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Intraspecific variation on epiphytic bacterial community from *Laguncularia racemosa* phylloplane

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Abstract

Mangroves are dynamic and unique ecosystems that provide important ecological services to coastal areas. The phylloplane is one of the greatest microbial habitats, and most of its microorganisms are uncultivated under common laboratory conditions. Bacterial community structure of *Laguncularia racemosa* phylloplane, a well-adapted mangrove species with salt exudation at foliar levels, was accessed through 16S rRNA amplicon sequencing. Sampling was performed in three different sites across a transect from upland to the seashore in a preserved mangrove forest located in the city of Cananéia, São Paulo State, Brazil. Higher bacterial diversity was observed in intermediary locations between the upland and the seashore, showing that significant intraspecific spatial variation in bacterial communities exists between a single host species with the selection of specific population between an environmental transect.

Keywords Epiphytic bacteria · Community structure · Intraspecific variation · Mangroves

Introduction

Mangroves are dynamic ecosystems, which provide important ecological services to coastal areas due to the high rates of primary production and harbor several marine organisms [1]. They are composed of a salinity tolerant vegetation that thrives in tropical and subtropical regions of the world under tidal influence [2]. This vegetation is one of the most expressive components of this ecosystem, and their leaves correspond to a major portion of the primary production in this environment [3]. Because of its location, mangrove forests function as an intermediary environment between marine, freshwater, and the terrestrial forest. In the Cananéia mangrove, three plant species are dominant. Near the shoreline, mangroves are mostly composed of *Rhizophora mangle* due to its capacity to withstand tidal action with its root system. The vegetation then transitions a higher abundance of *Laguncularia racemosa* and *Avicennia schaueriana* as the one gets landward, thus, forming an environmental gradient. Also, these plants have evolved to cope with various natural stresses [4], and the species *Laguncularia racemosa* is a well-adapted mangrove plant and an important component in the American continent [5].

Plants broadly influence the ambient by means of photosynthesis [6], and their leaves are responsible for a great part of the energy and organic matter input into planet Earth [7]. Healthy plants in nature live in association and actively interact with a multitude of microorganisms belonging to several microbial types, such as bacteria, archaea, fungi, and oomycetes, collectively called the plant microbiota [8]. Leaves are the dominant aerial plant structure with an estimated global area twice as great as the land surface [9]. The phyllosphere ecosystem is the aerial part of plants while the phylloplane is the foliar surface, and the organisms that thrive in this environment are called epiphytes [10, 11]. The

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microbial communities in this ambient are mainly composed of bacteria, archaea, filamentous fungi, and yeasts [10].

Because leaves have a relatively brief lifespan, the phylloplane is expected to be a transitory environment when compared with rhizosphere [9]. The microorganisms that colonize this environment are exposed to biotic and abiotic stresses like atmosphere, ultraviolet radiation, low or fluctuating water availability, plant metabolism [9], scarce and heterogenic nutrient condition [12], and presence of antimicrobial secondary metabolites of plant [13] or microbial origin [9]. In addition, epiphytic microorganisms of mangrove plant species like *Laguncularia racemosa* that exudate salt at foliar levels also have to deal with osmotic pressure [5].

Bacteria from phyllosphere generally have a well-defined backbone [14] comprising mainly Proteobacteria, Actinobacteria and Bacteroidetes [9]. To define a source for bacterial assemblages in the phylloplane is difficult because microbiota members can originate from rainwater, plant dispersal vectors [15], aerosols, animals, and soil as well as upward migration from the root [8].

Tropical ecosystems harbor a great epiphytic bacterial diversity with the potential to house new bacterial species [16], but most of the epiphytic microorganisms are uncultivated under commonly laboratory conditions when compared with other environments [17], and little is known about the epiphytic bacterial diversity on mangrove habitats. Studies on the ecology of these organisms must be further explored to help elucidate the structuring mechanism of these communities in this habitat and consequently increase the knowledge into the ecology of uncultivated microorganisms.

The aim of this work was to evaluate the spatial variation observed in the epiphytic bacterial community of the *Laguncularia racemosa* phylloplane across a transect from the upland to the seashore at Cananéia mangrove, through metagenomic 16S rRNA amplicon sequencing. We hypothesized that the spatial distribution of the plant species *Laguncularia racemosa* along a transect from the upland to the seashore in the Cananéia mangrove ecosystem could affect the dynamism of the epiphytic bacterial communities and that plants from different locations could house different assemblages of communities.

