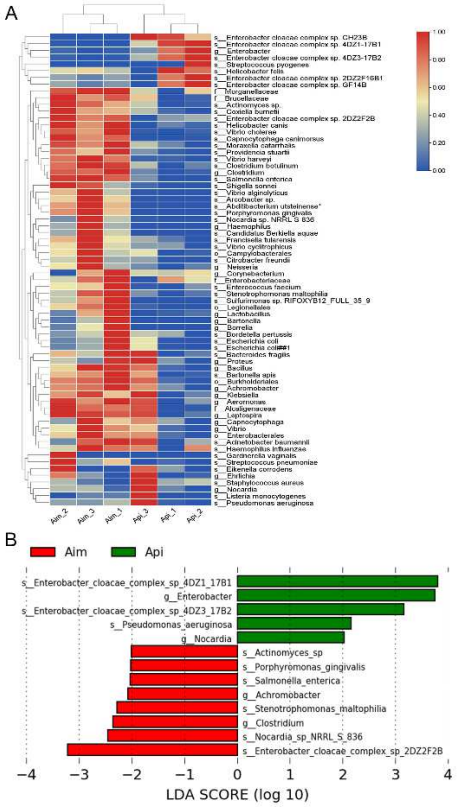

Figure 4 Pathogenic microorganisms analysis

(A) Heatmap of Pathogenic microorganism relative abundance in all samples. High abundance is denoted by the color red, while low abundance is represented by the color blue. (B) Graphical summary of LEfSe results. "Aim" represents *Azolla imbricata,* and "Api" represents *Azolla pinnata*.

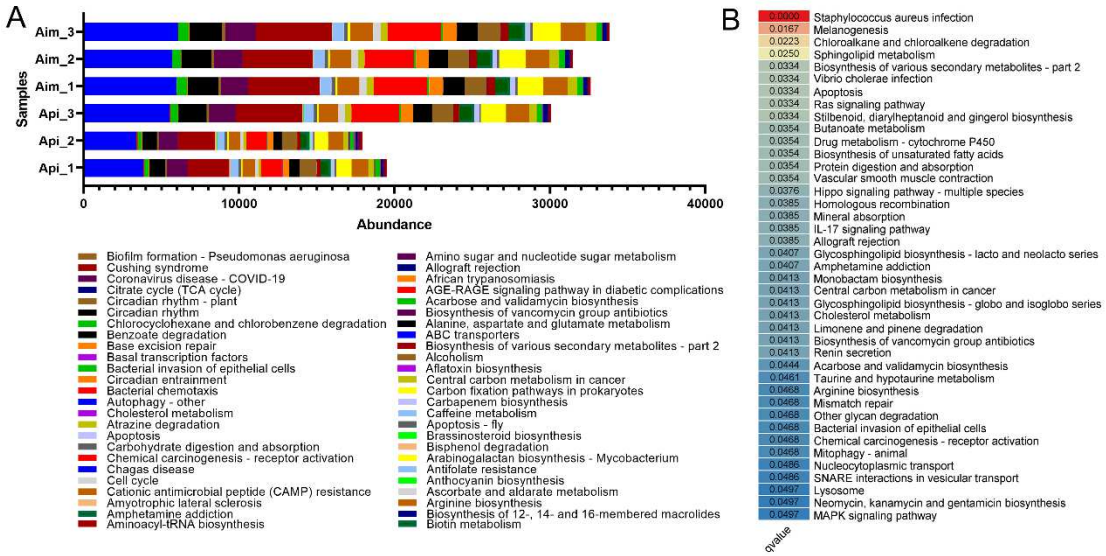

Figure 5 The KEGG functional analysis

(A) Dominant pathways are defined as those with a relative abundance greater than 1.00% of the total observed pathways. (B) The q-value of enriched pathways is represented by color, with a redder color indicating a smaller q-value. "Aim" represents *Azolla imbricata*, and "Api" represents

Azolla pinnata.

