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MICROBIAL GENES ASSOCIATED WITH *Tillandsia sp.:* ISOLATION AND SELECTION OF ENDOPHYTICS WITH AGRONOMIC POTENTIAL

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Josiane Soares Siqueira was born on October 20, 1993, in the municipality of Berilo, Minas Gerais, Brazil, the daughter of Adão Soares Siqueira and Maria Aparecida Soares Siqueira. She earned a degree in Biofuels Technology (2019–2022) from the Faculty of Technology - Nilo de Stéfani in Jaboticabal. In August 2022, she began her master's program in Agricultural Microbiology at the Faculty of Agricultural and Veterinary Sciences – UNESP, Jaboticabal Campus, under the supervision of Prof. Dr. Everlon Cid Rigobelo. Her research focuses on the project titled "Microbial Genera Associated with *Tillandsia sp.:* Isolation and Selection of Endophytes with Agronomic Potential," which has resulted in this document.

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SUMMARY

Page

Microbial Genes Associated With *Tillandsia sp*.: Isolation And Selection Of Endophytics With Agronomic Potential

Abstract

Tillandsia sp., an epiphytic plant, plays a crucial ecological role in forest ecosystems and is a valuable bioindicator for monitoring air pollution. The plant holobiont concept recognizes the complex relationships between plants and their associated microbiomes, which influence plant development, nutrition, fitness, and resilience. The microbiota and microbiome of Tillandsia spp. have been studied in various environments, including the rhizosphere, phyllosphere, and endosphere. Microorganisms associated with Tillandsia sp. contribute to nutrient acquisition, plant growth promotion, defense against pathogens, and tolerance to environmental stress. Factors such as plant genotype, environmental conditions, and spatial scale influence the composition and structure of the Tillandsia sp. microbiome. Diverse microorganisms have been isolated from Tillandsia sp., including nitrogen-fixing bacteria, aquatic microorganisms, and plant growth-promoting bacteria. The microbiome composition of Tillandsia sp. varies depending on growth support (trees vs. fences) and plant tissues (leaves vs. roots). Tillandsia sp. growing on trees exhibited higher microbial diversity and more complex microbial networks than those growing on fences. The microbiome of Tillandsia sp. may provide nutritional support in resource-poor environments and harbor bacteria of agricultural interest. Further research is needed to elucidate the contributions of the microbiome to the survival and adaptability of Tillandsia sp. under challenging conditions and to explore the potential of the isolated bacteria to enhance the growth of agricultural crops. This review aggregated the main studies presenting current information on the microbiome of *Tillandsia* spp. and suggested the potential application of these microorganisms to promote plant growth in agricultural crops.

Keywords Microbiome of Tillandsia sp. 2. Identification and Characterization Of Tillandsia Recurvata. 3. Plant growth-promoting microorganisms.

CHAPTER 1 - General Considerations

Introduction

Ecological Importance

Epiphytic plants play a crucial ecological role in forest ecosystems and contribute significantly to biodiversity and ecosystem functioning. Epiphytes are important primary producers and sources of oxygen, which modify the chemistry of their surrounding environment (Letáková et al., 2018). They represent a substantial component of forest biodiversity, particularly in tropical and temperate forests (Taylor et al., 2021). Epiphytic life forms are characterized by almost 10 % of all vascular plants. Defined by structural dependence throughout life and their non-parasitic relationship with the host, the term epiphyte describes a heterogeneous and taxonomically diverse group of plants (Zotz et al., 2023).

Interestingly, epiphytes can colonize not only native trees but also cultivated crops such as coffee shrubs, potentially serving as a conservation and restoration tool for biodiversity in heavily deforested regions (Hakobyan et al., 2023). However, epiphytes are highly vulnerable to deforestation and habitat transformation because of their canopy-dwelling nature (Magellan et al., 2021). *Tillandsia* is a genus of epiphytic plants known for their ability to absorb water and nutrients directly from the air, making them valuable bioindicators for monitoring air pollution (Van Hear et al., 2018). These plants, which belong to the Bromeliaceae family, exhibit various growth forms, including atmospheric juveniles and tank-forming adults, as observed in *Tillandsia deppeana* (Reyes-García et al., 2022). *Tillandsia* species have been successfully used to monitor air pollution, particularly trace metals such as Pb, Cu, and Cd (Morera-Gómez et al., 2024). Their effectiveness as biomonitors extends to urban environments, where they can detect structural alterations in response to pollutants (Shiva Nagendra et al., 2020). Interestingly, some *Tillandsia* species, such as *T. caput-medusae*, have been recommended for use in Latin American countries, owing to their widespread distribution (Piazzetta et al., 2018).

Different *Tillandsia* species exhibit different adaptations and preferences. For instance, *T. recurvata* shows host preference for certain tree species and is more likely to colonize larger trees (Bernal et al., 2005). *T. usneoides* demonstrates high tolerance to heavy metal accumulation, potentially owing to its unique leaf structure (Alves et al., 2007). Some species, such as *T. deppeana*, utilize C3 photosynthesis in both juvenile and adult forms, which is unusual in atmospheric *Tillandsia* (Adams & Martin, 1986). *Tillandsia recurvata*, commonly known as the ball moss, plays several important ecological roles in its habitat. *T. recurvata* serves as a critical refuge site for arthropods in tropical dry forests, especially during unfavorable periods. A study in Central Mexico identified 33 families, 15 orders, and two classes of Arthropoda using *T. recurvata* as a microhabitat (Luna-Cozar et al., 2020). This epiphyte augments the quantity and quality of habitats in tropical dry forests, potentially increasing arthropod survival and enhancing ecosystem resilience to disturbances and local extinction.

T. recurvata also serves as an effective biomonitor of air pollution in urban areas. Studies have shown that their abundance and metal accumulation correlate with the intensity of vehicular traffic and air pollution levels (Castañeda Miranda et al., 2015). This makes *T. recurvata* a valuable tool for passive biomonitoring of pollution, especially in cities in the Southern Hemisphere, where it is abundant.

Plant Holobiont

The plant is not the only individual. Plants carry many microorganisms on their surface and inside their tissues. This concept is known as a plant holobiont. The plant holobiont concept refers to an ecological entity comprising a plant and its associated microbiome, viewed as a single evolutionary unit rather than separate entities (Uroz et al., 2019). This holistic approach recognizes that plants interact with diverse microorganisms throughout their lives, forming complex relationships that influence plant development, nutrition, fitness, and resilience in dynamic environments (Middleton et al., 2020).

The plant holobiont includes microorganisms both inside (endosphere) and outside (ectosphere) of plant tissues, primarily consisting of bacteria and fungi. These microbes play crucial roles in plant nutrition, resistance to biotic and abiotic stresses, and overall plant performance (Vandenkoornhuyse et al., 2015). This concept

emphasizes that plant fitness is a consequence of both the plant itself and its microbiota, with the microbiome being shaped by host, microbial, and environmental factors (Kumar & Nautiyal, 2022). Interestingly, the plant holobiont concept challenges the traditional views of plant evolution and ecology. This suggests that plants and their associated microbes have co-evolved, developing cross-kingdom communication channels and metabolite-mediated strategies for selective recruitment of beneficial microorganisms (Morcillo & Manzanera, 2021). Some researchers have proposed that mitochondria and chloroplasts are entrained microbes within the phytomicrobiome, highlighting the profound influence of microbes on plant evolution (Lyu et al., 2021). This perspective emphasizes the inseparable nature of plants and their microbiomes, suggesting that without microbes, the concept of a "plant" may not be viable (Lyu et al., 2021).

Plant microbiota refer to a diverse community of microorganisms that live in association with plants, both on their surfaces and within their tissues. Microbial communities play a crucial role in plant health, growth, and overall ecosystem functioning (Arnault et al., 2023). Plant microbiota primarily consists of bacteria, fungi, archaea, and viruses. The composition varies depending on the plant species, environmental conditions, and plant tissues (Tanas et al., 2020).

Rhizosphere

The rhizosphere is a complex and dynamic zone of soil surrounding plant roots, typically extending approximately 2 mm from the root surface (Kuppe et al., 2022). This region is characterized by unique physical, chemical, and biological properties that differ significantly from those of the bulk soil (Zhang et al., 2022). The rhizosphere plays a crucial role in plant nutrition, microbial interactions, and overall ecosystem function. Root exudates are key factors that shape the rhizosphere environment. Plants release various compounds, including carbohydrates, amino acids, lipids, and vitamins, through their roots, which stimulate microbial activity in the soil (Schnepf, 2023). These exudates mediate complex interactions between plants and soil microorganisms, influencing nutrient availability, plant growth promotion, and pathogen defence (Bais et al., 2006; Jain et al., 2020). The composition and quantity of root

exudates can vary depending on plant species, environmental conditions, and soil properties.

Interestingly, the rhizosphere is not only important for plant nutrition but also plays a significant role in environmental processes, such as heavy metal (im)mobilization (Seshadri et al., 2015) and the biodegradation of pollutants (Alshaal et al., 2017). Climate change and global warming are expected to have substantial effects on rhizosphere processes, and consequently, on agricultural production (Alshaal et al., 2017). Advanced technologies such as metagenomics are now being employed to reveal the functional potential of rhizosphere microbiomes (Soni et al., 2017), providing new insights into this complex ecosystem (Ling et al., 2022).

Phyllosphere

The phyllosphere refers to the aboveground surfaces of plants, primarily the leaves, where diverse microbial communities reside. The phyllosphere is a dynamic and complex habitat that plays a crucial role in plant health, productivity, and ecosystem functioning (Zhan et al., 2022). It comprises the aerial parts of plants and plays a crucial role in plant health, productivity, and overall fitness. This microbial ecosystem contains diverse microorganisms that significantly contribute to plant well-being in several ways (Zhu et al., 2022).