Material and methods

Site description and collected material

Samples were collected from one mangrove forest in the city of Cananéia (25° 05′ 03″ S–47° 57′ 75″ W) that is located in a pristine area with little human influence. Fresh mature leaves that did not present any sign of lichen or lesion were collected directly from the mangrove plant species *Laguncularia racemosa* across a transect with three distinct sites: upland (S 25° 05′ 10.5” W 47° 57′ 42.6″), intermediary (S 25° 05′ 06.3” W 47° 57′ 44.1″), and seashore (S 25° 05′ 01.8” W 47° 57′ 45.7″) (Fig. 1). The distance between the sites is of 131 m between P1 and P2, 145 m between P2 and P3, and 281 m between P1 and P3.

These leaves were immediately placed in sterile bags and transported to the laboratory where they were processed within 24 h. The sampling was carried out in March, in the end of the summer at the Cardoso Island, in the Cananéia mangrove, a region which has a subtropical climate according to the Köppen-Geiger classification [18]. The day of the sampling was March 16th of 2016 that presented a climate media of 25 °C, with 182.2 mm of rainfall (http://www.ciiagro.sp.gov.br/), and it was not raining at the time of the sampling.

DNA extraction and bacterial community analysis

Five leaves were placed in 500-ml Erlenmeyer flasks containing 0.85% autoclaved saline solution, and this material remained under agitation at 135 rpm for the period of 2 h. This content was transferred to 50 ml of capacity Falcon tubes, containing 15 ml of the 10^{-1} dilutions of each of the samples, with three replicates each, and they were centrifuged at 16,000 rpm for 15 min. The pellets were recovered and suspended in 100 μl of autoclaved MilliQ water, and 0.25 g of this solution was used in the extraction. To obtain the nucleic acids (DNA) from the cell material found on the surface of the leaves of *L. racemosa*, DNA extraction was performed using the PowerSoil™ DNA Extraction (MoBio) Kit following the manufacturer's instructions. Quality and quantity of the DNA were evaluated in 0.8% agarose gels and in the NanoDrop spectrophotometer (Thermo Scientific 2000 spectrophotometer).

Three experimental samples of each location were PCR-amplified using the primer sets 967F [19] and 1193R [20] to generate V6-V7 region amplicons of the 16S rRNA gene. The PCR reactions were performed as described previously [21]. Sequencing was performed on Ion Torrent PGM system (Life Technologies) using the Ion 316™ Chip. The enrichment phase was performed by using the OneTouch 2 device with the Ion Sequencing 400 Kit according to the manufacturer's instruction (Life Technologies). Raw sequencing data obtained from the PGM system were processed using QIIME 1.9 software (Quantitative Insights Into Microbial Ecology) [22] following a modified version of the 454 Overview Tutorials as described previously [21]. After the filtering steps, we obtained 383,784 reads with an average of 26,239.78 reads per library (min. 11,388 and max. 41,974).

Nucleotide sequence accession numbers

Bacterial 16S rRNA gene sequences obtained in this study are publicly available in the Sequence Read Archive (SRA) server

Fig. 1 View of the three sites along the collection transept. Upland (P1), intermediary (P2), and seashore (P3)

(https://www.ncbi.nlm.nih.gov/sra) under the accession number SRP156580.

Statistical analyses

The alpha diversity was calculated considering Shannon and Simpson indexes, and significant differences were investigated by F test followed by Tukey test considering significant $p < 0.05$. The beta diversity was estimated by means of the principal coordinates analysis (PCoA) with the Bray-Curtis dissimilarity metric, to summarize the variation of phyllosphere community structure along the sampling sites. Alpha diversity and the constrained ordination analyses were performed with the function Ordinate in R software [23] using the Phyloseq Package [24]. Bar graphs with the dominant taxonomic groups, (i.e., at least 1% of relative abundance within a given sample) were generated to identify the contribution of different classes in each environment. To verify the effect of the three different sites in the structuration of the epiphytic community, we performed a PERMANOVA (a non-parametric analysis) with the ADONIS function of the Vegan package in R software.

