University of São Paulo
"Luiz de Queiroz" College of Agriculture

Diversity and interactions of bacteria from *Laguncularia racemosa* phylloplane

Marta Alves Moitinho

Thesis presented to obtain the degree of Doctor in Science. Area: Agricultural Microbiology

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DEDICATION

To my beloved mom, who are pure kindness and give me inconditional love.

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I would like to thank the Universe for all the opportunities and blessings I have had in life.

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EPIGRAPH

“Times are hard for dreamers.”.

Amelie Poulain.

CONTENTS

RESUMO

Diversidade e interações de bactérias do filoplano de *Laguncularia racemosa*

Os manguezais são ecossistemas dinâmicos, que prestam importantes serviços ecológicos às áreas costeiras devido às altas taxas de produção primária e por abrigarem vários organismos marinhos. Eles são compostos por vegetação tolerante à salinidade que prosperam nas regiões tropicais e subtropicais do mundo sob influência das marés. As plantas influenciam amplamente o ambiente por meio da fotossíntese e suas folhas são responsáveis por grande parte da energia e da matéria orgânica inserida no planeta Terra. Plantas saudáveis, na natureza, vivem em associação e interagem ativamente com uma infinidade de microorganismos pertencentes a vários tipos microbianos, coletivamente denominados como microbiota. Os ecossistemas tropicais abrigam uma grande diversidade de bactérias epifíticas com potencial para abrigarem novas espécies, mas a maioria dos microrganismos epifíticos não é cultivada sob condições comuns de laboratório quando comparada a outros ambientes, e pouco se sabe sobre a diversidade bacteriana epifítica em habitats de manguezais. A estrutura da comunidade bacteriana do filoplano de *Laguncularia racemosa*, uma espécie de mangue bem adaptada e com exsudação de sal em níveis foliares, foi acessada por meio do sequenciamento do amplicon 16S rRNA. A amostragem foi realizada em três locais diferentes, através de um transecto do continente até a beira-mar, em uma floresta de mangue preservada, localizada na cidade de Cananéia, Estado de São Paulo, Brasil. Observou-se maior diversidade bacteriana em locais intermediários entre o continente e a beira-mar, mostrando que existe uma variação espacial intraespecífica significativa nas comunidades bacterianas entre uma única espécie de planta, com a seleção de população específica entre um transecto ambiental. As bactérias residem em comunidades interativas complexas em estreita associação com possíveis espécies competidoras e parceiras. As comunidades microbianas são dinâmicas e sua estruturação é determinada pelas interações em pares que ocorrem entre diferentes espécies. Portanto, as células microbianas exibem comunicação intercelular e estão cientes de outras células próximas, produzindo respostas coordenadas. Neste trabalho, também avaliou-se as interações multiespécies entre dez linhagens isoladas do filoplano de *Laguncularia racemosa*. Todas as linhagens tiveram seu diâmetro de crescimento medido ao crescer em monoculturas, comparado com o seu próprio crescimento nas interações em pares e no consórcio de três bactérias. No total, quinze consórcios mostraram diferenças significativas no diâmetro de crescimento das bactérias em pelo menos uma combinação. No entanto, vinte combinações de consórcios não mostraram diferenças significativas no crescimento de bactérias durante as interações. Em conclusão, este trabalho mostrou que as bactérias do filoplano de *L. racemosa* podem detectar outras cepas próximas e alterar suas taxas de crescimento em resposta às co-culturas.

Keywords: Manguezais. Diversidade de bactérias. Interações bacterianas. Filosfera

ABSTRACT

Diversity and interactions of bacteria from *Laguncularia racemosa* phylloplane

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 . They are composed by a salinity tolerant vegetation that thrives in tropical and subtropical regions of the world under tidal influence. Plants broadly influence the ambient by means of photosynthesis and their leaves are responsible for a great part of the energy and organic matter input into planet Earth. Healthy plants in nature live in association and actively interact with a multitude of microorganisms belonging to several microbial types, collectively called the plant microbiota. Tropical ecosystems harbor a great epiphytic bacteria diversity with the potential to house new bacteria species, but most of the epiphytic microorganisms are uncultivated under commonly laboratory conditions when compared to other environments and little is known about the epiphytic bacterial diversity on mangrove habitats. 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 bacteria diversity was observed in intermediary locations between the upland to the seashore, showing that exists significant intraspecific spatial variation in bacteria communities between a single host species with the selection of specific population between an environmental transect. Bacteria reside in complex interactive communities in close association with competitors and partners. Microbial communities are dynamic and their structuration are determined by the pairwise interactions that occur between different species. Therefore, microbial cells exhibit intercellular communication and are aware of other cells in their vicinity, producing coordinated responses. In this work we also evaluated the multispecies interactions among ten strains isolated from *Laguncularia racemosa* phylloplane. All the strains had their growth diameter measured when growing in monocultures compared against their own growth in the pairwise interactions and in the consortium of three bacteria. In the total, fifteen consortia showed significant differences in the growth diameter of the bacteria in at least one combination. However, twenty-one consortia combinations did not show significant differences in the bacteria growth while interacting. In conclusion this work has showed that bacteria from *L. racemosa* phylloplane can sense other strains nearby and alter their rates of growth in response to the co-cultures.

Keywords: Mangroves; Bacteria diversity; Bacteria interactions; Phyllosphere

1. Introduction

Mangroves are ecosystems composed of vegetation that thrives in intertidal zones in tropical and subtropical regions of the planet. It is considered a productive biome that interacts with adjacent coastal areas as they serve as shelter for different marine species and participate in the renewal of coastal biomass. Despite their ecological, social and economic importance, mangroves have been declined and fragmented. Deforestation is a major reason for mangrove areas reduction in many tropical and subtropical countries.

The plant species *Laguncularia racemosa*, known as white mangrove, is an important component of the mangrove forests of the American continent and in West Africa. Its leaves are simple, have abundant deposits of epicuticular wax on the epidermis, as well as salt glands that are distributed on both sides of the petioles. The phyllosphere comprises the aerial part of the plants, while the phylloplane is the surface of the leaves and the organisms that inhabit this habitat are called epiphytes. The microbial community of these environments is mainly composed of bacteria, archaea, filamentous fungi and yeasts.