Resistance Gene Analysis

A total of 122 contigs were identified, of which 19 were linked to the Antibiotic Resistance Ontology (ARO). The analysis of resistance gene abundance revealed that the most prevalent resistance gene was *adeF* (as illustrated in Figure 6A). Furthermore, to compare the various resistance genes between Api and Aim, we conducted a Metastats analysis using the CARD database. This analysis identified five distinct genes: *AAC6-IIa*, *cmlA9*, *qacEdelta1*, *tetD*, and *ANT3-Ii-AAC6-IId_fusion_protein* (as depicted in Figure 6B). Notably, both *AAC6-IIa* and *qacEdelta1* were found to be present within the phylum Proteobacteria.

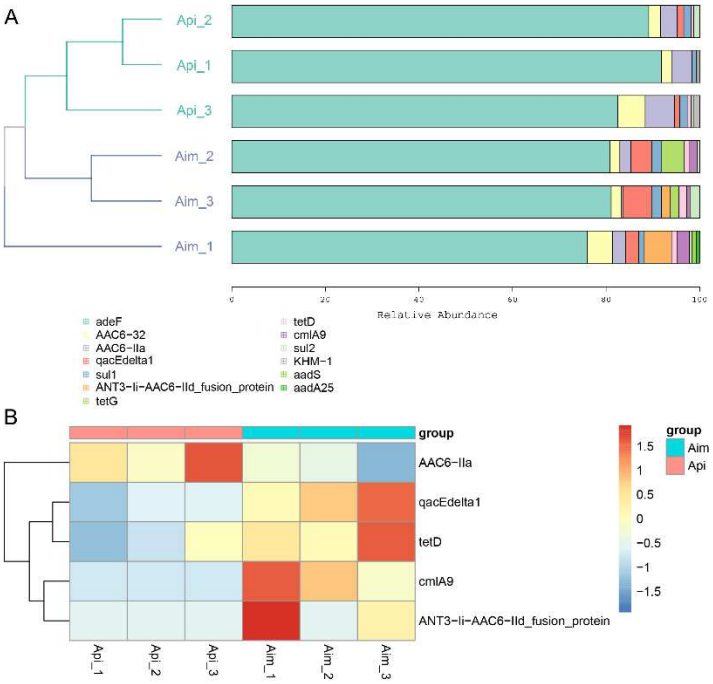

Figure 6 Resistance gene analysis

(A) Resistance gene in different samples. (B) Heatmap of the significantly different genes, Redder color indicates higher abundance. “Aim” represents *Azolla imbricata*, and “Api” represents *Azolla pinnata*.

Discussion

This study conducted a thorough examination of the phyllosphere microbial communities present in two species of *Azolla*, *Azolla imbricata* (Aim) and *Azolla pinnata* (Api), uncovering notable interspecific variations in both microbial structure and function. These findings highlight the importance of species-specific interactions in shaping microbial diversity, which subsequently affects ecological adaptation and the utilization of resources. The unique microbial profiles of

each *Azolla* species align with extensive studies on plant-microbe interactions, where host species apply selective pressures on microbial communities to optimize adaptability and functionality [21,22].

In Aim, Proteobacteria, specifically Burkholderiales, were notably abundant, whereas Api predominantly exhibited a higher presence of Cyanobacteria, particularly from the Nostocales order. This microbial differentiation is likely a result of the unique resource needs and ecological preferences of each species, allowing them to support specific microbial communities [23]. The significant presence of Proteobacteria in Aim may facilitate nitrogen cycling and foster growth, which correlates with research indicating that Burkholderiales contribute positively to plant health and enhance resilience against soil-borne pathogens. In contrast, the elevated levels of Cyanobacteria in Api reflect its adaptability to aquatic environments, potentially boosting its nitrogen fixation and photosynthetic efficiency.

The analysis of KEGG pathways further clarified the functional differences among the microbial communities. The presence of enriched ribosomal and oxidative phosphorylation pathways in Aim's microbial community points to an increase in metabolic activity, which supports stable growth amid fluctuating environmental conditions [24]. Conversely, the enrichment of photosynthesis and antioxidative pathways in Api suggests an enhanced microbial role in energy acquisition and resistance to stress, thereby improving the plant's capacity to adapt to various environments [25]. Additionally, quorum sensing and two-component systems play crucial roles in microbial communication and adaptation to environmental changes, underscoring the importance of microbial partnerships in the physiological responses of *Azolla*. The differences noted in antibiotic resistance genes across Aim and Api highlight potential microbial adaptations to environmental pressures. The increased incidence of resistance genes in Aim, save for *AAC6-IIa*, may reflect more intense selective pressures, possibly associated with the intricate aquatic habitats occupied by these species.