Phyllosphere microorganisms enhance the host's genomic and metabolic capabilities, including defense against pathogens (De Mandal & Jeon, 2023). They are involved in nutrient uptake, disease resistance, and growth promotion (Danso Ofori et al., 2024). The microbiome also plays a vital role in plant fitness, and some studies have shown that adding a synthetic microbial community to greenhouse-grown plants can lead to increased fruit production (Mehlferber et al., 2023). Phyllosphere microbes have various functions such as fixing nitrogen and promoting plant growth (Huang et al., 2023). Microorganisms that inhabit the phyllosphere are crucial for various functions, including enhancing plant productivity and fitness by influencing leaf operation and lifespan, seed weight, top growth, flowering, and fruit formation, and they play essential roles in pollutant removal (Thapa & Prasanna, 2018). For example, certain growth-promoting bacteria found in the phyllosphere, such as *Microbacterium*, *Stenotrophomonas*, and *Methylobacterium*, can enhance the growth and nutritional

conditions of the host plant by generating natural growth regulators (e.g., indole acetic acid IAA) and fixing nitrogen (Zhan et al., 2022). The phyllosphere microbiome also contributes significantly to reducing plant emissions of methanol (e.g., methylotrophs) and isoprene (e.g., isoprene-degrading bacteria of the genus *Variovorax*) into the atmosphere (Stone et al., 2018). Furthermore, the phyllosphere microbiome plays a vital role in sustaining plant health and inhibiting excessive growth of plant pathogens. As an illustration, the phyllosphere microbiome can safeguard *Arabidopsis* plants against fungal pathogens and dysbiosis (a disturbance in microbiota balance), which could negatively impact the health of the host (Beilsmith et al., 2019). Recent studies have shown that bacteria and yeasts colonizing nectar can alter their chemical composition, subsequently influencing insect pollinator visitation and foraging behavior (Beilsmith et al., 2019).

Microbiota and Microbiome

The terms microbiota and microbiome are often used interchangeably, but they have distinct meanings in the context of host-associated microbial communities. Microbiota refers to the collection of microorganisms that inhabit a specific environment, such as a plant tissue or a particular organ. For example, plant root microbiota consists of trillions of commensal microorganisms (Uhr et al., 2019). The plant leaves microbiota, as another instance, comprises over 700 microorganisms, including bacteria, fungi, and viruses (Xiao et al., 2020).

On the other hand, the microbiome encompasses the totality of genes that the microbiota can express, representing its genetic heritage (Di Domenico et al., 2022). This is essentially the collective genome of all microorganisms in a given environment. For instance, the plant microbiome contains at least 100 times more genes than our own genome (Gill et al., 2006). It encompasses the entire genetic material of all microorganisms associated with a plant as well as their interactions with the plant and each other (Hao et al., 2024). The microbiome concept considers the functional potential and activity of the microbial community as a whole (Compant et al., 2019).

Interestingly, the plant microbiome is sometimes referred to as the "extended plant genome," highlighting its importance in plant function and fitness. This perspective emphasizes the integrated nature of plants and their associated

microorganisms, leading to the concept of plants as ecological entities or "plant holobionts" (Mesny et al., 2023). Plant microbiomes perform a wide range of essential functions that contribute to plant growth and health. These functions can be broadly categorized into several key categories (Qu et al., 2020). Plant microbiomes play a crucial role in promoting plant growth and enhancing nutrient acquisition. They facilitate the uptake of essential nutrients, particularly through nitrogen fixation by rhizobia, and improved phosphorus absorption by mycorrhizae (Compant et al., 2024). Additionally, microorganisms in the rhizosphere can solubilize nutrients, making them more readily available for plant uptake (Qu et al., 2020). These interactions significantly contribute to plant nutrition and overall productivity. Interestingly, plant microbiomes also serve as defense mechanisms against pathogens and environmental stresses. They help improve plant resistance to diseases and abiotic stresses, thereby maintaining plant health (Song et al., 2020). Some microorganisms act as biocontrol agents, suppress potential pathogens, and offer protection against harmful bacteria, fungi, and herbivores (Zhang et al., 2023). Furthermore, the microbiome can help to restore dysbiosis or compensate for pathogen-induced shifts in the microbial community (Berg et al., 2021).

Plant microbiomes are influenced by a complex interplay of various factors, with both biotic and abiotic elements playing crucial roles in shaping microbial community composition and structure. Plant genotype has been identified as a significant factor that influences microbiome diversity and structure. Research has shown that different plant genotypes can have varying effects on microbial communities in different plant compartments, with a stronger effect observed in fruits than in leaves and soil (Malacrinò et al., 2022). Additionally, plant species identity has been found to have a stronger influence on the microbial community structure than bioaugmentation in contaminated sediments (Dagher et al., 2019). Environmental factors and spatial scales also play an important role in shaping plant microbiomes. Studies have demonstrated that microhabitat and location can differentially affect bacterial and fungal communities, with microhabitats better explaining bacterial community composition and location and better predicting fungal compositional variance (Bernard et al., 2020). Soil type and composition have been shown to exert a strong influence

on the bacterial community structure of seedlings, and the extent of this influence varies depending on the soil type (Walsh et al., 2021).

Other factors influencing plant microbiomes include agricultural practices, herbivory, and the initial soil microbial diversity. Research has shown that soil microbial diversity can affect both plant- and herbivore-associated microbial communities (Malacrinò et al., 2021). Additionally, the interplay between plants and their microbiomes is affected by plant domestication, with insights from wild plant relatives and native habitats potentially contributing to the restoration of beneficial microorganisms (Cordovez et al., 2019).

Microbiome of *Tillandsia* sp

Tillandsia recurvata is an epiphytic plant that harbors a diverse microbiome with important ecological functions. The interior of *T. recurvata* has been found to hosts nitrogen-fixing bacteria, specifically *Pseudomonas stutzeri*, which may play a crucial role in nutrient acquisition by plants (Mukherjee et al., 2024). This finding suggests a potential close association between bromeliad plants and nitrogen-fixing bacteria, highlighting the importance of the plant microbiome in supporting *T. recurvata* growth in nutrient-poor environments.

Interestingly, although the microbiome of *T. recurvata* contributes to its survival, the plant itself serves as a refuge site for various arthropods in tropical dry forests, demonstrating its role in biodiversity. This dual relationship between T*. recurvata* and its associated organisms underscores the complex interactions within the plant microbiome and its extended ecosystem (Luna-Cozar et al., 2020).

One study compared the performance of *T. flexuosa* populations growing on cables with those growing on trees in the eastern part of the Peninsula de Azuero, Panama. The researchers monitored the in-situ growth of naturally established populations on electrical cables in three villages and compared it with data from a companion study of conspecifics growing on trees in nearby areas. The study found that *T. flexuosa* can successfully establish and grow on electrical cables, with densities of approximately one individual per meter of cable. However, plants growing on cables had slower growth rates than those grown on trees. This study provides unique insights into the adaptability of epiphytic bromeliads to artificial substrate. This demonstrates

that, while *T. flexuosa* can survive on electrical cables, these plants face challenges such as slower growth and lower recruitment rates compared to their tree-dwelling counterparts. This research suggests that the main differences between cable and tree populations are related to water availability, with cable-dwelling plants being more exposed to wind and sun, leading to faster drying after rain events. This study also highlights the importance of survival, particularly for large individuals, as the most critical demographic process for population maintenance on cables. These findings contribute to our understanding of epiphyte ecology and adaptation to non-natural substrates in modified human environments (Wester & Zotz, 2009).

A previous study evaluated 20 *Tillandsia* species and isolated several other microorganisms. The predominant groups isolated include: Bacillus species: *B. megatherium*, *B. licheniformis*, *B. subtilis*, and *B. brevis* were identified in 75% of the examined *Tillandsia* species. Additional bacteria, including *Pseudomonas*, *Vibrio*, *Agrobacterium*, *Aeromonas*, *Xanthomonas*, and *Rahnella* species, were also isolated from various *Tillandsia* species. Aquatic microorganisms: Notably, certain aquatic microorganisms, such as *Rahnella aquatica*, *Vibrio fluvialis*, and *Aeromonas hydrophila*, were found exclusively in atmospheric *Tillandsia* species. Among these, only *Bacillus megatherium* exhibited nitrogen-fixing activity in pure culture. The diversity of bacteria isolated from *Tillandsia* collected from different environments was relatively low, suggesting a lack of specificity between epiphytic microorganisms and *Tillandsia* species (Brighigna et al., 1992). Another study selected *Tillandsia landbeckii* plants from seven different populations in the northern Chilean Atacama Desert and identified several microorganisms associated with these plants in the Atacama Desert. Some of the key microorganisms isolated included the dominant bacterial classes Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Bacteroidia. Well-known phyllosphere colonizers include *Pseudomonas*, *Massilia*, *Ralstonia*, and *Hymenobacter* spp. Actinobacterial genera: *Modestobacter* and *Kineococcus*. Arthropod endosymbionts: *Wolbachia* and *Candidatus*. Other notable genera: *Acidiphilium* and members of the Acidobacteriae subgroup2. It is important to note that this study found different bacterial communities in the phyllosphere (aboveground plant parts) and laimosphere (buried shoots) of *T. landbeckii*, indicating that these plants

provide distinct habitats for microorganisms in hyperarid desert environments (Hakobyan et al., 2023).

A study on *T. utriculata* demonstrated that tank development was responsible for multiple plant adaptations and capabilities pertaining to nutrient uptake, physiology, metabolism, nitrogen assimilation, and modulation of the plant microbiome. This study shows the effects and contributions of plant anatomy and physiology on microbiome modulation. This study confirmed the presence of significant bacterial groups, including those with various plant growth-promoting capabilities. These groups encompassed nitrogen-fixing bacteria such as *Beijerinckia*, which exhibited higher abundance in smaller plants. Additionally, nitrogen-fixing methanotrophs, specifically *Methylosinus* and *Methylocystis*, were more abundant in smaller plants. Potential ammonium-associated bacteria, namely *Blattabacterium* (order Flavobacteriales), were observed in all small and some intermediate plants, but were absent in large plants. Nitrate reducers such as *Conexibacter* demonstrated an increased relative abundance with plant size. Other bacteria that were more abundant in the larger plants included *Cellulomonas*, *Rhodanobacter*, and *Fulvimonas*. Bacteria were present in nearly all samples, but were more abundant in smaller plants, including *Sphingomonas* and *Methylobacterium*. The study observed that the bacterial community composition changed as the plants developed, with various bacterial types exhibiting fluctuations in abundance depending on the plant size and developmental stage (Stryker et al., 2024).