The microbial ecology world was rocked by fast advances in technology and computational power, mainly guided by the progress in next generation sequencing. Driven by the fuel of curiosity, scientists have been studying the microbial communities, the called microbiome, from different parts of the world like forests, deserts, air, seas, extreme environments and from different hosts like plants, human beings, non-rational animals and even from the International Space Station. The advantage of the methodologies independent of cultivation is that it does not require any prior knowledge about the communities and enable the discovery of new groups. The knowledge about microbial diversity is important to know the main members of the microbial communities in different habitats.

A fundamental question in ecology is how different species manage to coexist in nature. The ability to perceive neighboring cells and respond to stimuli from the environment reflects information contained in the bacterial genome. This ability is especially important when these organisms are found in natural habitats, which requires from these species a vast number of genes that act as a signaling system and help to interpret changes in environmental conditions and the presence of possible competing species. Populations form networks of relationships that include competition for nutrients, biofilm formation, amensalism and quorum sensing. Microorganisms live within complex and interactive communities and chemical signaling allows bacteria to transmit information and coordinate behaviors. These relationships between different species are mediated by molecules that diffuse into the

common environment and are important in interactions with some spatial distance between the microorganisms.

Therefore, the goals of this work were to study the bacteria diversity on the *Laguncularia racemosa* phylloplane by means of the 16S rRNA amplicon sequencing at three different sites in the Cananéia mangrove, and to build a synthetic community to study the multispecies interactions between ten different epiphytic bacteria strains isolated from *L. racemosa* phylloplane.

2. THE UNEXPLORED BACTERIAL LIFESTYLE ON LEAVES SURFACE

Abstract

Social interactions impact microbial communities and these relationships are mediated by small molecules. The chemical ecology of bacteria on the phylloplane environment is still little explored. The harsh environmental conditions found on leaves surface requires high metabolic performances of the bacteria in order to survive. That is interesting for both scientific fields of prospecting natural molecules and for the ecological studies. Important queries about the bacterial lifestyle on leaves surface remains not fully comprehended. Does the hostility of the environment increase the populations' cellular altruism by the production of molecules, which can benefit the whole community? Or does the reverse occur and the production of molecules related to competition between species is increased? Does the phylogenetic distance between the bacterial populations influence the chemical profile during social interactions? Are phylogenetically related bacteria tending to cooperate more than the distant ones? The phylloplane contains high levels of yet uncultivated microorganisms, and understanding the molecular basis of the social networks on this habitat is crucial to gain new insights into the ecology of the mysterious community members due to interspecies molecular dependences. Here, we review and discuss what is known about the bacteria social interactions and their chemical lifestyle on leaves surface.
Keywords: epiphytic bacteria, secondary metabolites, interactions

2.1Introduction

In the social context the ecological relationships are shaped by the behaviour of an organism in response to an interaction with another organism, which is strongly influenced by the environmental conditions in what they are found (1). Microorganisms in a community are linked in a social network that can vary regarding strength and type, and this dynamic affect the ecology and evolution of species (1). So, a fundamental question in the ecological studies is how different organisms live together in nature (2). Over the years microbial interactions were surveyed by different approaches like by the direct interactions between two cultivable microorganisms (3), or between mixed populations in a microbial consortia 4,5, and also by using computational models 6 and game theories (7).

The ecological social studies argue when should organisms cooperate or when should they be selfish when interacting with other organisms (8). Why should an individual cell carry out a costly cooperative behavior for the benefit of all the community? (8,9). This answer is more complex than the simple perspective that cooperation can increase the populations' fitness, mainly because individuals die and reproduce way faster then populations 8. Because cooperation among individuals affects natural selection, understanding the evolutionary origins and maintenace of cooperation is a primary theme in biological research (10).

In the last years the bacterial community members of the extreme phylloplane habitat were broadly studied by using next generation sequencing (11–13), but considerable part of the microorganisms that thrives on phylloplane still remains uncultivated in commonly used media and culture conditions compared with other natural environments (13–16). That is especially true when considered the number of plant species in the world that was estimated to be 374,000 (17) and only a portion of these plants had their epiphytic bacteria community studied (18). The phylloplane of tropical forest trees remains largely unknown despite the rainforests being regarded as the climax of biodiversity (13,19). Only Brazilian Atlantic forest can harbour between 2 and 13 million undescribed epiphytic bacterial species (13,16). Thus, identity, social interactions, as well the metabolic potential of epiphytic organisms are not fully understood 20, and this dynamic environment can reveal enormous genetic and metabolic microbial diversity (13,21).

Few studies paid attention to the chemical potential of the epiphytic bacteria (16,18,21–24) and to their social interactions (21,25,26); and most of those investigations focused in interactions with the intent to control plant diseases (27,28), or frost injury (29) or within bacteria-host interactions (30,31). Much less understood are the non-pathogenic microorganisms that inhabit the phylloplane and their chemical potential (16,20,21). Even reviews about interactions among microorganisms paid little or no attention to the chemical potential of the epiphytic bacterial populations (32).

The power of small molecules in the microbial world is great (33) and the most important challenge for the ecological studies on the phylloplane habitat is to understand the metabolic networks between epiphytic individuals and the type of interactions that structure the communities. Here, we review and discuss the recent studies about the chemical ecology of the epiphytic bacteria, which may help to unveil the chemical lifestyle on leaves surface.

2.2 Bacterial assembly on the phylloplane habitat

Healthy plants in nature live in association with a multitude of microorganisms of several microbial types, such as bacteria, archaea, fungi, and oomycetes, collectively called the plant microbiota (34). The phyllosphere comprises the areal part of plants while the phylloplane is the surface of the leaves and the microorganisms that thrives on this environment are called epiphytes (11).

Many microorganisms can be associated with the phylloplane as transients and residents, but the environmental conditions select few groups that persist as true epiphytic populations 35. Bacteria are the dominant microorganisms on the phylloplane (11,12,36) and,

until now, the most identified bacterial groups are from the phyla Proteobacteria, Bacteroidetes and Actinobacteria; and among the classes, Alphaproteobacteria and Gammaproteobacteria are the dominants (11,12,37).

To define a source for bacterial assemblages on the phylloplane is difficult because microbiota members can originate from rainwater, plant dispersal vectors (38), aerosols, animals (39), soil and even by upward migration from the roots (34). The most colonized spaces on the leaves by bacteria are the grooves, trichomes, vein cells (25) and regions nearby the stomata (40). Site (26), plant species (40), soluble carbohydrates, calcium, phenolic compounds (41) and the plant genotype (42) are also important determinants of the bacteria community composition on the phylloplane.