Differences in antibiotic resistance genes (ARGs) between Aim and Api reveal microbial adaptation strategies. The greater abundance of ARGs in Aim may indicate heightened selective pressures within its habitat, potentially due to the complex aquatic environments and competitive microbial dynamics. Antibiotic resistance is widely recognized as a mechanism that supports microbial resilience and competitive advantage, suggesting that Aim's microbial community may

experience intensified environmental or biotic stress [26]. This finding aligns with research showing that ARGs in environmental microbiomes help microbial communities adapt to ecological challenges, including interspecies competition and environmental stressors [27].

Our research contributes to a deeper comprehension of plant-microbe relationships by clarifying the species-specific contributions of *Azolla* phyllosphere communities to ecological adaptation and productivity. Supporting previous findings that plant species can shape their microbiomes to enhance health and growth, these results reveal the opportunity to optimize the functional traits of *Azolla* by altering its microbial composition. Furthermore, the unique diversity of microbes found in the phyllosphere of *Azolla* suggests possible uses in environmental remediation and agricultural productivity, where microbial communities might be designed or selected for particular ecological roles[28].

Conclusions

The analysis of composition revealed that Api exhibited a higher abundance of the phylum Cyanobacteria, specifically within the order Nostocales, family Nostocaceae, and genus Trichormus. In contrast, Api demonstrated a lower abundance of the phylum Proteobacteria, particularly within the order Burkholderiales and class Beta-proteobacteria, when compared to Aim. Additionally, the identification of drug-resistant genes across several genera suggests the presence of drug-resistant bacteria in both Aim and Api plants. This finding highlights the critical need for further research to understand the complex interactions between plants and microorganisms.

Materials and methods

Plant materials

Two species of *Azolla*, namely *Azolla pinnata* R.Brown (accession number, IRRI 7001, referred to as “Api” for short) and *Azolla imbricata* (Roxb.) Nakais (accession number, NARC 500, referred to as “Aim” for short), were selected as plant materials. Api was originally collected from Australia in 1982 and introduced from International Rice Research Institute (IRRI) in Philippines in 1984. Aim was collected from China in 1980. Both *Azolla* species were identified by Longfei Tang and preserved at the National Azolla Germplasm Resource Nursery (Fuzhou), in China.

Experimental settings

The experimental site was situated in Fuzhou City, China (26°7'58"N, 119°19'58"E). The air-dried

paddy soil was crushed and sieved to eliminate impurities before being utilized as the cultivation substrate in each small pond. Each pond was filled with tap water, maintaining a 5cm layer of soil and a water depth of 7cm. The composition of the cultivation substrate is presented in Supplementary Table 1. Throughout the experiment, natural illumination was employed with an average daily light duration of 8–10 hours, while the ambient temperature was maintained at 20–25°C, and the relative humidity was stabilized at 80% via an automatic humidification system.

Sample collection

We collected 10 grams of *Azolla* plants from each species that had been cultured for one month as samples for sequencing. To ensure the samples were free from contaminants. The whole plants were carefully rinsed under running water for 10 minutes, and the roots were removed to obtain frond-rich tissue. The fronds were then washed with double-distilled water (ddH2O) to eliminate epiphytes and contaminants. For further disinfection, the fronds were soaked in 70% ethanol for 40 seconds, followed by immersion in 2.5% sodium chloride for 10 minutes. Subsequently, the fronds were immersed in sterile Milli-Q water for 5 minutes, a process that was repeated three times. After washing, the fronds were carefully dried with sterile filter paper and briefly frozen with liquid nitrogen for 30 second. To ensure accuracy, each sample was replicated three times to obtain reliable results. Finally, the sediment-free fronds were stored at -80°C for further analysis. To confirm sterility, the final rinse water from the surface sterilization process was plated on nutrient agar and incubated at 28°C for 7 days. No microorganism was observed, indicating the successful elimination of surface contaminants.