A recent study investigated the microbial communities associated with *T. recurvata* growing on different supports (trees and fences) and plant tissues (leaves and roots). The findings showed that *T. recurvata* growing on trees exhibited a significantly higher microbial diversity than those grown on fences. This was observed in both the bacterial and fungal communities. Regarding the taxonomic composition Proteobacteria was the dominant bacterial phylum across all samples, with Actinobacteria and *Sphingomonas* also playing important roles. For fungi, Ascomycota was the predominant phylum, with *Paraconiothyrium* and *Nigrospora* showing significant differences between trees and fences. The study also showed that, despite differences in microbial composition, the functional profiles of microbial communities were similar across different growth supports and plant tissues. Furthermore, plants on trees showed more complex and interconnected microbial networks than those on

fences, suggesting a more intricate ecological relationship. *T. recurvata* growing on trees harbors bacteria of agricultural interest, such as *Bradyrhizobium*, *Azospirillum*, and *Pseudomonas*, suggesting their potential as reservoirs of beneficial microbes. This study suggests that the microbiome of *T. recurvata* may provide nutritional support to compensate for the lack of nutrients in particular growth supports, especially in resource-poor environments such as fences. The research concluded that growth support (trees vs. fences) significantly influences the structure, diversity, and interactions of microbial communities associated with *T. recurvata*. This study provides insights into the complex relationships between epiphytic plants, their microbiomes, and their growth environments, with potential implications for understanding plant ecology and identifying beneficial microorganisms for agricultural applications (Siqueira et al., 2024).

Conclusion

Tillandsia sp. is an epiphytic plant that inhabits diverse environments, including trees, cables, and fences. This species possesses various mechanisms for water and nutrient acquisition, without the need for mineral fertilization. On the other hand, numerous studies have demonstrated the importance, effect and influence of the plant microbiome on several plant capabilities. What are the contributions of the microbiome of *Tillandsia* sp. to environmental stresses? Is it possible that many of its abilities or its capacity to survive in challenging conditions are not attributable to the microbiome? Few studies have demonstrated the isolation of bacteria with various capabilities to promote plant growth from Tillandsia sp.. It is worth investigating whether these bacteria can be used to enhance the growth of agricultural crops. Further research is required to address these questions. Moreover, the mechanisms and contributions of microbiota to the lifestyle of these plants are intriguing and warrant further investigation.

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CHAPTER 2 – Influence Of Growth Support On The Diversity, Composition And Functionality Of Microbial Communities Associated With Tillandsia Recurvata[1]

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Abstract

Tillandsia recurvata is an epiphytic plant commonly found in tropical regions and colonizes tree trunks, fences, and power wires. This plant plays an important role in interacting with trees, sharing microorganisms, and performing specific functions in the process of tree colonization. The objective of this study was to evaluate and compare the microbiomes of *T. recurvata* collected from two different locations (trees and fences) and two plant tissues (leaves and roots). The hypothesis of this study was that the microbiome of *T. recurvata* is composed of microorganisms that would provide nutritional support to compensate for the lack of nutrients in a particular growth support. The results showed significant differences in microbial diversity between trees and fences, with trees exhibiting higher richness and more complex microbial networks. *Proteobacteria* was the most prevalent bacterial phylum, with *Actinobacteria* and *Sphingomonas* also playing key roles in nitrogen fixation and plant growth. Fungal communities were similar across locations, with *Ascomycota* and *Basidiomycota* being

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predominant, but *Paraconiothyrium* and *Nigrospora* showed significant differences in abundance between trees and fences. Functional analysis indicated similar metabolic profiles across leaf and root samples, with key functions for *T. recurvata* including carbohydrate and amino acid metabolism, stress control, and biofertilization.
Keywords: Microbial Ecology; epiphytic; plant growth-promoting; trees; fence

Introduction

Tillandsia recurvata is an atmospheric epiphyte that occupies canopy trees in many parts of tropical America and play a crucial role in a rain and cloud forests [1]. Epiphytes are a significant element of the forest canopy, and they not only interact with one another, but also with their host plant and the surrounding wildlife [2]. The coexistence of various species that occupy similar ecological roles necessitates precise differentiation of their respective niches, which in turn helps reduce competition between them. This differentiation is often brought about by life-history trade-offs, wherein competitive advantages are gained by superior competitors confined to fewer locations, while colonizers with higher fecundity and broader dispersal ranges are better suited to exploit harsh environments [3].

Bromeliaceae represents the only epiphytic lineage within the order Poales and comprises a highly diverse group of plants that encompasses both grasses and sedges [4]. Most epiphytic Bromeliaceae obtain mineral nutrients through their leaves via modified trichomes, a process that is facilitated by atmospheric deposition or rainwater flow over their host. These and several other morphophysiological characteristics are extensively examined in the comprehensive review conducted by Benzing [5], which also addressed the crucial ecological functions of bromeliad in the environment.

Colonization of plants by microorganisms is a widely recognized phenomenon, both aboveground in the phyllosphere and belowground in the rhizosphere [6, 7]. However, studies examining the bacterial communities of epiphyte plants have been conducted under adverse environmental conditions and have primarily focused on specific plant species [6]. These studies revealed differences in microbial community composition between plant compartments, species, temporal changes, and biogeographic patterns [8]. The distribution of T. recurvata colonization usually occurs on trees, fences, or power wires. Numerous studies have employed T. recurvata to

identify regions where pollution is caused by human activities and to distinguish areas with superior soil and air quality [9].

Limited information is available regarding the microbiome of T. recurvata, and it remains uncertain whether this plant serves as a reservoir for microorganisms of agronomic interest, particularly in areas with limited available nutrients, such as fences. Joseph et al [10], evaluated some endophytes fungi isolated from T. recurvata and this research demonstrated that of the seven fungal morphotypes analyzed, five were identified as Sordariomycetes through DNA sequencing, revealing a substantial representation of this class within the plant. Phylogenetic analysis using ITS and β-tubulin sequences corroborated the taxonomic classification and uncovered hidden diversity among the isolates, indicating intricate fungal relationships. This study highlights the varied and potentially distinct fungal endophytes present in epiphytic bromeliads such as T. recurvata that can be used for agricultural purposes. Previous research has concentrated on the dynamics of bacterial communities in the phyllosphere to assess the influence of deposited bacteria on plant growth and to enhance the comprehension of their significance in biogeochemical processes [11].

The present study aimed to evaluate and compare the microbiome of T. recurvata obtained from two distinct locations, trees and fences, and two different plant tissues, specifically the leaves and roots. The hypothesis underlying this study posits that the microbiome of plants located on fences consists of microorganisms with high diversity and specialized functions capable of providing nutrients and conditions for plant growth and development to compensate for the lack of nutrients and adverse conditions in the microenvironment.

Methods

Collection Of *Tillandsia Recurvata* Samples

Ten specimens of T. recurvata were collected: five from the fence and five from phorophyte trees. Each plant was carefully collected using gloves and was transported to the laboratory belonged to the São Paulo State University (UNESP), Jaboticabal City, Brazil. The plants of T. recurvata were collected from an iron fence (-21,2421684, -48,2895118) and from three different phorophyte trees: Andira surinamensis (-21,2465929, -48,2940464), Stryphnodendron pulcherrimum (-21,2439687, -

48,2931995), Cedrela fissilis (-21,2447914, -48,2937735). The trees were near to each other and subjected to identical climatic conditions. The climate is characterized by hot, rainy summers, and cold, dry winters. According to Köppen-Geiger the climate classification is Aw.

Characteristics Of Phorophyte Trees

The T. recurvata specimens were collected from three distinct phorophyte trees. These trees were: (1) Andira surinamensis (Fabaceae): a medium to large-sized tree species typically found in tropical forests, particularly near rivers and swampy areas. This species can reach heights of 20-30 meters, making it a significant component of its ecosystem. A. surinamensis produces fragrant flowers that attract pollinators, notably bees, which may play a role in the plant's reproductive success and local pollinator dynamics. The tree bears large, fibrous fruits and is valued for its durable wood, which finds applications in construction and furniture-making. Additionally, the species has reported medicinal properties; (2) Stryphnodendron pulcherrimum (Fabaceae): a medium-sized tree species adapted to tropical and subtropical regions, typically thriving in well-drained soils. This species reaches heights of 15-20 meters, contributing to the mid-canopy structure of its native habitats. *S. pulcherrimum* produces small, whitish-yellow flowers and elongated seed pods, characteristics that may influence its interactions with pollinators and seed dispersers. Notably, the bark of *S. pulcherrimum* has documented medicinal properties, particularly in traditional medicine practices where it is employed for wound healing and as an astringent; and (3) Cedrela fissilis (Meliaceae): a large tree species native to tropical and subtropical regions, typically found in fertile, well-drained soils. This species can attain heights of 20-35 meters, establishing itself as a dominant canopy component in its habitat. *C. fissilis* produces small, inconspicuous flowers and woody capsules containing winged seeds, adaptations that likely influence its reproductive strategies and dispersal mechanisms. This tree is particularly notable for its high-quality wood, which is characterized by its workability and pleasant aroma. These properties make *C. fissilis* timber highly valued for furniture, cabinetry, and musical instrument construction [12].

The phorophyte species selected for this study, despite belonging to different families (A. surinamensis and S. pulcherrimum from Fabaceae, and C. fissilis from

Meliaceae), share several ecological and economic characteristics. These medium to large trees, typically reaching heights of 15-35 meters, are common components of tropical and subtropical forest ecosystems. All three species have recognized economic value, particularly in timber production, construction, and traditional medicine. Notably, the Fabaceae species likely form symbiotic associations with nitrogen-fixing bacteria, potentially influencing soil fertility and nutrient cycling in their habitats. Furthermore, these trees produce flowers and seed pods that play crucial roles in local ecosystems by attracting pollinators and seed dispersers [12].

Identification And Characterization Of Tillandsia Recurvata Specimens

Plants of T. recurvata were collected and identified for this study using taxonomic keys [13] at the Plant Taxonomy Laboratory of the Department of Biology. Only fully developed plants were collected and the uniformity of specimen size between groups was considered. This epiphytic bromeliad, commonly known as "ball moss", adapted to arid environments, forms distinctive spherical or rounded clumps measuring 10-20 cm in diameter. *T. recurvata* specimens exhibit narrow, grayish-green leaves that bend backward (recurve), contributing to the plant's characteristic ball-like structure. The leaves are thin, pointed, and covered with trichomes, microscopic hair-like structures that impart a silvery appearance and likely play a role in water and nutrient absorption [5, 13, 14].