Knowledge about the mechanisms and compounds involved in interactions between microorganisms from the plant microbiome is essential for practical use in biological control programs and in the biotechnological aspects for natural molecules prospection (15). Previous studies showed that many epiphytic bacteria establish benign commensal associations with contributions to the health of the ecosystem and the host plant (28,43).

Epiphytic bacteria present potential to be used as bio-inoculants for sustainable cultivation and biological control (44,45), they are metabolically capable of degrading phenol and then could potentially contribute to the natural attenuation of organic air pollutants (46), they are capable of fixing atmospheric nitrogen, thus providing significant nitrogen input into ecosystems (47) and microbial interactions on the phylloplane can increase plant performance under herbivore biotic stresses (28).

2.3 The phylloplane as a harsh habitat for microbial life

Extreme conditions are in the eye of the beholder and harsh environments are those that make a metabolism difficult to function (48); and as life is governed by organic chemistry, such chemistry must be allowed to operate (48). The low and heterogenic levels of nutrient and moisture combined with the incidence of high levels of ultraviolet radiation and the oxygen exposure make the atmosphere a severe environmental aspect for microbial life (49) and that cause enormous stresses to microorganism’s survival (50). Besides the aerobic metabolism is far more efficient than the anaerobic (33), the exploitation of oxygen metabolism has its costs and thus, all aerobic organisms can be considered extremophiles (48).

Leaves are the dominant aerial plant structure, with an estimated global area greater than the land surface (11,12) and because they have a relatively brief lifespan (12), the phylloplane ecosystem is highly dynamic and the microorganisms that colonize this habitat are exposed to cyclic and noncyclic environmental variables as atmosphere exposure, atmospheric pollutants (51); wind and rain (14), low or fluctuating water availability, desiccation (52); ice (29), a scarce and heterogenic nutrient condition (37,53), the presence of antimicrobial secondary metabolites of plant (30,54) and in dynamic coastal ecosystems like mangroves they are also exposed to salinity (55).

Leaves are the main photosynthetic organs of plants and therefore their conformation and positioning allows an optimal capture of solar energy (56,57); and the ultraviolet radiation on phylloplane can reach temperatures of 40–55 °C under intense sunlight (58). This direct exposition to ultraviolet radiations influence the diversity of epiphytic communities with increase in the UV tolerant groups or a decrease in the non-tolerant ones (51,57,59). Pigmentation and the DNA repair are the two most well-known mechanisms for UV resistance (59).

The nutrients available on phylloplane are composed by sugars (37,60), amino acids, organic acids, alcohols (54), mineral trace elements, vitamins, hormones (14), Chloromethane gas (61) as well antimicrobial compounds (12,14,41). These molecules can be originated from the plant itself (54) and also from soil particles, dust, solutes in rainwater, dead microorganisms, bird and insect excrement and pollen (39). But the phylloplane cannot be described as a nutrient rich environment because all these compounds can be easily removed from leaves either by the leaching and other environmental actions as fog and dew (14). Epiphytic bacteria are mainly found in aggregates (12) and they are capable of growing on low nutrient concentrations but they preferentially grow on high nutrient conditions (52).

Because of all these dynamic and harsh conditions on the phylloplane habitat (Figure 1), epiphytic bacteria present mechanisms to mitigate the environmental adversities like by means of the syntheses of proteins to deal with environmental stresses (20,52,62); by the production of biosurfactants that benefits the bacteria by both attracting moisture and facilitating access to nutrients (63); and by the production of pigments that confer UVR tolerance and give the bacteria the ability to maintain their population sizes (57,64).

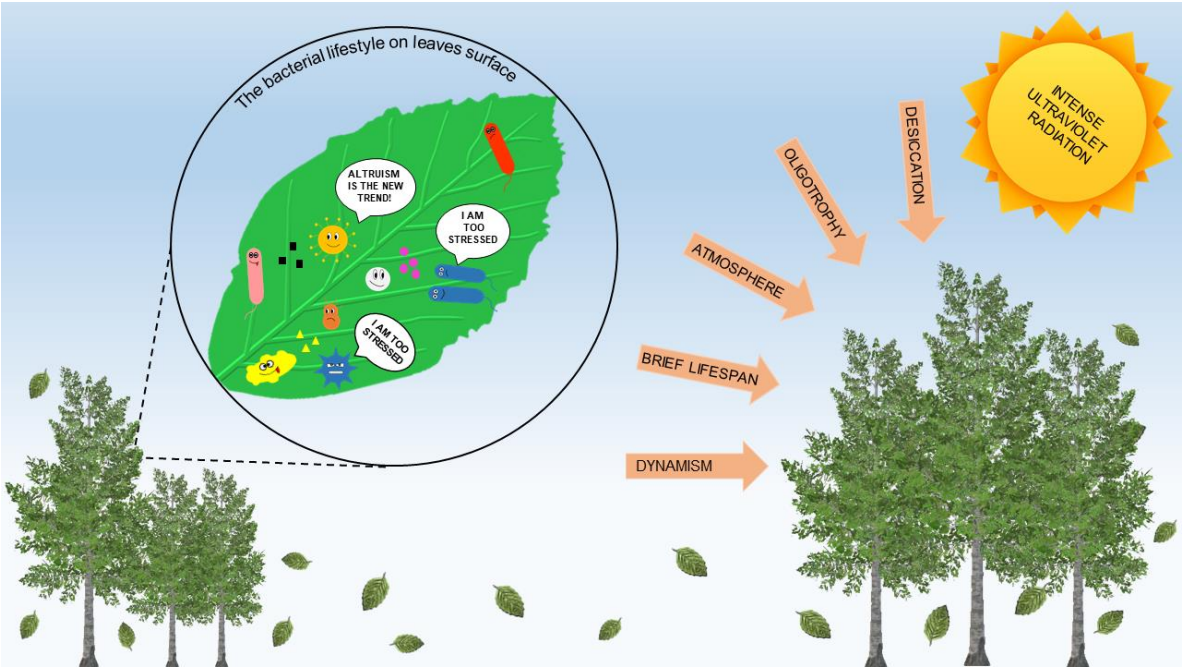

Figure 1: The dynamic and harsh phylloplane environment

2.4 The unexplored face of the chemical interactions among epiphytic populations of bacteria

The capacity to perceive neighbouring cells and answer to environmental stimuli is contained in the bacteria genome (6,65). This ability is important when microorganisms are found in their natural habitats, which requires a huge number of genes that act as signalization systems and help them to interpret the environmental conditions and the presence of competing species (65). The soluble and volatile secondary metabolites are the mainly mediators of antagonistic and synergists relationships between microorganisms (5,66,67). The molecule produced by one species can serve of nutrient or cause damage to others (33). But not every molecule that affect the behaviour can be considered a true signal from the social aspect; for that, they must have consequences in the fitness of the sender and of the receiver (10).