Once the effect of the sampling sites in the assembly of phyllosphere microbial community was verified, differently abundant OTUs between the sites were assessed through DESeq2 Package [25], in R environment [23]. The input data consisted in a matrix containing raw counts of sequencing of reads [25, 26], after removing OTUs with less than 15 reads in each treatment. All the p values of the differential analysis were adjusted to the false discovery rate (FDR) according to the Benjamin-Hochberg correction [26].

Results

The total community 16S rRNA gene sequencing of the intermediate site (P2) along the transect, which represents the transitional zone between the sea and the continent, showed the higher bacterial diversity, followed closely by the seashore site (P3), while the less diverse site was the upland (P1). The two diversity indexes (Shannon and Simpson) showed that the intermediate site (P2) and the seashore site (P3) were significantly more diverse than the upland point (P1) ($p < 0.05$). Sites two (P2) and three (P3) did not present significant differences among them, although the two indexes revealed that the intermediate one (P2) was more diverse than the seashore (P3) (Fig. 2). The PERMANOVA analysis (Table 1 of the supplementary material) showed that the sites had a marginal effect of in the structure of the epiphytic bacterial community ($p = 0.1$).

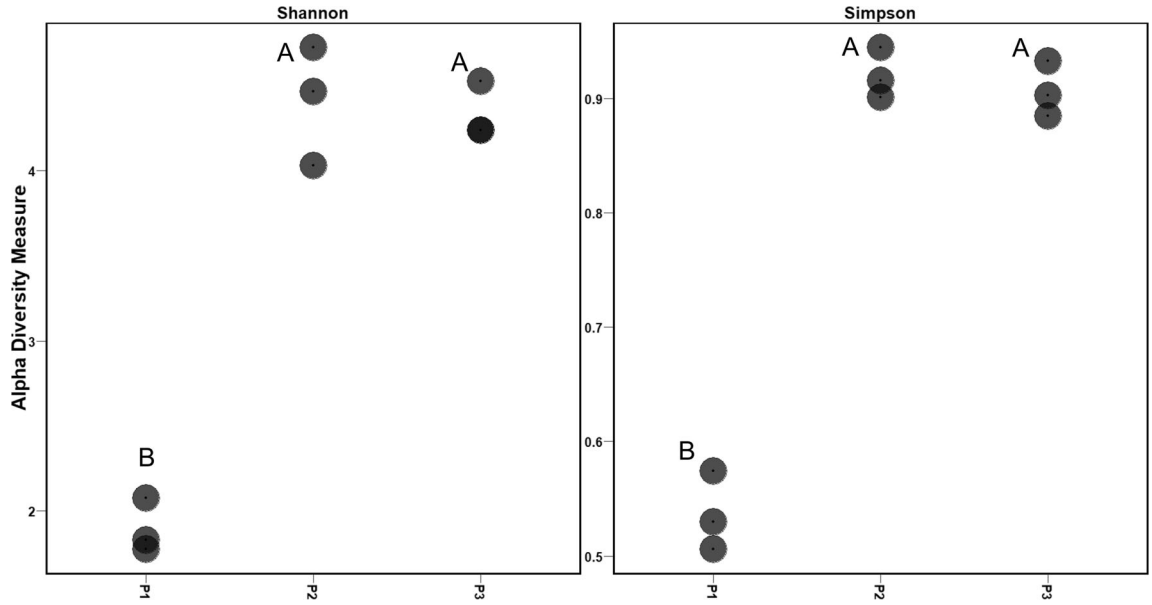

Fig. 2 The α diversity indexes **a** Shannon and **b** Simpson in the three sites: upland (P1), intermediary (P2), and seashore (P3). Each dot represents α diversity index from a sample. Different letters correspond to significant differences in Tukey test ($p < 0.05$)

The microbial community structure was differently assembled when comparing the phylloplane of *L. racemosa* in the three sampling sites (Fig. 3). The first axis explained 58.3% of the data while the second axis explained 25.7% (Fig. 3). The results showed the total separation of the samples based on the location, which suggests the effect of sampling sites on structure and composition of the epiphytic bacterial community of *L. racemosa* in Cananéia mangrove of São Paulo State, Brazil.