DNA extraction from the leaves and roots

The leaves and roots were collected manually using surgical gloves and immediately deposited in sterilized plastic containers. Subsequently, the leaves and roots were placed in a 50 ml conical tube containing 35 ml of phosphate buffer with 0.02% surfactant (Tween 20). The tubes were vortexed for 2 min to separate the root system from the rhizosphere. Then, using sterilized tweezers, the leaves and roots were placed on paper towels and transferred to centrifuge tubes (50 ml). Superficial sterilization of the leaves and the roots were performed according to the method described by [15], with modifications. The tissues were maintained in 100% ethanol for 3 min, followed by 2% sodium hypochlorite for 2 min, and 70% ethanol for 3 min. The disinfected plant tissues were washed thrice with sterile distilled water, and the last

wash was inoculated onto nutrient agar plates to validate the effectiveness of the superficial sterilization procedure. Sterilized leaves and roots were macerated in liquid nitrogen using a sterile mortar and pestle. A PowerMax soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used to extract genomic DNA from all samples, according to the manufacturer's instructions. The concentration of the extracted DNA was determined by fluorometry (Qubit™ 3.0, Invitrogen), and the purity was estimated by calculating the A260/A280 ratio via spectrophotometry (NanoDrop™ 1000, Thermo Fisher Scientific). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') [16]. Also, the Internal transcribed spacer (ITS) region was amplified using primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to investigate the diversity of fungal communities in the samples [17]. PCR was performed in 30 cycles using the HotStarTaq Plus Master Mix kit (Qiagen) under the following conditions: 94 °C for 3 min, followed by 28 cycles at 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. PNA clamp sequences (PNA Bio) were added to block amplification of the 16S rRNA gene from the ribosomes and mitochondria. The amplification products were analyzed on a 2% agarose gel to determine the success of amplification and relative intensity of the bands. Amplicons were sequenced using 2x300bp paired-end protocol in the Illumina MiSeq™ platform at the GoGenetic facility (Curitiba, Brazil).

Data Processing

Initial quality assessment of sequencing data was performed using FastQC (v. 0.11.9) [18]. For further analysis, USEARCH (version 11.0.667) [19] was employed, utilizing the "fastx_info" and "fastq_eestats2" functions to examine quality distribution, sequence length, and expected errors. The "search_oligodb" function in USEARCH was used to identify the presence and location of the primers 341F and 805R, targeting the V3-V4 region of the 16S rRNA gene, as well as primers ITS3 and ITS4 for ITS sequences. Primers and adjacent barcodes were removed using Atropos (version 1.1.31) [20]. To ensure data quality, Fastp (version 0.23.2) [21] was used to remove sequences with an average Phred quality score below Q25, using the parameter "--

average_qual 25". Given the paired-end sequencing approach, sequences were merged using PEAR (version 0.9.11) [22] with a minimum overlap criterion of 10 base pairs (--min-overlap 10).

Merged reads were processed using the DADA2 pipeline [23], implemented through the dada2 package (version 1.22.0) in R (version 4.1.2) [24]. The process began with filtering and truncation of reads using the "filterAndTrim" function, with a maximum expected error threshold of 2 ("maxEE = 2"). Error probabilities per base were estimated using the "learnErrors" function. Based on this error model, sequences were corrected with the "dada" function, leading to the identification of Amplicon Sequence Variants (ASVs) specific to each sample. Potential chimeric sequences were removed using the "removeBimeraDenovo" function. Taxonomic classification of 16S rRNA ASVs was performed against the "RefSeq + RDP" database (RefSeq from the National Center for Biotechnology Information [NCBI] supplemented with sequences from the Ribosomal Database Project [RDP]) (version 16.0) [25]. ITS ASVs were compared against the UNITE database (version 2022.11.29) [26]. ASVs that could not be classified to the respective kingdoms or identified as potential contaminants, including chloroplast and mitochondrial sequences, were excluded from the analysis.

ASV counts and taxonomic annotations were exported in "phyloseq" format using the phyloseq package (version 1.38.0) [27]. The phyloseq object was transformed into compositional data using the "phyloseq_standardize_otu_abundance" function from the metagMisc package (version 0.04) [28] for downstream microbiome analyses.

Descriptive and Statistical Analyses of the Microbiome

Sampling efficiency was assessed using rarefaction curves generated by the "amp_rarecurve" function in the ampvis2 package (version 2.7.17) [29]. Alpha diversity was quantified by examining species richness and diversity indices (Shannon and Gini-Simpson), using the "alpha" function in the microbiome package (version 1.16.0) [30]. Comparative analysis of means was conducted using Student’s t-test, with a 95% confidence interval (p-value ≤ 0.05). Additionally, alpha diversity metrics were calculated for rare ASVs, defined as those with an average relative abundance of less

than 0.001% within a group or present in only one sample. Beta diversity was analyzed by calculating Bray-Curtis dissimilarity between samples using the "distance" function in the phyloseq package. Significant differences between leaves and roots samples of *T. recurvata* collected from trees and fences were assessed using PERMANOVA, implemented via the "adonis" function in the vegan package (version 2.6.2) [31], with significance set at p-value ≤ 0.05. Principal Coordinate Analysis (PCoA) was performed to interpret multidimensional distances, and results were visualized in subsequent plots.

Differentially abundant taxa between leaves samples of *T. recurvata* collected from trees and fences were identified using the DESeq2 methodology (R package version 1.34.0; Love et al., 2014), which applies a negative binomial model to compare means, with the Wald test used for significance (adjusted p-value ≤ 0.05). The resulting analyses were visualized using the ggplot2 package (version 3.3.6) [32] in R.

To evaluate the structural characteristics of microbial communities in response to growth support, co-occurrence network analysis was performed at the Genus taxonomic level. Pearson correlation coefficients were calculated using the "corr.test" function in the psych package (version 2.2.5) [33]. Only significant correlations (p-value ≤ 0.05) with a Pearson coefficient of ±0.75 or greater were considered, focusing on strong positive or negative relationships. Network construction and analysis of topological properties were conducted using the igraph package (version 1.3.4) [34]. Topological properties included the total number of correlated genera (nodes), total number of connections (edges), average and maximum degrees, and centrality measures (average and maximum betweenness centrality). Key hubs were identified by calculating the Kleinberg's hubbiness score [35], highlighting the most influential genera in the networks. The mean values of degrees and betweenness centrality were compared with Student’s t-test, with a 95% confidence interval (p-value ≤ 0.05).

To infer the functional potential of bacteria (16S rRNA data), PICRUSt2 (version 2.5.2) [36] was used to predict the functional capacity in terms of KEGG Orthology (KO) counts per sample. Additionally, KOs were mapped to the PLaBAse database (version 1.0) [37] to associate functions with pathways relevant to plant-microbe interactions. Differentially abundant functions between conditions were identified using

the DESeq2 methodology (R package version 1.34.0) [38], with significance determined at an FDR adjusted p-value ≤ 0.05.

Results

The high-throughput sequencing process generated a total of 3,726,967 (16S rRNA) and 3,161,214 (ITS) reads distributed among the different samples analyzed, including the leaves and roots of epiphytic plants with growth support on trees or fences. The data were organized into two sets, 16S rRNA and ITS, each grouped by plant tissue and growth support conditions. For leaf samples obtained from *T. recurvata* collected from trees, the average raw reads were 295,565.8 for 16S rRNA and 232,207.4 for ITS, while for roots, the total values were 391,499 and 334,619 for roots, respectively. After quality control, the retained 16S rRNA sequences were 272,080.60 for leaves and 347,050 for roots. Additional filtering to remove contaminants resulted in 69,549.80 usable reads for leaves and 336,515 usable reads for roots. For samples collected from fences, the average raw reads were 349,855.80 for 16S rRNA and 265,528.80 for ITS, 108,360, and 337,914, respectively, for roots. The valid reads post-filtering was 20,982.00 for 16S rRNA and 233,110.60 for ITS in leaves, and 68,909 and 281,795 for roots, respectively. The complete read counts for all libraries sequenced are detailed in Table S1 of the Supplementary Material.

The remaining reads, after quality control, processing, and filtering of Amplicon Sequence Variants (ASVs), proved to be adequate for capturing the microbial diversity present under different conditions. This conclusion is supported by the stabilization of rarefaction curves (Figure 1), indicating that the sequencing depth achieved was sufficient to represent the diversity of microbial communities under the evaluated conditions. Therefore, even considering the inherent losses during quality control and subsequent data filtering, the obtained sequencing coverage ensures representative sampling of the biodiversity present on leaves and roots samples of *T. recurvata*. Individualized rarefaction curves for bacterial and fungal domains further confirmed this conclusion, with each data set demonstrating stabilization, as shown in the supplementary material (Figure S1).

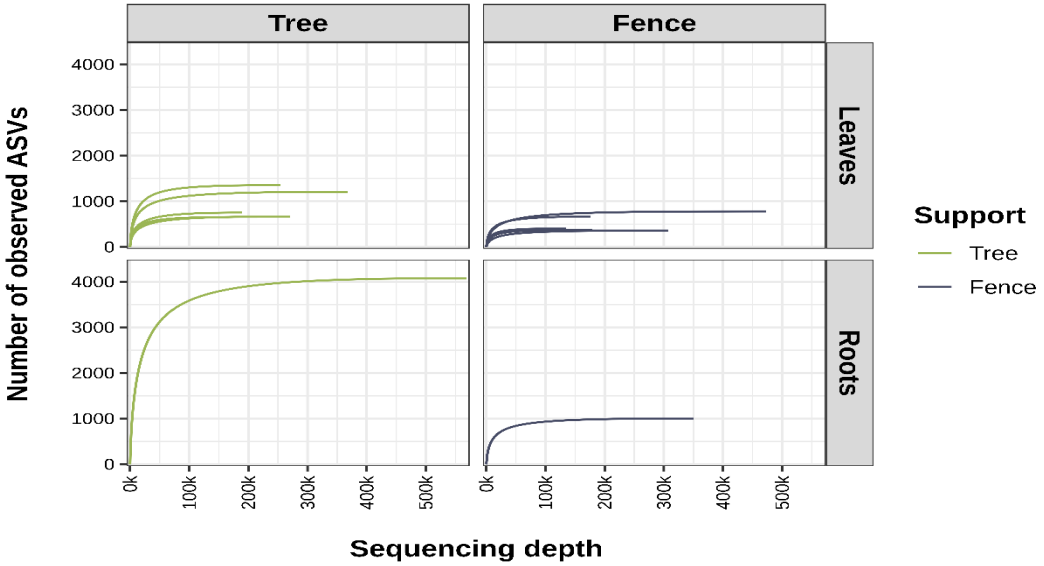

Figure 1. Rarefaction curves illustrating the observed Amplicon Sequence Variants (ASVs) of microbial (bacterial and fungal) communities associated with *Tillandsia recurvata* growing on trees and fences, separated by plant tissue (leaves and roots). The y-axis represents the number of observed ASVs, while the x-axis denotes sequencing depth. The curves show the stabilization into a plateau, indicating sufficient sequencing coverage to capture the microbial diversity within each sample.