Besides their importance in microbial interactions, biochemically diverse compounds have a wealth of different bioactivities, many of which have been exploited as drugs in human and veterinary medicine (21). But the production of siderophores (68), quorum sensing related molecules (69), quorum quenching enzymes (70), peptides (21), exopolysaccharides production (71) biofilms' formation (72) and antibiotics production (73) are some of the various ways in which bacteria can interact by means of the secondary metabolism. Antibiotics are the most known examples, and they form only a part, perhaps a smaller part,

of the possible bioactive metabolites of microorganisms; they represent only the top of the iceberg (67).

The social interactions in the bacterial communities can alter the production of secondary metabolites (5). The environmental aspect is the main trigger of cooperation's and competitions among species (32) and most of the secondary metabolites are silent under laboratory conditions (74). It is the phylloplane environment that determines the morphological and primary metabolic properties of the epiphytic communities (21,75) and these microorganisms have various lifestyles and modes of interactions (76). In the harsh conditions of the phylloplane the movement of bacteria are restricted and they only perceive signals such as sugar, amino acids and volatiles that diffuses in the surrounding environment (77).

Competition for space and nutrient resources, production of antibiotics and interference with cell-signalling systems in microbial communities are the main mechanisms by which epiphytic bacteria interact (21,23,78). A study that evaluated competitiveness of diverse Methylobacterium strains on the phylloplane of Arabidopsis thaliana showed that epiphytic bacteria are actively interacting during growth in mixed cultures and that they have distinct metabolites strategies to explore the nutrients in the milieu, which enable then to compete successfully with each other and coexist (52). From a biological perspective, this harsh environment of the phylloplane as a poor nutrient condition might have selected for highly competitive species engaged in chemical warfare (21); but it also could be a great place to favour cooperative strategies and altruistic behaviours.

Epiphytic communities are important for the metabolic function of plants (16,75,76) and some epiphytic isolates have the expression of gallate decarboxylase that present antifungal activity (75). Epiphytic bacteria are capable of detoxify secondary metabolites of plant origins and the resulting molecules can present allelochemical roles against another phylloplane competing species (22). And besides presenting antifungal activity, epiphytic bacteria also present proteolytic activity and siderophores production (3). The complex phylloplane environment requires unique adaptations for microbial survival, and that impact their interactions with each other and also with their hosts (79). The production of proteins related with methanol utilization and stress responses were most prominent on the phylloplane then in normally medium culture conditions (62).

A study that searched antimicrobial activity among epiphytic bacteria from four different plant species, showed that 26% of the strains had good antimicrobial activities against one or more tested pathogen (18). In a bioprospecting study with isolates from

phylloplane and rhizosphere, the greater number of antagonistic bacteria against the phytopathogen Rhizoctonia solani was found on the phylloplane (50). But in general, in this same experiment, epiphytic bacteria produced less antimicrobial compounds than organisms from rhizosphere and the authors concluded that this could be due to the enormous stresses that they suffer in these harsh environmental conditions. In fact, a study with 224 strains of epiphytic bacteria from Arabidopsis leaf microbiome showed that among over 50,000 combinations of pairings interactions only 1,4% were inhibitory (21). Which may suggest that the phylloplane habitat may induce more cooperation from epiphytic populations to survive than antagonism.

The discovery of the bacteria communication by means of diffusible signal molecules known as quorum sensing (69,80,81) revolutionized the way scientists see bacterial populations (for a review of this theme see (82)). Although not being required fot all cooperative interactions, communication among neighbouring individuals is considered a fundamental mechanism to coordinate cooperative strategies (10). Epiphytic bacterial populations live in aggregates on leaves surface (11,16) and then the phenomenon of quorum sensing which affects the multicellular behaviour in a community gains importance (83). In a study with bacteria isolated from the phylloplane of wheat heads, about 33% of the strains showed the production of quorum sensing related molecules (84). These quorum sensing molecules may affect polysaccharide production, and both polysaccharides and quorum sensing molecules may be involved in the survival and growth of bacteria on leaves surface (76).

The process that disturbe quorum sensing are named quorum quenching, which often involves enzymes (33,70). This is a natural mechanims by which quorum sensing producers recicle or clear their own signals or as a competitive action of quorum quenching organisms against quorum sensing producers (70). A study with epiphytic bacteria from tobacco leaves showed that 14% of the isolated species presented production of quorum quenching molecules, with higher values on the phylloplane than in soil and rhizosphere (78). And this values can be even higher when considering the large amount of yet uncultivated bacteria existing on leaves surface (13,16,78). The authors concluded that quorum quenching could be a strategy for bacteria to survive on the phylloplane, where they can acquire the nutrients via signal interference degrading quorum sensing molecules as an energy source.

In a metaproteomic study of the phylloplane of four plant species from Atlantic forest in Brazil, a total of 4413 peptide mass spectra did not have significant matches in the

chemical databases, and those molecules may represent proteins from yet unknown microorganisms (16). The most abundant proteins found in this study were from the glycolytic pathway, anaerobic carbohydrate metabolism, solute transport, protein metabolism, cell motility, stress and antioxidant responses, nitrogen metabolism, and iron homeostasis. In this work, the authors concluded that the protein profiles of microorganisms from the phylloplane may depend on the plant taxon and of the environmental conditions; and that epiphytic bacteria sampled from phylogenetically divergent hosts with similar functional niches have resembling core proteins necessary for survival, growth and maintenance of biofilms on leaves surface (16).