The nine most abundant bacterial classes in the phylloplane of *L. racemosa* were Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Betaproteobacteria, Actinobacteria, Cytophagia, Clostridia, Bacilli and Oscillatoriophycideae, respectively

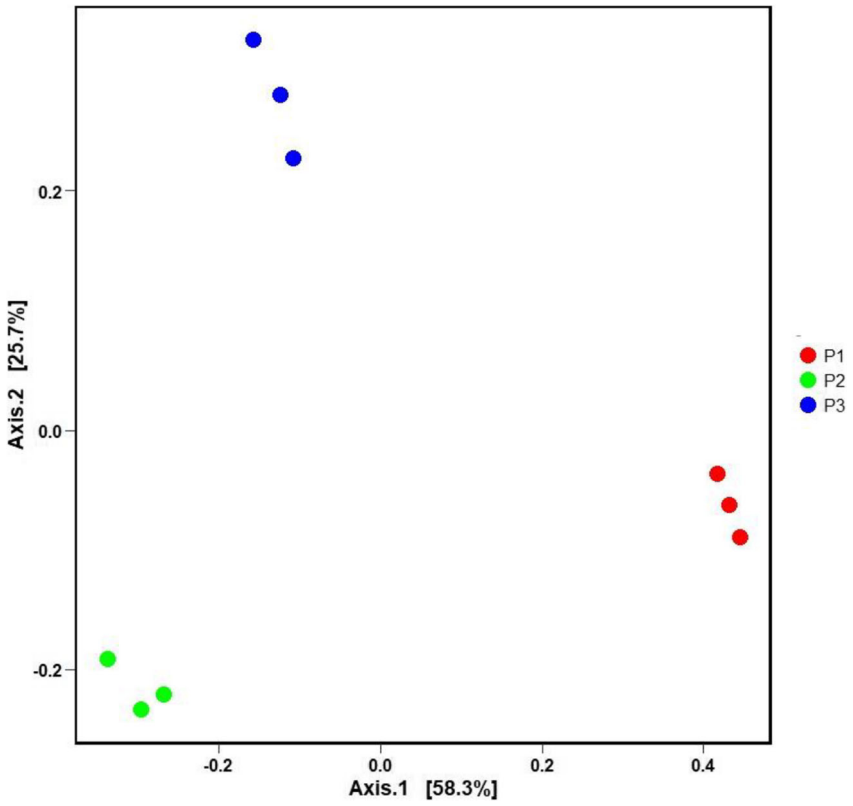

Fig. 3 Principal coordinates analysis showing the β diversity in the three collected sites: upland (P1), intermediary (P2), and seashore (P3)