Taxonomic assignments of the obtained ASVs demonstrated a variation in the classification efficiency across different taxonomic levels and marker types. For 16S rRNA sequences, 95.56% of the reads were classified up to the phylum level, 77.40% up to the family level, 68.46% up to the genus level, and 4.91% up to the species level. In contrast, ITS sequences showed even greater efficiency at higher levels, with 99.98% of the reads classified up to the phylum level, 93.27% to the family level, and 90.01% to the genus level. Species-level classification also performed better than 16S rRNA, with 24.39% of the reads correctly assigned. These results suggest high accuracy at higher taxonomic levels but considerable limitations in species-level classification, highlighting the difficulties of using taxonomic markers for finer taxonomic resolution. Additionally, cumulative relative abundance curves presented in the Supplementary Material (Figures S2 and S3) indicate that a significant portion of the microbial abundances is concentrated within a relatively small number of taxa or ASVs in both the 16S rRNA and ITS datasets. This pattern suggests that while a broad

diversity of taxa is present, the community structure is dominated by a few highly abundant groups.

The analysis of Venn diagrams (Figure 2) revealed significant taxon sharing at higher taxonomic levels, as well as substantial variations at the ASV level across different conditions. At the phylum level, of a total of 33 phyla, 17 (51.52%) were shared among all conditions, while 10 (30.30%) and 1 (3.03%) were exclusive to trees and fences, respectively. At the genus level, out of 681 genera, 182 (26.73%) were shared among all conditions, while 485 (71.22%) and 144 (21.14%) were exclusive to trees and fences, respectively. Regarding ASVs, out of 8677, 118 (1.36%) were shared among all conditions, while 6197 (71.42%) and 1711 (19.72%) were exclusive to trees and fences, respectively. This pattern was consistent across both microbial domains (bacteria and fungi) studied, as shown in the supplementary material (Figure S4). These results indicate significant conservation of major taxonomic groups among the conditions, whereas differences at the ASV level reflect substantial variations in population composition.

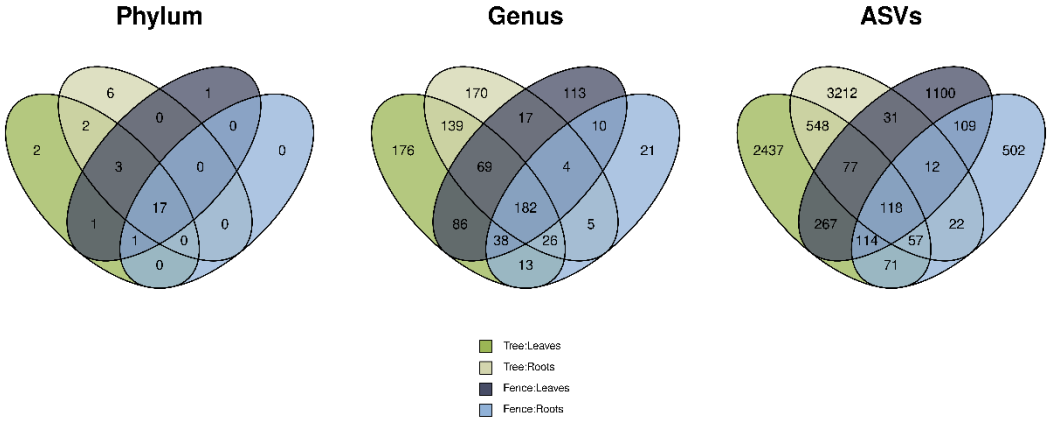

Figure 2. Venn diagrams depicting the shared and unique microbial (bacterial and fungal) taxa across taxonomic ranks (Phyla, Genera, and ASVs) among *Tillandsia recurvata* samples collected from two different growth supports (trees and fences) and two plant tissues (leaves and roots). Each diagram represents the overlap and exclusivity of microbial diversity across the specified groups, illustrating the extent of taxonomic sharing among the samples.

Detailed taxonomic analysis revealed the most prevalent taxa on the studied samples, ranging from the phylum to the species level. As an illustrative example of

the observed taxonomic diversity, the levels of "Phylum" and "Genus," grouped by condition, are highlighted in Figure 3.

The composition of the bacterial community varied significantly among the different sampling environments (Figure 3A). In the leaf samples, Proteobacteria was the dominant phylum, representing 69.37% of the relative abundance in tree epiphytes and 54.20% in fence epiphytes. This dominance is primarily due to the Sphingomonadaceae family, which constitutes a large part of the community. Actinobacteria and Acidobacteria were also notable, with 6.35% and 11.53% in tree plant leaves and 7.37% and 15.53% in fence plant leaves, respectively. In the root samples, Proteobacteria still dominated, but to a lesser extent, with 46.79% in trees and 62.28% in fences. Actinobacteria made significant contributions, with 27.71% in tree epiphyte roots and 21.14% in fence epiphyte roots, while Acidobacteria made contributions of 6.58% and 1.88% in tree and fence plants, respectively. At the genus level (Figure 3B), Sphingomonas was the dominant genus in the leaves, with 11.89% in the trees and 14.80% in the fences. Lichenibacterium was also prevalent, at 8.08% in tree plant leaves and 6.87% in fence plant leaves. Granulicella accounted for 4.09% of tree leaves and 10.37% of fence leaves. In roots, Sphingomonas represented 6.02% of trees and 7.66% of fences, whereas Lichenibacterium and Granulicella had lower proportions.

The fungal community composition varied among sampling conditions (Figure 3C). In the leaf samples, Ascomycota was the predominant phylum, comprising 98.56% of trees and 98.64% of fences. Basidiomycota were present in lower proportions, with 1.37% in trees and 1.35% in fences. In the roots, Ascomycota dominated with 99.85% of trees and 99.94% of fences, while Basidiomycota had 0.13% and 0.05%, respectively. At the genus level (Figure 3D), Paraconiothyrium was the dominant genus in the leaves, with 31.01% in the trees and 59.46% in the fences. Nigrospora was prevalent in 15.45% of the tree leaves and 9.93% of the fence leaves. Diaporthe was notably present in roots, with 19.42% in trees and 3.62% in fences.

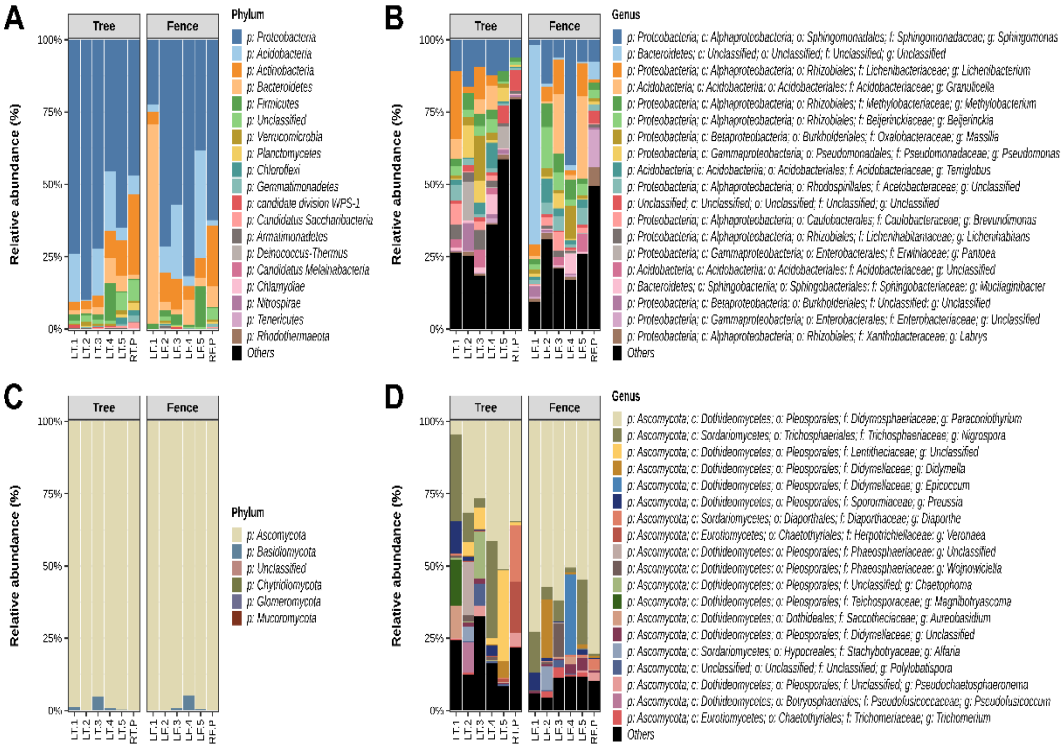

Figure 3. Taxonomic profile representing the distribution of relative abundances of major microbial taxa identified in *Tillandsia recurvata* samples. The plots are separated into Bacteria (A, B) and Fungi (C, D), displaying the relative abundance at the Phylum (A, C) and Genus (B, D) levels for samples collected from trees and fences. Up to 19 of the most abundant taxa are shown in each plot, with taxa of lower abundance grouped under the category "Others" to enhance the visualization of dominant taxa.

The results of the alpha diversity analysis showed significant differences in richness and Shannon and Gini-Simpson indices between leaf samples of epiphytic plants grown on trees and fences (Table 1; Figure 4). The leaves of plants growing on trees had higher average values for richness (928.8), Shannon (3.62), and Gini-Simpson (0.89) compared to the leaves of plants grown on fences, which had averages of 518.2, 2.18, and 0.67, respectively (Table 1). These differences were statistically significant, with p-values of 0.05 (Richness), 0.003 (Shannon), and 0.014 (Gini-Simpson), indicating greater diversity and balance in the microbial community associated with the leaves of plants on trees (Figure 4).