A robust and recent study of binary interactions with more than 200 bacteria isolated from the phylloplane of Arabidopsis thaliana showed that 196 strains (88%) engaged in inhibitory interactions; and that epiphytic bacteria tend to inhibit distinct phylogenetic groups rather than closely related strains (21). The most frequently molecules ribosomally synthesized and post-translationally modified peptides (RiPPs) produced by this synthetic community are of the families bacteriocins, lanthipeptides, lassopeptides, microviridins, linaridins, thiopeptides, thiopeptide-linaridin hybrids and lantipeptide-proteusin hybrids. The results of the chemical ecology from this study indicated a broad structural diversity of ribosomally encoded peptides from epiphytic bacteria (21).

2.5 Conclusions and perspectives

The molecular strategies to survive in harsh environmental conditions are not fully comprehended, but it is known that microorganisms from severe habitats have developed interesting biomolecules and biochemical pathways for biotechnological purposes (85). The studies of bacterial populations from little explored habitats represent a promising path to identify new natural products and also to understand their roles in the social aspect (21).

The scientific works mentioned above showed how metabolically rich are the bacterial populations from phylloplane, and that this habitat represents a promising and unique source for the isolation and discovery of bacterial natural products with a large and distinct biosynthetic repertoire, with unprecedented scaffolds (21). Thus, the investigations of the chemical ecology on phylloplane environment have the potential to contribute with researches in both fields of social ecology and in the bioprospecting of compounds (21).

Although the molecular approach have increased the knowledge about the diversity of the microorganisms that thrives on phylloplane in the last years (12), little is known about the chemical ecology of the epiphytic bacterial communities and much more phylogenetic and

metabolic diversity still needs to be discovered (13,16). Important aspects of microbial communities' ecology and structure cannot be inferred by genomic techniques alone. It is of great importance to have a holistic view of how microbial populations interact directly or indirectly, instead of considering the study of isolated groups (86).

Recent advances in metabolomics technologies like imaging mass spectrometry, secondary ion mass spectrometry, stable isotope probing, Nanospray desorption electrospray ionization (NanoDESI), Global Natural Products Social (GNPS) molecular networking project and many other chemometric approaches have been helping scientists to visualize the chemical world of microorganisms even directly from environmental samples (87–91).

Therefore, scientists around the world should look to the phylloplane environment as a great model for the exploration of the social interactions and the chemical ecology among epiphytic bacterial populations to gain insights into the social behaviours of the already cultured organisms and also possibly to improve the knowledge into the ecology of the mysterious community members of this habitat that we still do not know.

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3. INTRASPECIFIC VARIATION ON EPIPHYTIC BACTERIA COMMUNITY FROM *LAGUNCULARIA RACEMOSA* PHYLLOPLANE

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 bacteria diversity was observed in intermediary locations between the upland to the seashore, showing that exists significant intraspecific spatial variation in bacteria communities between a single host species with the selection of specific population between an environmental transect

Keywords: Epiphytic bacteria; Community structure; Intraspecific variation; Mangroves

3.1 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 by 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 *Aviccennia 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 microbial communities in this ambient are mainly composed by 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 to 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 mangroves 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 bacteria diversity with the potential to house new bacteria species [16], but most of the epiphytic microorganisms are uncultivated under commonly laboratory conditions when compared to 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.

3.2 Material and Methods

3.2.1 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") (Figure 1). The distance between the sites are of the 131 meters between P1 and P2; 145 meters between P2 and P3 and 281 meters between P1 and P3.

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

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

3.2.2 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 hours. 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 minutes. The pellets were recovered and suspended in 100 µl of autoclaved MilliQ water and 0.25 g of this solution were 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) DNA extraction 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 primers set 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 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 26239.78 reads per library (min. 11388 and max. 41974).

3.2.3 Nucleotide sequences accession numbers.

Bacterial 16S rRNA gene sequences obtained in this study are publicly available in the Sequence Read Archive (SRA) server (https://www.ncbi.nlm.nih.gov/sra) under the accession number SRP156580.

3.2.4 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 analyze) 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].

3.3 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 bacteria 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 significant more diverse than the upland

point (P1) ($p<0.05$). Site 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) (Figure 2). The PERMANOVA analysis (Table 1) showed that the sites had a marginal effect of in the structure of the epiphytic bacterial community ($p=0.1$).

Table 1: Analyse of variance of the epiphytic community of the three different sites: Upland, intermediary and seashore

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
meta$Local	2	0.8254	0.41269	1.7472	0.18488	0.086 **.**
meta$Treatment	3	0.8046	0.26821	1.1353	0.18024	0.306
Residuals	12	28.344	0.23620		0.63488	
Total	17	44.644			1.00000	

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

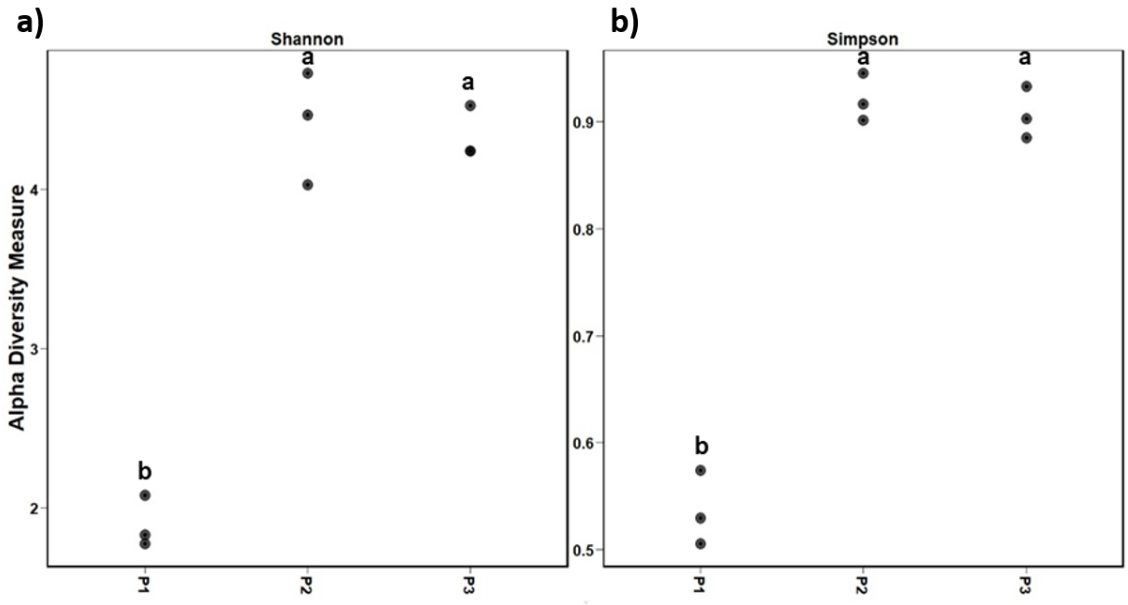

Figure 2: The α diversity indexes a) Shannon and b) Simpson in the three sites: upland (P1), intermediary (P2) and seashore (P3). Each dot represents α diversity index from a sample. Different letters correspond to significant differences in Tukey Test ($p<0.05$).