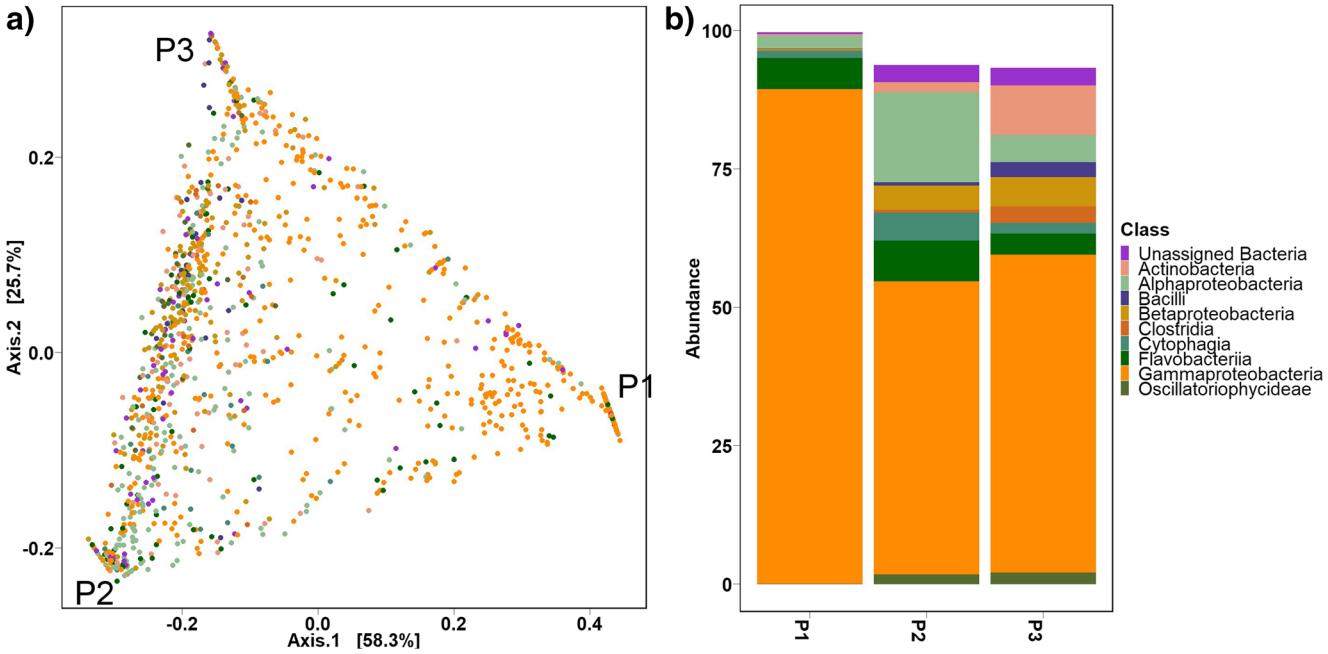

Fig. 4 *Laguncularia racemosa* phylloplane microbial community assembly in different locations in Cananéia mangrove showed by **a** principal coordinates analysis showing the β diversity of the ten most abundant classes in the three collected sites: upland (P1), intermediary (P2), and seashore (P3), where each dot represent an OTU colored according to class level of taxonomic classification, and **b** relative abundance of the ten most abundant bacterial classes in the three collected sites

(Fig. 4a). Gammaproteobacteria was the most abundant class in the three collected sites despite presenting significant differences in their frequency among the locations (Fig. 4a, b). While this group decreased their abundance in the intermediary and seashore areas, a visible increase in the abundance of Alphaproteobacteria occurred in P2 and Actinobacteria in P3 (Fig. 4b). Higher abundance of Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Cytophagia, Betaproteobacteria, Actinobacteria, and Clostridia was observed in intermediary site (P2). Alphaproteobacteria and Cytophagia were enriched in this site compared with the upland and seashore (Fig. 4b).

Between upland (P1) and intermediary (P2) sites, 247 OTUs were significantly enriched, but after the false discovery rate (FDR) correction, 137 OTUs remained as significant ($p < 0.05$) (Supplementary File Table 2). Between upland (P1) and seashore (P3), 140 OTUs were enriched but after FDR correction, only 12 were significant ($p < 0.05$) (Supplementary File Table 3). And comparing intermediary (P2) with the seashore site (P3) before the correction, 82 OTUs were significantly enriched and after, 11 OTUs remained (Supplementary File Table 4).

The DESeq2 results showed that when compared with upland (P1), which is the most distant local from sea water, intermediary site (P2) presented an enrichment of OTUs belonging to ten classes: Alphaproteobacteria, Betaproteobacteria, Bacteroidia, Saprospirae, Oscillatoriophycideae, Bacilli, Clostridia, Actinobacteria, Opitutae and Nitrospira, while upland presented enriched OTUs belonging to Gammaproteobacteria and Flavobacteria (Fig. 5).

When comparing the seashore site (P3) with the upland site (P1), it is possible to see an enrichment of classes belonging to Actinobacteria, Clostridia, and Bacilli in the seashore location. Only one OTU of the classes Alphaproteobacteria and Gammaproteobacteria was differentially enriched in the upland site (Fig. 6).

In the seashore, there was a significant differential enrichment of Actinobacteria and Clostridia compared with the intermediary site (P2). Only one OTU belonging to Alphaproteobacteria and three belonging to Gammaproteobacteria was differentially enriched in the intermediary site compared with the seashore (Fig. 7).

Discussion

The phylloplane of *L. racemosa* in the studied conditions was mainly composed of the bacterial classes Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Betaproteobacteria, Actinobacteria, Cytophagia, Clostridia, Bacilli, and Oscillatoriophycideae. These groups are characteristic of the mangrove microbiota and are important in the ecological maintenance of this biome [21, 27–31].