For roots, richness was considerably higher in plants grown on trees (4,077) compared to those grown on fences (1,005). The Shannon and Gini-Simpson indices were also higher in the roots of plants on trees, with values of 5.73 and 0.97,

respectively, compared to values of 2.54 and 0.59 in plants on fences (Table 1). These results suggest that epiphytic plants growing on trees support a more diverse and balanced microbial community in both leaves and roots, reflecting the distinct influences of different growth supports on the associated microbial communities. Individualized values for each sample and microbial domain are provided in the Supplementary Material (Table S2). These data show that bacterial richness is more significantly affected by the growth support, while fungal diversity metrics exhibit more pronounced differences (Figure S5). Additionally, the analysis of rare species (ASVs with a mean relative abundance < 0.001% or present in only one sample) revealed that the diversity (Shannon index) of rare bacterial ASVs is higher in samples from plants grown on trees, whereas fungal diversity appears unaffected by the different growth supports (Table S3 and Figure S6).

Table 1. Summary of alpha diversity metrics—Richness, Shannon, and Gini-Simpson Index— of microbial (bacterial and fungal) communities across leaves and roots samples of *Tillandsia recurvata* collected from trees and fences. Averages are accompanied by ± standard deviation (only for leaves).

Group	Richness	Shannon	Gini-Simpson
Tree (Leaves)	928.8 ± 328.58	3.62 ± 0.60	0.89 ± 0.07
Tree (Roots)	4077	5.73	0.97
Fence (Leaves)	518.20 ± 192.44	2.18 ± 0.48	0.67 ± 0.12
Fence (Roots)	1005	2.54	0.59

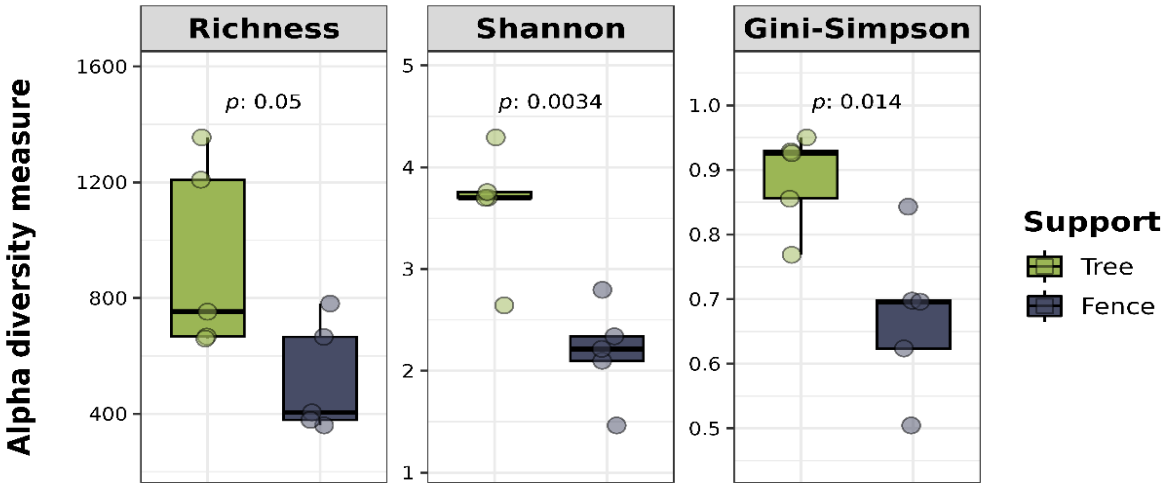

Figure 4. Box plots displaying alpha diversity metrics—Richness, Shannon, and Gini-Simpson Index— of microbial (bacterial and fungal) communities across leaves samples of *Tillandsia recurvata* collected from trees and fences. The box plots illustrate the variation in diversity within these samples. Statistical comparisons were performed using Student's t-test to identify significant differences ($p \leq 0.05$) between the two groups.

Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances was used to determine the similarity of microbial compositions between epiphytic plant samples grown on trees and fences (Figure 5). The distances from the centroid indicated a greater dispersion of samples from trees compared to those from fences, suggesting more variability within the microbial communities associated with tree-supported plants (Figure 5A). The PCoA results showed significant separation of samples according to growth support, explaining 26.69%, 20.21%, and 12.66% of the total variability observed in the first three principal axes, respectively, totaling 59.56% of the explained variability (Figure 5B and 5C). PERMANOVA analysis confirmed the statistical difference between the microbial compositions of epiphytic plants from trees and fences, with a p-value of 0.006, indicating that growth support significantly influenced the structure of microbial communities. Although there was some overlap between the groups, the separation trend observed in the PCoA plots suggests distinct patterns in microbial composition associated with each type of support. The results, when individualized by microbial kingdom (Figure S7), revealed that the effect of

growth support was more pronounced in fungal communities (Figure S7D-F) compared to bacterial communities (Figure S7A-C).

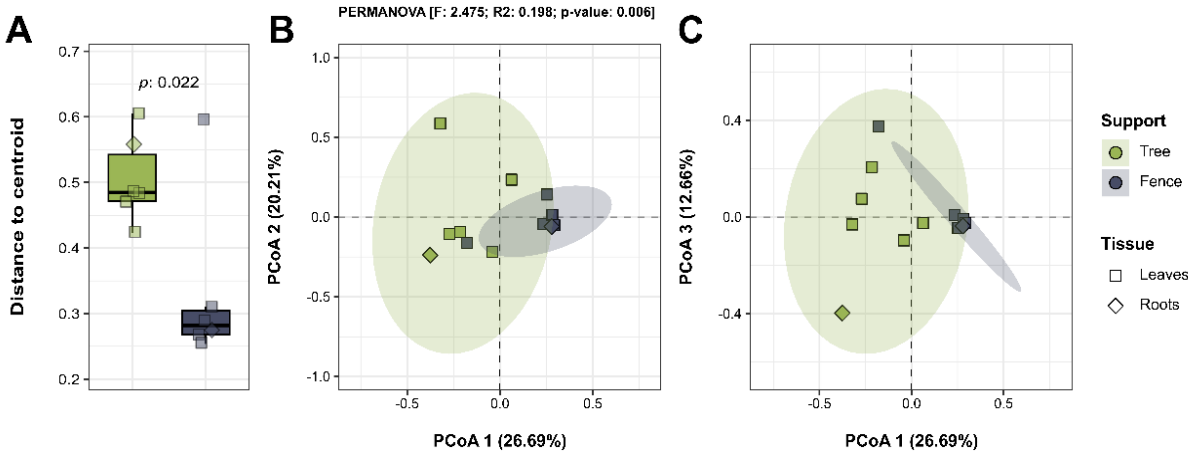

Figure 5. Beta diversity analysis of microbial communities (Bacteria and Fungi) associated with *Tillandsia recurvata* growing on trees versus fences. The panel includes (A) box plots showing the distribution of centroid distances based on Bray-Curtis dissimilarities, with statistical comparisons performed using Student's t-test ($p \leq 0.05$), and Principal Coordinate Analysis (PCoA) plots displaying the dispersion of leaves and roots samples across combinations of axes 1 and 2 (B), and axes 1 and 3 (C). The influence of growth support on community composition was assessed using permutational multivariate analysis of variance (PERMANOVA) ($p \leq 0.05$).

Differentially abundant (DA) analysis revealed 180 DA taxa between epiphytic plants grown on trees and fences, comprising 65 bacteria and 115 fungi. These taxa were categorized into one phylum, seven classes, nine orders, 28 families, 76 genera, and 59 species. Among the identified genera, 67 (42 bacterial and 25 fungal) were more abundant in the tree epiphytic plant samples, while nine fungal genera were more abundant in the fence samples (Figure 6). Most DA taxa from tree epiphytes were exclusive to this condition and were not detected in fence samples.

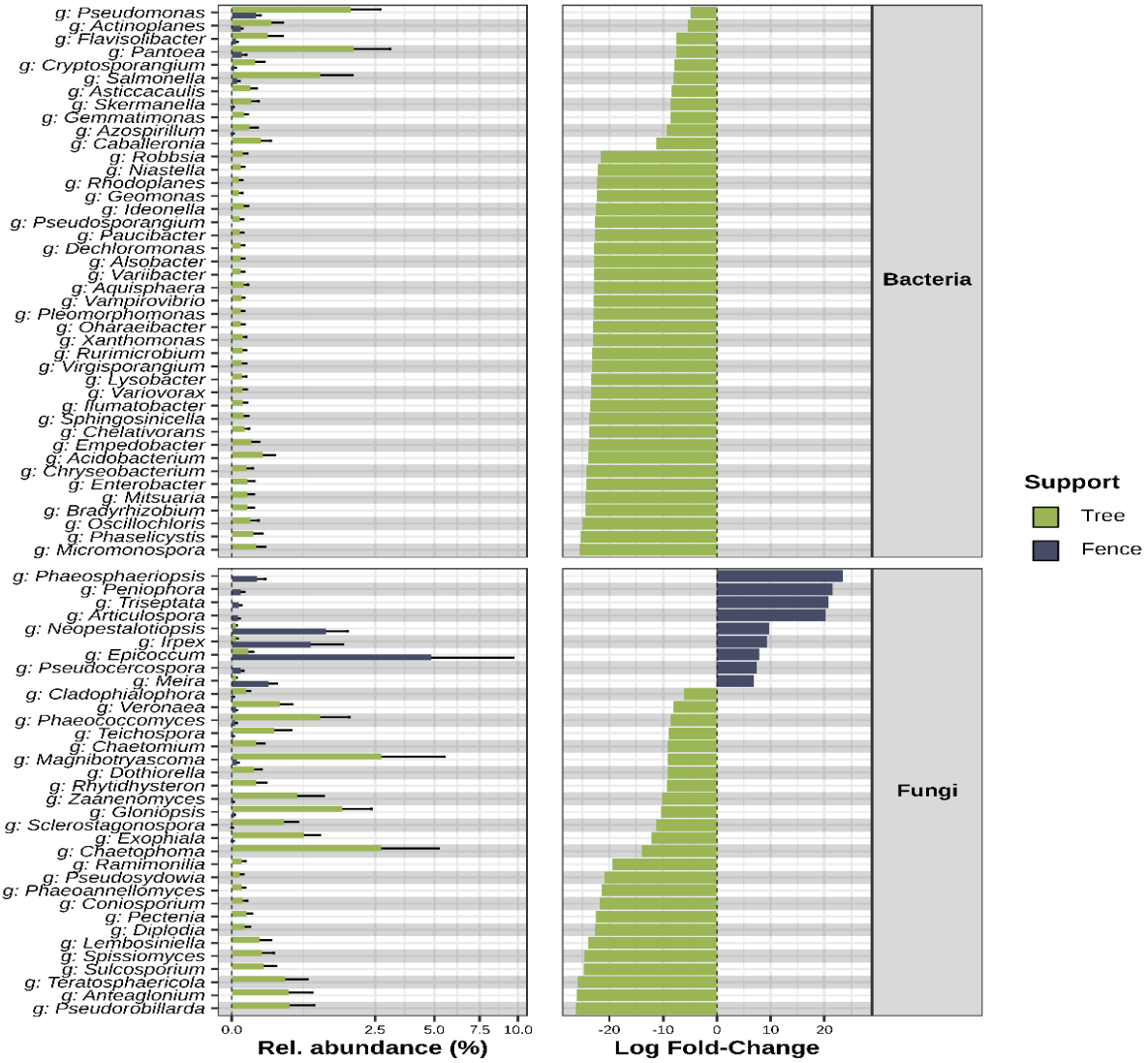

Figure 6. Differential abundance analysis of Bacterial and Fungal genera from leaves' samples of *Tillandsia recurvata* collected from trees and fences. The left panel shows the relative abundance of differentially abundant (DA) genera, with the x-axis transformed using a square root scale to enhance the visualization of less abundant genera. The right panel presents the mean intensity of the difference in abundance, expressed as Log2 Fold-Change, for genera that exhibited statistically significant differences between the two groups (FDR corrected $p \leq 0.05$).