The microbial community structure was differently assembled when comparing the phylloplane of *L. racemosa* in the three sampling sites (Figure 3). The first axis explained 58.3% of the data while the second axis explained 25.7% (Figure 3). The results showed the total separation of the samples based on the location, what 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.

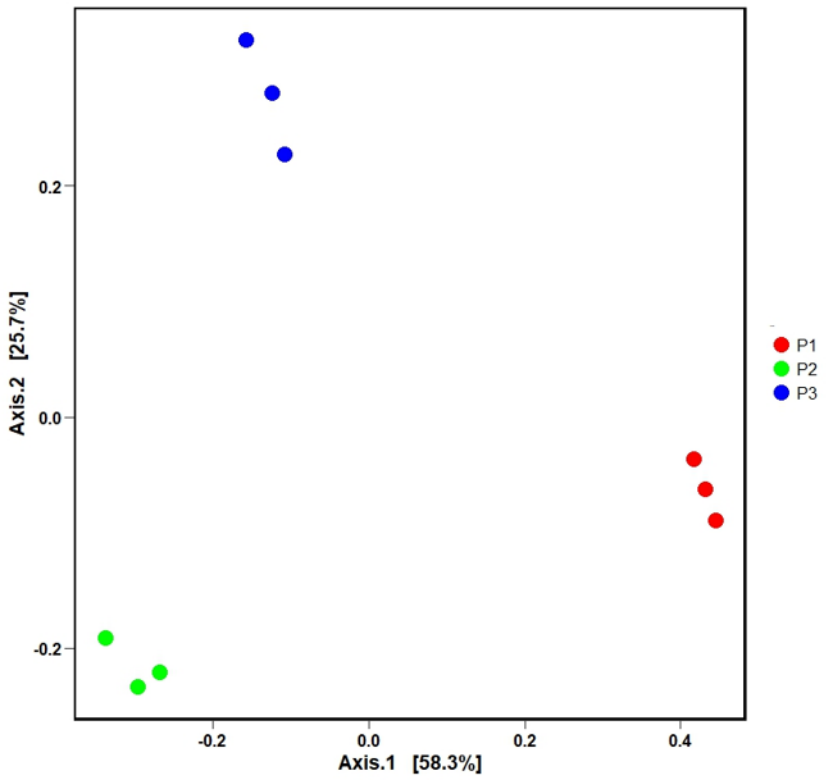

Figure 3: Principal Coordinated Analysis showing the β diversity in the three collected sites: upland (P1), intermediary (P2) and seashore (P3).

The nine most abundant bacteria classes in the phylloplane of *L. racemosa* were Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Betaproteobacteria, Actinobacteria, Cytophagia, Clostridia, Bacilli and Oscillatoriophycideae respectively (Figure 4a). Gammaproteobacteria was the most abundant class in the three collected sites despite presenting significant differences in their frequency among the locations (Figures 4a and 4b). 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 (Figure 4b). Higher abundance of Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Cytophagia, Betaproteobacteria, Actinobacteria and Clostridia were observed in intermediary site (P2). Alphaproteobacteria and Cytophagia were enriched in this site compared with the upland and seashore (Figure 4b).

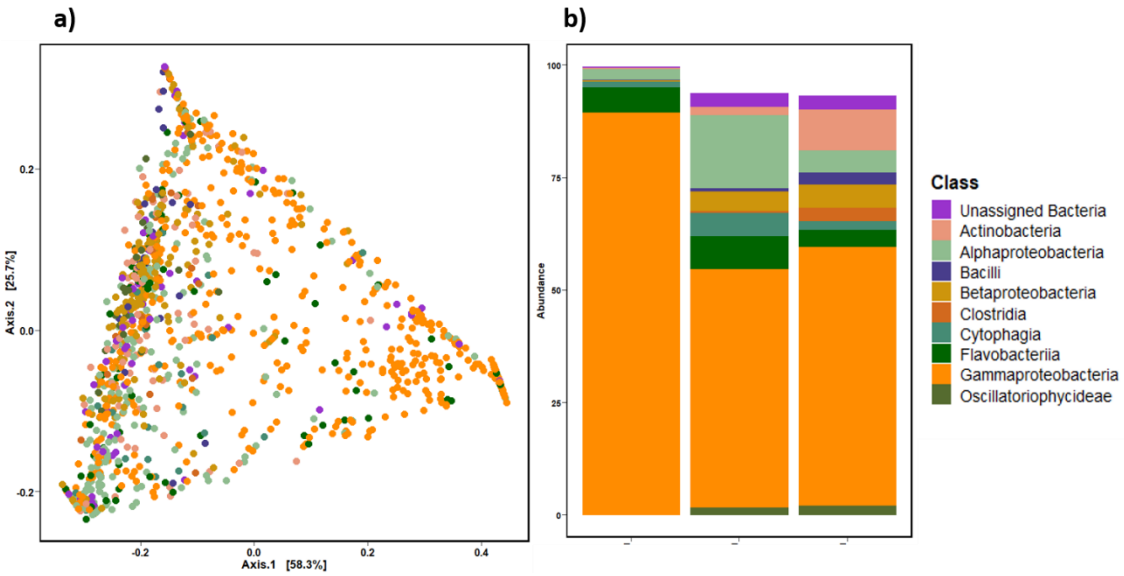

Figure 4: *Laguncularia racemosa* phylloplane microbial community assembly in different locations in Cananéia Mangrove showed by a) Principal Coordinated analysis showing the β diversity of the ten most abundant classes in the three collected sites: upland (P1), intermediary (P2) and seashore (P3); where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

Between Upland (P1) and Intermediary (P2) sites, 247 OTUs were significantly enriched, but after the False Discovery Rate (FDR) correction137 OTUs remained as significant ($p<0.05$). Between Upland (P1) and Seashore (P3) 140 OTUs were enriched but after FDR correction only 12 were significant ($p<0.05$). And comparing Intermediary (P2) with the Seashore site (P3) before the correction 82 OTUs were significantly enriched and after 11 OTUs remained.