Proteobacteria is the dominant phylum on the leaf surface and in marine environments [28, 32, 33], followed by the phylum Bacteroidetes, Actinobacteria, Firmicutes, and Cyanobacteria [28, 31–34]. In mangroves, Proteobacteria and Firmicutes were associated with important biogeochemical transformations [27] while Alphaproteobacteria and

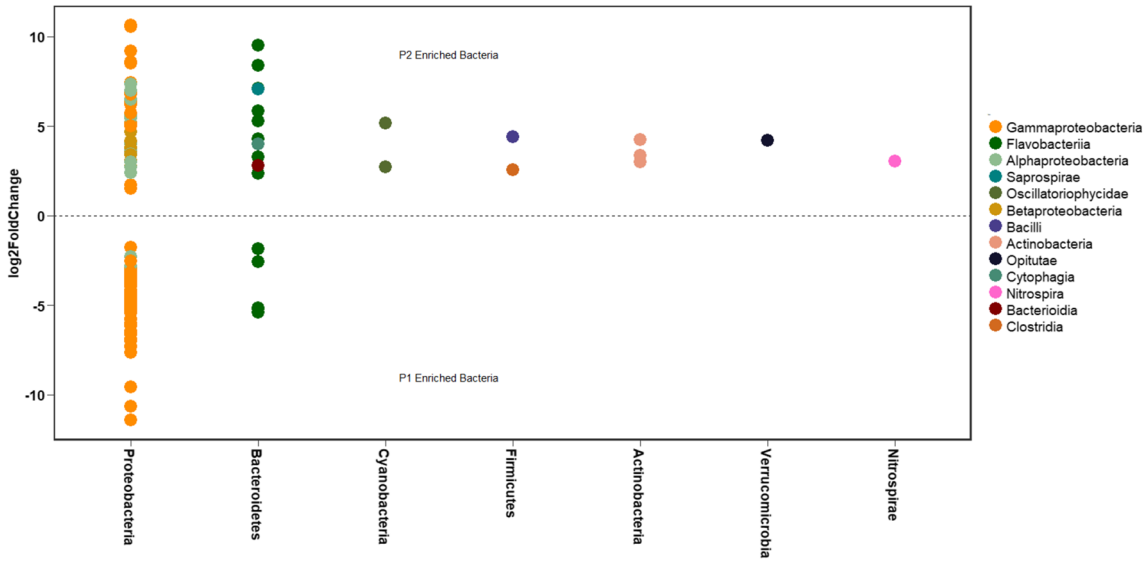

Fig. 5 Differential analysis comparing the bacterial classes enriched in the intermediary (P2) site vs the upland site (P1). Each dot represents an OTU colored according to class level in taxonomic classification and distributed according to phylum level in the *x*-axis. The larger the distance from the dotted line, the greatest is the enrichment. False discovery rate control (Benjamin-Hochberg), considering $p < 0.05$

Betaproteobacteria were linked with nitrogen transformations [27]. Cyanobacteria groups were already observed in mangrove phyllosphere with different abundances in a sampling transect [31].

Previous culture-independent approaches showed that Alphaproteobacteria and Gammaproteobacteria are generally the dominant groups of bacteria colonizing leaf surfaces, and Betaproteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria are also found in high proportions, although their abundance level varies depending on plant species and circumstances [35, 36]. Deltaproteobacteria that is one of the most abundant classes in mangrove sediments, because they act as important sulfate reducing agents [21, 37], was not observed in any of the three studied sites (Fig. 4b).

Gammaproteobacteria was the most abundant class of bacteria on the phylloplane of *L. racemosa* and also occurs in abundance in mangroves' sediment [27, 30, 38]. This group presents approximately 250 genera that contain species that are morphologically and metabolically well diversified and that can colonize since from the human gastrointestinal tract, as well as live in symbiosis with insects and also inhabit the phyllosphere of