Metagenomic library construction and sequencing

To isolate metagenomic DNA from frond-rich tissues, we employed the PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc.). The DNA samples were collected and stored at -80°C until the extraction process. To minimize potential bias during DNA extraction, we established three biological replicates for each group, with each replicate consisting of DNA extracted from three samples. The extracted DNA was subsequently sheared into fragments of approximately 300 bp using an M220 Focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) to facilitate the construction of a paired-end library. The DNA templates were then processed using the TruSeqTM Kit in accordance with the manufacturer's instructions (Illumina, CA, USA). Finally, paired-end sequencing (2 * 150 bp) was performed at Allwegene Technology Co., Ltd. (Beijing, China)

following standard protocols.

Sequence data analysis

To ensure data integrity, the raw readings were trimmed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with parameters set for a minimum quality score of 20 and a minimum length of 50 bp. Subsequently, the SOAPdenovo software (http://soap.genomics.org.cn/) was utilized to assemble the cleaned readings. After quality control of the sequences, metagenomic assembly analysis was performed to obtain more effective genomic information and improve the accuracy of functional annotation. Specifically, all sample reads were merged and assembled using the MEGAHIT or IDBA_UD software, which is based on the De Bruijn graph principle. This method constructs a De Bruijn graph by establishing overlaps between k-mers, thereby generating contigs. Subsequently, contigs longer than 800 bp were selected for statistical analysis and used for further functional annotation and bioinformatics research. For the prediction and annotation of open reading frames (ORFs) in each sample, Prodigal was employed. The CD-HIT tool was then applied to predict non-redundant ORFs and clusters of non-redundant ORFs . Taxonomic annotation was conducted by blasting samples against the NCBI-NT database. The BLAST program was utilized to match protein-coding reads against protein sequences in the NCBI-NR database, with results compared to the NCBI microbial taxonomy database based on the best hits. Clusters of Orthologous Groups of Proteins (COGs) were generated by aligning the ORFs to the eggNOG database (Version 4.0) using BLASTP (BLAST Version 2.2.28+) with an optimal E-value threshold of 1e-5. STAMT was employed for pairwise statistical comparisons of COG categorization, while the Mann-Whitney rank sum test in SigmaPlot (Version 12.5, Systat Software, USA) was used to assess significance between samples. To identify KEGG pathways, a BLAST search (BLAST Version 2.2.28+) was conducted with an optimized E-value threshold of 1e-5 against the Kyoto Encyclopedia of Genes and Genomes database (KEGG GENES, Kyoto Encyclopedia of Genes and Genomes). Functional annotation of genes was performed using KOBAS 2.0 (KEGG Orthology-Based Annotation System). Statistical analysis of the sequencing data was carried out in accordance with the parameters specified by the software.

Declarations

Ethics approval and consent to participate

The plant materials used in this research complies with international, national and/or institutional guidelines. All experiments on plants were conducted in accordance with the cultivars protection regulations of the Agricultural Ecology Institute, Fujian Academy of Agricultural Sciences.The *Azolla pinnata* was originally preserved in the International Rice Research Institute (Philippines), and it was legally introduced by the National Azolla Germplasm Resource Center (China) in the late 1980s. The *Azolla imbricata* was collected from Fujian and kept in the National Azolla Germplasm Resource Center (China) in the late 1980s. All of the *Azolla* varieties have been identified by researcher Longfei Tang, and plant samples were kept in the Agricultural Ecology Institute, Fujian Academy of Agricultural Sciences. National Azolla Germplasm Resource Nursery (Fuzhou) is a legal institution in China to legally collect and preserve *Azolla* plants.

Consent for publication

Not applicable.

Availability of data and materials

The sequence data generated and analyzed in this study are available at NCBI (https://www.ncbi.nlm.nih.gov) under accession numbers (PRJNA983057).

Competing interests

The authors declare that they have no competing interests.

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Author’s Contributions

Conceived and designed the study: Yan-Qiu Yang. Performed research: Yan-Qiu Yang, Su-Fang Deng, You-Quan Yang. Analyzed the data and Wrote the paper: Yan-Qiu Yang. Contributed materials/reagents: Zhao-Yang Ying.

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