The DESeq2 results showed that when compared to 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 (Figure 5).

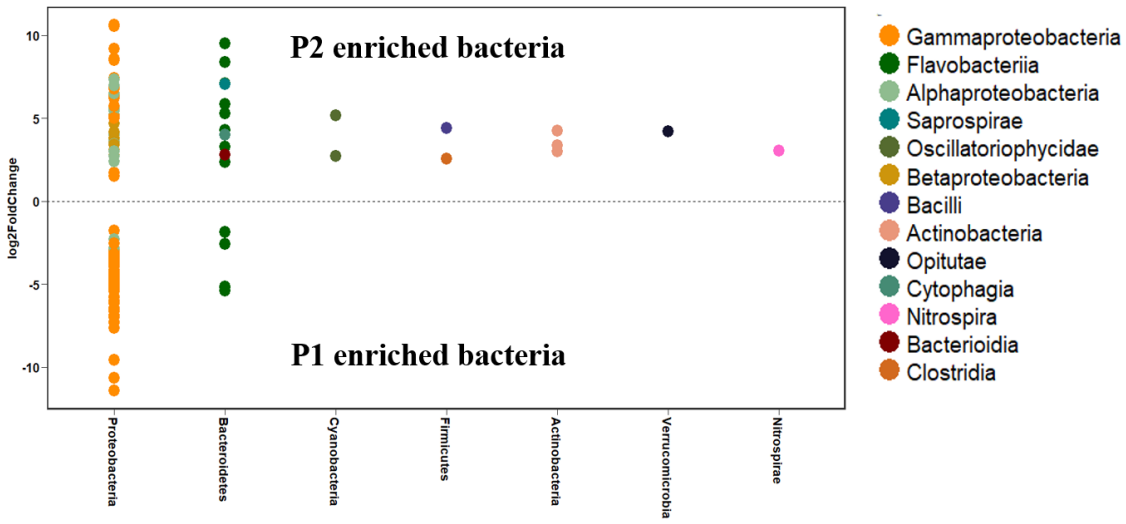

Figure 5: Differential analysis comparing the bacterial classes enriched in the intermediary (P2) site vs the upland site (P1). Each dot represents an OTU colored according to Class level in taxonomic classification and distributed according to Phylum level in the x axis. The larger the distance from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

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 were differentially enriched in the upland site (Figure 6).

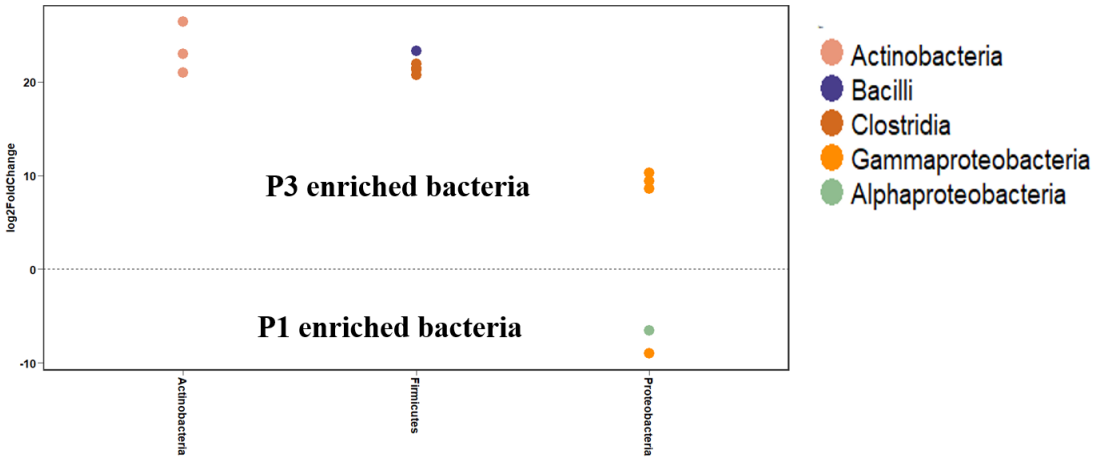

Figure 6: Differential analysis comparing the bacteria classes enriched in the seashore site (P3) vs the upland site (P1). Each dot represents an OTU colored according to Class level in taxonomic classification and distributed according to Phylum level in the x axis. The larger the distance from the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

In the seashore, there was a significant differential enrichment of Actinobacteria and Clostridia compared to intermediary site (P2). Only one OTU belonging to

Alphaproteobacteria and three belonging to Gammaproteobacteria was differentially enriched in the intermediary site compared to the seashore (Figure 7).

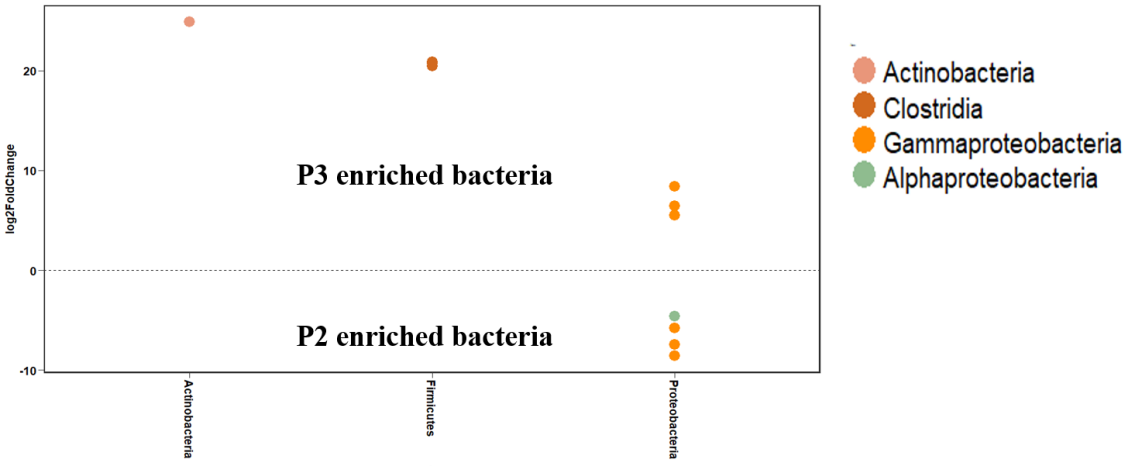

Figure 7: Differential analysis comparing the bacteria classes enriched in the seashore (P3) site vs intermediary local (P2). Each dot represents an OTU colored according to Class level in taxonomic classification and distributed according to Phylum level in the x axis. The larger the distance from the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

3.4 Discussion

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

Proteobacteria is the dominant phylum on the leaves 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 Betaproteobacteria were linked with nitrogen transformations [27]. Cyanobacteria groups were already observed in mangroves 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 [Figure 4 b].