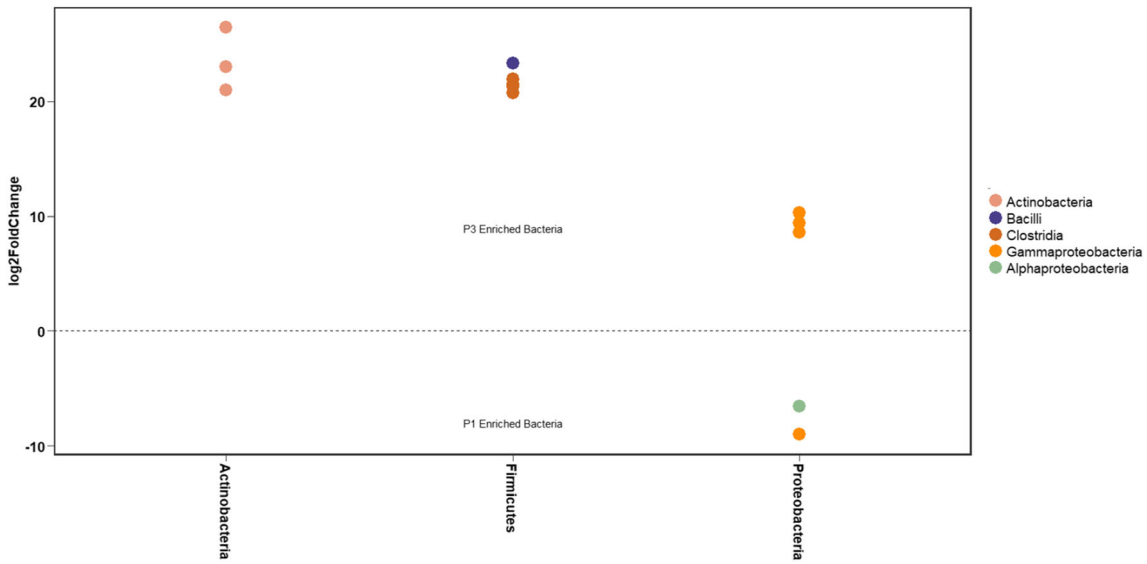

Fig. 6 Differential analysis comparing the bacterial classes enriched in the seashore site (P3) vs the upland site (P1). Each dot represents an OTU colored according to class level in taxonomic classification and distributed according to phylum level in the *x*-axis. The larger the distance from the dotted line, the greatest is the enrichment. False discovery rate control (Benjamin-Hochberg), considering $p < 0.05$

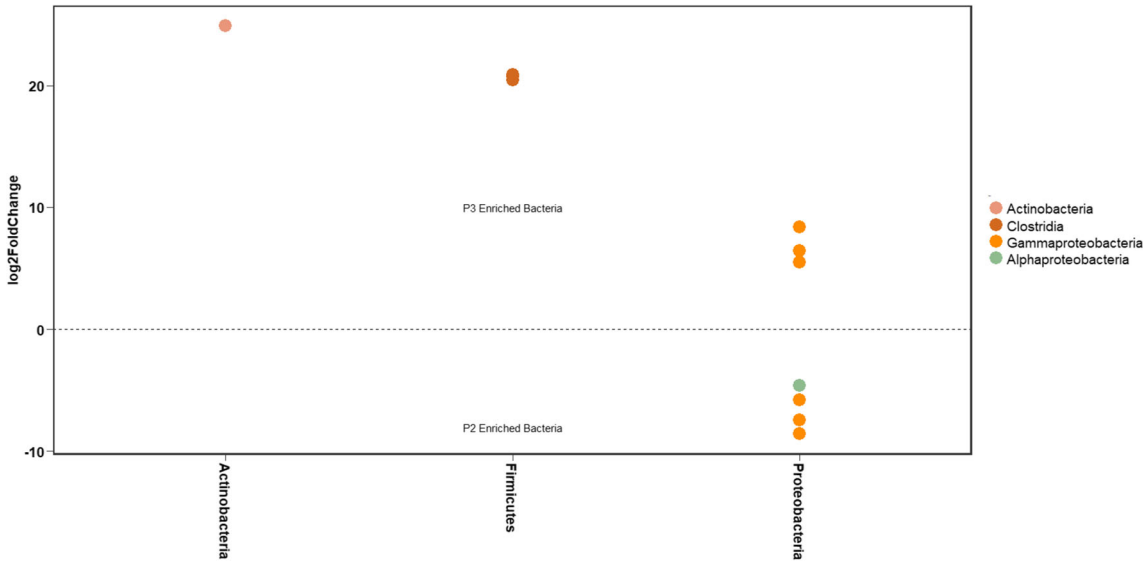

Fig. 7 Differential analysis comparing the bacterial classes enriched in the seashore (P3) site vs intermediary local (P2). Each dot represents an OTU colored according to class level in taxonomic classification and distributed according to phylum level in the *x*-axis. The larger the distance from the dotted line, the greatest is the enrichment. False discovery rate control (Benjamin-Hochberg), considering $p < 0.05$

different plant species and mangrove sediment [34, 39, 40].