Co-occurrence network analysis, based on Pearson correlation coefficients ($r \geq 0.75$ or $r \leq -0.75$) and a 95% confidence level ($p \leq 0.05$), revealed considerable differences in the structures of microbial networks associated with epiphytic plants grown on trees and fences at the genus level (Figure 7, Table 2). The network associated with trees showed a higher number of nodes (548) and twice the number of edges (22,986) compared to the fence network, which had 396 nodes and 11,369

edges (Table 2). Both networks had a low number of negative edges, although the ratio of positive to negative edges was higher in the tree-plant samples network. Additionally, the tree network exhibited higher values in terms of average degrees, with an average degree of 83.89, compared to the fence network, which had an average degree of 57.42. Statistical comparison indicated that these differences were significant ($p < 0.001$) (Figure 7C). Betweenness centrality measures, which indicate the extent to which a node lies on the shortest path between other nodes, were relatively similar between the two networks, with an average betweenness of 0.005 in the tree network and 0.007 in the fence network (Figure 7D, Table 2).

The main hubs differed between the networks, with the genus Marmoricola being the primary hub in the tree network and Mucilaginibacter in the fence network (Table 2). Taken together, these differences reflect the influence of growth support on the structure and interactions of microbial communities associated with epiphytic plants. Thus, the analysis suggests a more complex and interconnected network in trees.

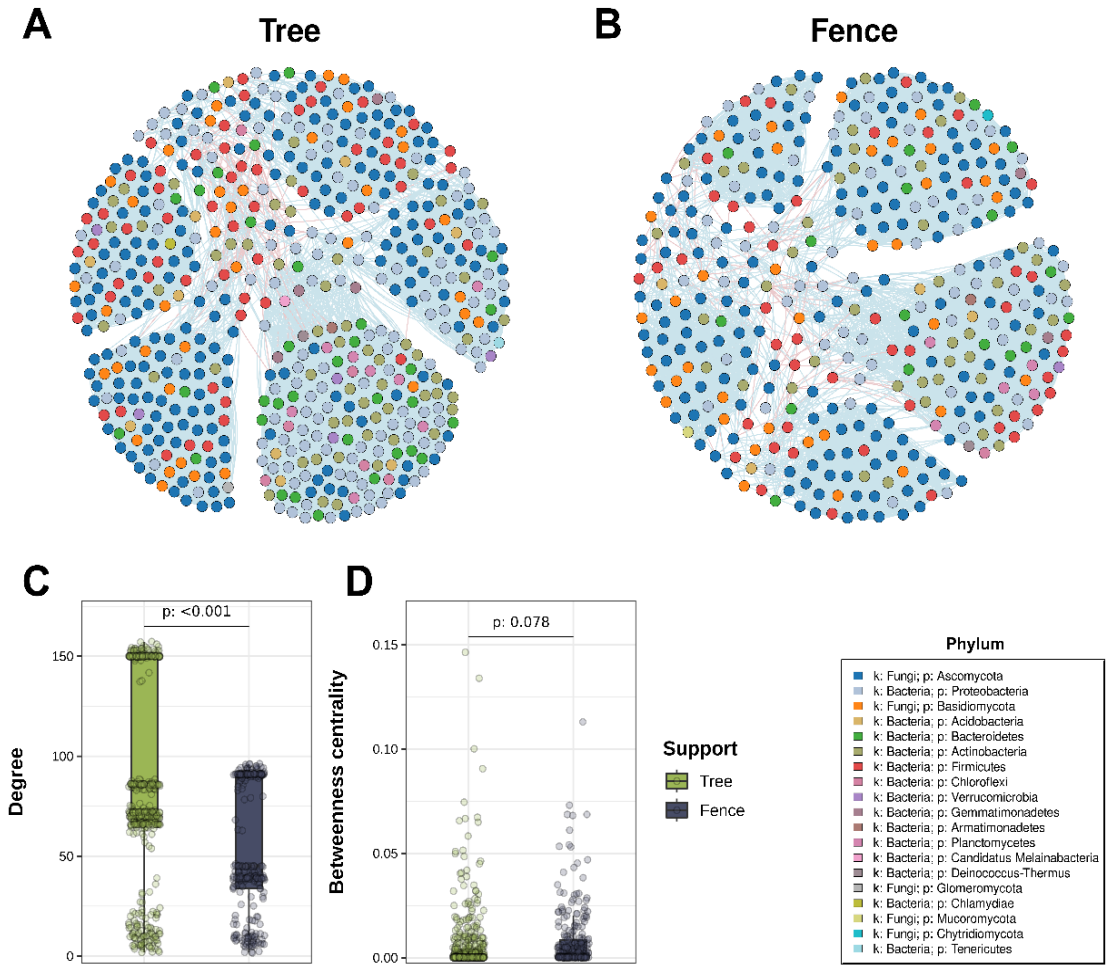

Figure 7. Co-occurrence networks of microbial genera (Bacteria and Fungi) in leaves and root samples of *Tillandsia recurvata* collected from trees (A) and fences (B). The networks are constructed based on Pearson correlation coefficients ($r \geq 0.75$ or $r < -0.75$) with a 95% confidence level ($p \leq 0.05$). Positive correlations are represented by blue edges, and negative correlations by red edges, with node fill color indicating the phylum to which each genus belongs. The average number of connections (Degree) (C) and betweenness centrality (D) of the groups were statistically compared using Student's t-test ($p \leq 0.05$).

Table 2. Summary of the main topological characteristics and centrality measures of co-occurrence networks for microbial (bacterial and fungal) genera identified in leaf and root samples of *Tillandsia recurvata* grown on trees and fences.

Attribute	Tree	Fence
N. of nodes	548	396
N. of edges	22,986	11,369
Positive edges	22,885	11,287
Negative edges	101	82
Clustering coefficient	0.971	0.948
Mean degree	83.89	57.42
Max. degree	157	96
Mean betweenness	0.005	0.007
Max. betweenness	0.146	0.113
Main hubs	*Marmoricola*	*Mucilaginibacter*

Functional prediction analysis of the microbial communities was performed using the PICRUSt2 program, which predicts functions from 16S rRNA sequences. Principal Component Analysis (PCA) of the annotated functions (KOs) revealed considerable overlap in functional composition across different conditions (Figure 8A). The first three principal components explained 24.07%, 16.71%, and 14.05% of the total variance, respectively, accounting for 54.83% of the explained variance.

The functional profile of the KOs classified in the metabolism class indicated similar profiles between leaf and root samples (Figure 8B), as well as between plants grown on trees and fences. The most abundant classes were carbohydrate metabolism (23.45%), amino acid metabolism (19.49%), cofactor and vitamin metabolism (11.88%), and energy metabolism (11.32%). Regarding the microbial traits annotated with the PLaBAse database (Figure 8C), the functional profile was also similar and conserved among the evaluated conditions. The main functional categories were plant system colonization (27.4%), stress control or biocontrol (19.12%), competitive exclusion (17.98%), and bio-fertilization (13.9%).

The volcano plot (Figure 8D) highlights the differentially abundant functions between the leaf samples of plants grown on trees and fences. Although the overall profile is conserved, tree-grown plants exhibited 546 differentially expressed functions (representing 7.74% of the 7050 detected KOs), in contrast to only 170 functions

(2.41% of the 7050 detected KOs) that were more expressed in fence plants, indicating a greater functional diversity in T. recurvata plants grown on trees.

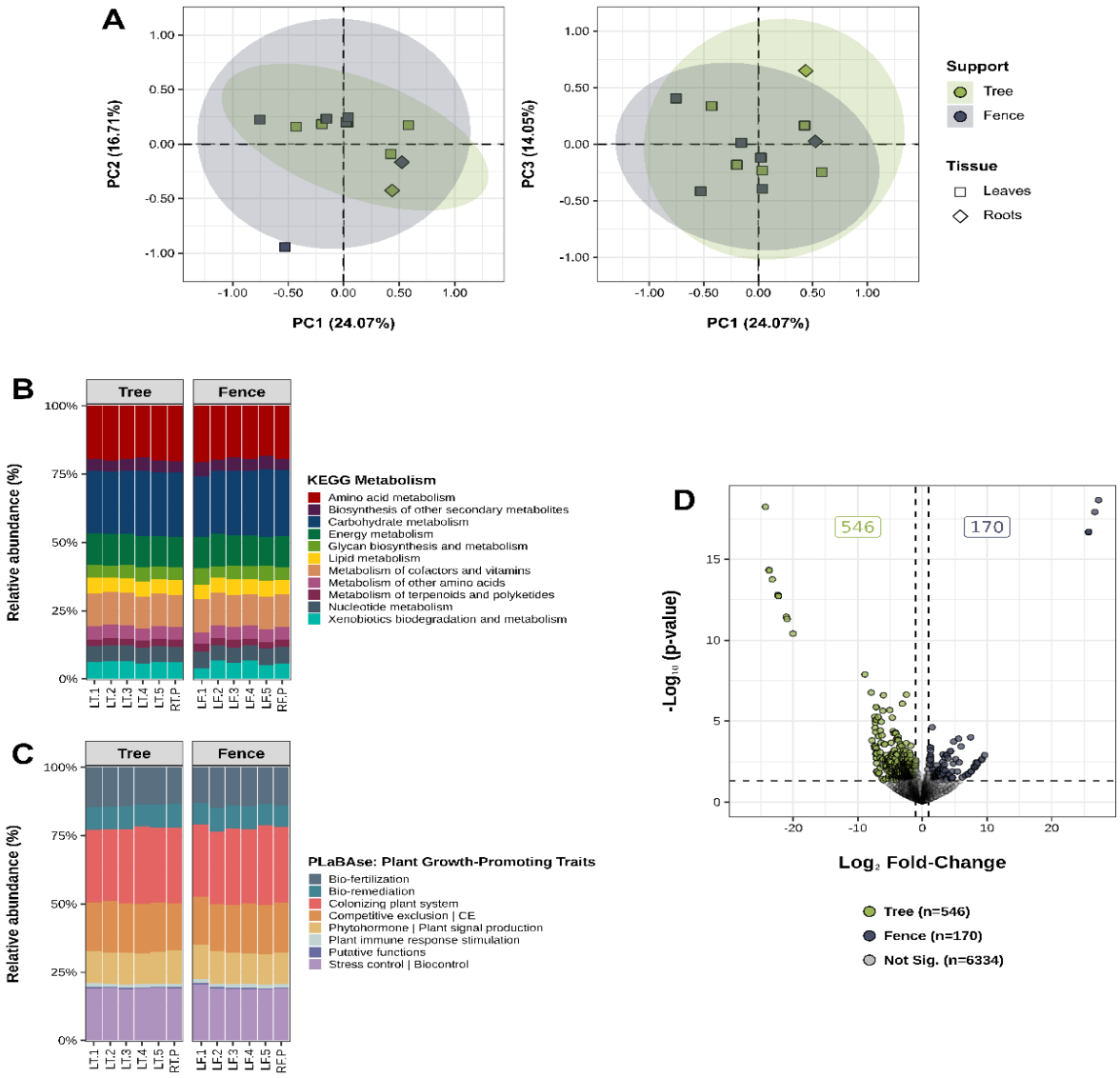

Figure 8. Functional composition and differentially abundant predicted genes in bacterial communities associated with *Tillandsia recurvata* growing on trees versus fences. The panel includes: (A) Principal Component Analysis (PCA) of the functional composition (KEGG Orthologs [KOs]) predicted using PICRUSt2 on 16S rRNA data. (B) Functional profile of KOs associated with metabolic classes from the KEGG database. (C) Functional profile of microbial traits annotated using the plant-associated bacteria web resources database (PLaBAse). (D) Volcano plots showing differentially abundant KOs between leaf samples from plants grown on trees and fences, with statistical significance set at FDR corrected p ≤ 0.05 and a minimum fold-change difference of 2 (Log2FC ±1).

Discussion

Taxonomic classification determines the degree of relatedness among organisms recorded on the leaves and roots of the epiphyte T. recurvata growing on trees and fences. At the phylum level, 33 major taxonomic groups were identified. In all conditions, 17 phyla (51.52%) were shared. Of these, 10 phyla (30.30%) were exclusive to trees and one phylum (3.03%) was exclusive to fences. At the genus level, 681 specific phyla were identified. Under all conditions, 182 genera (26.73%) were shared. A total of 485 genera (71.22%) were exclusive to trees and 144 genera (21.14%) were exclusive to fences. These findings indicate that fences provide less microbial diversity and sharing than trees. Therefore, it has been suggested that colonizing trees is more advantageous than fencing for epiphytic plants. Many studies have evaluated the distribution of colonization by Tillandsia sp. The study examined the intricate dynamics governing the habitat occupancy of epiphytes, such as T. recurvata, highlighting the significance of host traits, tree size, and spatial configuration in shaping the distribution and abundance of these species [1]. Another study showed that T. flexuosa growing on electrical cables in Panama showed slow growth and less successful colonization of plants on cables compared to trees, indicating suboptimal conditions for cable-inhabiting populations [39]. The present study reinforces that the microbial diversity was lower than that of the tree beyond the suboptimal conditions of cable-inhabiting. It is fascinating to consider that understanding the complex relationships among various bromeliads can yield valuable insights into the patterns and dynamics of natural communities, particularly in environments with high and low tree densities. The results of this study indicate that positive interactions and high levels of dispersal may have a significant impact on the assembly of atmospheric bromeliads than local competitive interactions [2].

Although there was a statistically significant difference in the prevalence of bacterial groups between different locations (trees and fences) and plant tissues (leaves and roots), the bacterial communities were fairly conserved at broader taxonomic levels, such as the Phylum (Figure 3). These similarities between the microbiomes of plants from different locations were found in another study. Aguiar-Cruz [40] evaluated the microbiome of bromeliad plants from five different forests in Mexico and found that despite the environmental differences, there was a high

redundancy in the putative metabolic functions of the prokaryotic communities. This suggests that certain metabolic functions, particularly those related to organic carbon and nitrogen cycling, remain relatively constant in these microecosystems. However, differences in microbial communities may be influenced by spatial variability [41]. The prevalent phylum is Proteobacteria, which is important in soil ecosystems [42]. Actinobacteria contribute to the ecosystem by being involved in atmospheric nitrogen fixation and plant growth [43]. Sphingomonas is also present, and some members of this genus may have the ability to fix atmospheric nitrogen and promote plant growth [44]. The presence of nitrogen-fixing microflora on the leaves of Tillandsia sp. is potentially significant for the nutrition of these plants, especially in relation to the absorbing role of foliar trichomes. These microorganisms also contribute to the nutrition of other phyllospheric microorganisms, such as yeasts and fungi [45] Lichenibacterium is another genus that might play a role in plant growth. It is worth noting that the phylum Granulicella has a lower prevalence in tree leaves (4.09%) and a higher prevalence in fence leaves (10.37%) and plays an important role in the health and ecology of lichens [46]. The presence of these phylum could explain the plant growth-promoting traits including colonization plant system and stress control as can be seen in Figure 8C. Likewise, this phylum is the most important when plants live in fences.

Regarding the prevalence of fungi, it was observed that the similarity between the locations and plant tissues was higher for fungi than for bacteria. At the phylum level, no significant differences were observed in the prevalence of Ascomycota and Basidiomycota. Ascomycota include species that are either plant pathogens or edible fungi [47], whereas Basidiomycota comprises fungi that play important ecosystem functions and can be both plant pathogens and beneficial fungi [48]. However, there was a statistically significant difference in the phylum Paraconiothyrium, with a 31.01% prevalence in tree leaves and 59.46% in fence leaves. This genus may play a role in biological control, bioremediation, and antibiotic production [49]. Additionally, Nigrospora was present in tree leaves at 15.45%, and in fence leaves at 9.93%. This genus may also have biocontrol potential or produce secondary metabolites [50]. The genus Diaporthe was notably present in the roots, with 19.42% prevalence in tree roots and 3.62% in fence roots. This genus includes endophytic, saprobic, and plant

pathogenic fungi, with some species transforming infection-inhibiting factors into their derivatives. This genus includes temperate and tropical species [51]. The fungal species identified in the present study were found in the endosphere (leaves and roots). However, the phyllosphere also shows high fungal diversity. Felix et al. [52] found that more than 180 species of yeasts and yeast-like fungi were recorded from the bromeliad phyllosphere. At least 50 yeast species with biotechnological potential have been isolated from bromeliads, and over 90% of these species are capable of producing extracellular enzymes, indicating significant biotechnological applications.

It is noteworthy that the plants situated in the tree exhibited a greater significance in terms of microbial diversity than those placed in the fences, as evidenced by the higher values observed in all the indices assessed, namely Richness, Shannon, and Gini-Simpson. These findings raise the question of why plants choose to grow in trees rather than fences? This outcome suggests that several factors are involved in this process, and it underscores the importance of trees in maintaining ecological balance, as has been discussed in some studies [2, 17]. Including genera of agronomic interest, Bradyrhizobium (Log2FC: 24.45; $p < 0.001$), Azospirillum (Log2FC: 9.29; $p = 0.011$), and Pseudomonas (Log2FC: 4.82; $p = 0.029$), the results indicated that these taxa were significantly more abundant in the plant from the trees. These findings suggest that the growth conditions of trees promote a greater diversity of microbial genera, including taxa with potential agronomic benefits [53]. This observation highlights the influence of the support environment on the microbial ecology profiles of epiphytic plants.

Co-occurrence analysis revealed that trees displayed a more intricate microbial network with greater connectivity than fences. This suggests that the way plants develop, whether on trees or fences, influences the structure and interactions of associated microorganisms. The microbial communities associated with trees form a more complex network. A study shows that mixed-species plantations exhibit more robust co-occurrence networks than monocultures, indicating stronger microbial interactions. Furthermore, the study revealed that afforestation with functional traits of different tree species significantly enhanced the microbial structures associated with soil carbon and nitrogen cycling [54]. The results of the present study suggest that the microbial community on plants located on the tree trunk may play an important role in

tree health, whereas the microbial community on plants located on the fence is only necessary to support plant growth. On the other hand, the analysis of metabolism class (KOs) results revealed that there were similar profiles between leaf and root samples, as well as between plants on trees and fences. The conservation of these skills is more prevalent in microorganisms, resulting in no discernible variation between the locations where the plants were collected.

Conclusion

This study suggests that T. recurvata individuals growing on trees have a higher microbial diversity and distribution than those growing on fences. Plants on trees are carriers of bacteria, such as Bradyrhizobium, Azospirillum, and Pseudomonas, which are of agricultural interest. In addition, the growth conditions of trees appeared to encourage a greater variety, and co-occurrence analysis revealed that trees formed a more complex microbial network with greater connectivity than that of fences. This suggests that the growth support, whether on trees or fences, affects the structure and interactions of associated microorganisms and that the plants on trees could be a reservoir of microbes of agricultural interest.

Author Contributions

JSS: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. LALC: Data curation, Formal analysis, Methodology, Investigation, Writing – original draft, Writing – review & editing. CHB: Data curation, Methodology, Investigation, Writing – review & editing. ETF: Data curation, Methodology, Investigation, Writing – review & editing. DGP: Data curation, Investigation, Writing – review & editing. DN: Data curation, Investigation, Writing – review & editing. ND: Data curation, Investigation, Writing – review & editing. ECR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Data Availability

The raw data and analyzed data used during the current study are available from the corresponding author on reasonable request. The raw data can be found in the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA1134710 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1134710).

Ethical Approval

Not applicable.

Competing Interests

The authors declare no competing interests.

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