Local communities are not stagnant and isolated; instead, they are dynamically interacting between then and with the environmental aspects in a wider scale, which defines the concept of a metacommunity [41]. Bacteria from the phylloplane can come from different sources such as water, soil, neighboring vegetation, and animals [42]. However, despite soil and air being the great sources of epiphytic microorganisms, studies showed that bacterial communities from phyllosphere present a well-defined core of microorganisms that differ from surrounding soil and air samples [34, 36, 43].

The diversity of the bacterial communities varied throughout the three collection sites (Fig. 2). The upland site, the most distant site from the sea water and closest of the continent, presented the lowest bacterial diversity. Differences in the diversity of microbial populations that make up the phyllosphere communities of different mangrove plants are described in the literature [2]. This type of variation in the composition of microbial communities between plants of the same species is not uncommon and is called intraspecific variation [42], despite intraspecific variation on epiphytic bacteria is far lower than the variability between samples from different tree species [44].

Intraspecific variation occurs even within different locations in the canopy of a single tree, and most of the factors that could explain that differences are not well understood. Epiphytic communities are exposed to different degrees of ultraviolet radiation, wind, and moisture, and therefore, community structure could change depending on the position of the leaves sampled [38].

A robust ecological study with the bacterial communities from ten different plant species from several locations across the globe showed that bacterial communities were organized in patterns predictable from the relatedness of the trees and that the interspecific variability exceeded intraspecific variability, a pattern observed even across continents with minimal geographic differentiation in the bacterial communities on *Pinus ponderosa* needles [38].

Mangroves present constant fluctuations in their environmental aspects along their geographic distribution [39, 40], and this dynamism has a strong influence in the composition and organization of local bacterial biodiversity [40], where different localities produce communities with different profiles. Therefore, the marine influence could be an important aspect in the structuration of the epiphytic communities [2, 28, 30, 45].

Estuaries have a vigorous circulation of salt and water, but a typical characteristic of this ambient is the horizontal salinity stratification, where generally the salinity decreases from the ocean toward the continent due to fresh water input, which make this ecosystem unique [46]. The variation in the bacterial communities on *L. racemosa* phylloplane collected from Cananéia mangrove, which present the influence of both fresh and salt water [47], could be due the differences in salt exudation by leaves (which was not measured here) along the collection transept. This plant species present salt excretion at foliar levels, and the rates of salt secretion enhance according to the increase in soil salinity [5].

This aspect of the plant metabolism could explain the differences in the abundance of bacterial classes as well as the unidentified groups. In mangroves, the salinity variation that occurs as a result of tidal oscillations is one of the main aspects

in shape biodiversity [48, 49]. Therefore, the possible increase of salinity on *L. racemosa* leaves in the transept could have a great impact in the community's organization, acting as a selective pressure and presenting a positive effect in the abundance of some of the major groups and in the species richness.

Although Gammaproteobacteria presents groups of organisms that are capable of supporting the salinity levels found in mangroves environments [49], this group was already correlated with lower salinity gradient [50], while Bacteroidia, Clostridia [51], and mainly Alphaproteobacteria [50, 51] were correlated with increased salinity levels. The salinity aspect also was positively correlated with Actinobacteria diversity in lakes [52]. Thus, in a condition of less salinity in the leaves surface, organisms like Gammaproteobacteria can have more success in the colonization of the environment, while with a relative little increase in the salinity level, as supposed to occur in the intermediate site and mainly in the seashore site, the growth of this group could diminish and enable the thrive of groups like Alphaproteobacteria, Actinobacteria, and even unassigned microorganisms.

Increase in prokaryotic communities along salinity gradients was observed in salinity pounds [53], in sediment surfaces [51], in estuary areas [50], and in saline lakes [52]. But other important aspects of the ecosystem could influence the pattern of the epiphytic communities like geographic distances [31]; site [54]; plant species [28, 44]; the accessibility of nutrients [9], soluble carbohydrates, calcium, and phenolic compounds [55], plant genotype [34]; and the environmental aspects like humidity and climate [10]. We began this study with the hypothesis that the intraspecies differences in phyllosphere communities from *Laguncularia racemosa* may be related to the environmental gradient characteristic from mangrove environments, where we have a vegetation that in one side suffers influence from restinga forest and in other extreme is subject to the marine influence like tidal oscillations and aerosols from sea waves [56]. The results presented in this work showed that significant intraspecific variation in bacterial communities exists between a single host species and can contribute with the knowledge about the dynamics driving intraindividual variability in epiphytic community's structure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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