Gammaproteobacteria was the most abundant class of bacteria on the phylloplane of *L. racemosa* and also occur in abundance in mangroves' sediment [27,30,38]. This group presents approximately 250 genera that contain species that are morphologically and metabolically well diversified, that can colonize since from the human gastrointestinal tract, as well as live in symbiosis with insects and also inhabit the phyllosphere of 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 differs from surrounding soil and air samples [34,36,43].

The diversity of the bacterial communities varied throughout the three collection sites (Figure 2) The upland site, the most distant site from the sea water and closest of the continent, presented the lowest bacteria 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 are 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 intra-specific 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; what make this ecosystem unique [46]. The variation in the bacteria 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 [what 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 the increase in soil salinity [5].

This aspect of the plant metabolism could explain the differences in the abundance of bacteria 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 present groups of organisms that are capable of support the salinity levels found in mangroves environments [49], this group was already correlated with lower salinities 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 diminishes and to 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 are subject to the marine influence like tidal oscillations and aerosols from sea waves [56]. The results presented in this work showed that exists significant intraspecific variation in bacterial communities between a single host species and that can contribute with the knowledge about the dynamics driving intra-individual variability in epiphytic community's structure.

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4 MULTISPECIES INTERACTIONS OF TEN BACTERIA ISOLATED FROM THE *LAGUNCULARIA RACEMOSA* PHYLLOPLANE

Abstract

The phylloplane is one of the greatest microbial habitats. Bacteria reside in complex interactive communities in close association with competitors and partners. Microbial communities are dynamic and their structuration are determined by the pairwise interactions that occur between different species. Therefore, microbial cells exhibit intercellular communication and are aware of other cells in their vicinity, producing coordinated responses. Then, the aim of this work was to evaluate the multispecies interactions among ten strains isolated from *Laguncularia racemosa* phylloplane. Two hundred and forty-three bacteria were isolated from *Laguncularia racemosa* phylloplane, and one hundred phylloplane isolates of different morphotypes were subjected to a genotypic screening by the BOX-PCR technique. After that, ten bacteria were chosen to have their 16S rRNA gene partially sequenced. These ten strains were used to perform assays of multi-species interactions in all possible combinations. The consortia of three bacteria were done by inoculating 15 µL of the suspensions of each strain in Petri dishes containing TSA 20%, with a distance of 1cm between each other in a triangle conformation. For the pairwise combinations the bacteria suspensions were inoculated side by side also with 1 cm of distance between them. For the monocultures 15 µL of the suspensions of each strain were inoculated on the middle of the plates. All the strains had their growth diameter measured when growing in monocultures compared against their own growth in the pairwise interactions and in the consortium of three bacteria. In the total, fifteen consortia showed significant differences in the growth diameter of the bacteria in at least one combination. However, twenty consortia combinations did not show significant differences in the bacteria growth while interacting. In conclusion this work has showed that bacteria from *L. racemosa* phylloplane can sense other strains nearby and alter their rates of growth in response to the co-cultures.

Keywords: bacterial growth, interactions, mangroves

4.1 Introduction

Mangroves are ecosystems composed of salinity tolerant vegetation that thrives in intertidal zones in tropical and subtropical regions of the planet (1). It is considered a productive biome that interacts with adjacent coastal areas (2). Plant and animal species that survive in these regions can tolerate atypical environmental conditions, which gives them an advantage over other competing species that do not thrive at these extremes (2).

Mangrove vegetation is one of the most significant components of the mangrove ecosystem and the leaves of this vegetation correspond to most of the primary production of this environment (3). *Laguncularia racemosa*, known as white mangrove, is an important component of the mangrove forests in the Americas and West Africa (4). Leaves constitute an extensive plant mass that is responsible for much of the energy and matter input into planet Earth (5).

The phyllosphere comprises the aerial part of the plants, while the phylloplane is the surface of the leaves and the organisms that inhabit this habitat are called epiphytes (6–8). The microbial communities of these environments are mainly composed of bacteria, archaea, filamentous fungi and yeasts (7).

In general, Bacteria do not behave as solo free-living planktonic organisms, but rather reside in interactive multicellular communities (9). They adhere to surfaces and act in a coordinated and efficient way to capture nutrients, to protect themselves from harsh environmental conditions, and to launch expeditions in search of new territories (10).

Microbial communities are dynamic and the structuration of these communities are determined by the pairwise interactions that occur between different species (11). Microorganisms have developed different strategies to improve nutrient acquisition. Mobility, antibiotic production, coordinated behavior, predation, space competition and rapid growth rates can be interpreted as ways to maximize nutrient acquisition by certain organisms over others (12,13).

Interspecific interactions affect the production of secondary metabolites by bacteria (14). Antagonistic and synergistic relationships connect microorganisms and secondary metabolites are the main mediators of these ecological interactions (15). The molecule produced by one species can serve as nutrient or cause lethal damage to others (13). Microorganisms interact in their environment and influence the chemistry of oceans, soils and different environments (10).

All microbial cells respond to chemical signals, whether produced by organisms of the same or other species, and these signals usually culminate in new cellular reactions (13). Bacteria residing in multispecies communities show spatial positioning in response to interspecific interactions, which is crucial for community function and structuration (16). In some biofilms bacterial species tend to keep proper distance, thereby avoiding strong substrate competition or toxic compounds secreted by others (16). Therefore, microbial cells exhibit intercellular communication and are aware of other cells in their vicinity, managing to produce coordinated responses (13).

Then, the aim of this work was to evaluate the multispecies interactions among ten strains isolated from *Laguncularia racemosa* phylloplane. The hypothesis of this work is that interactions between more than two populations prompt changes the morphological response of some of these populations when we compare to the normal solo morphology and in pairwise interactions.

4.2 Material and Methods

4.2.1 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.

4.2.2 Isolation of bacteria from *Laguncularia racemosa* phylloplane

For isolation of the bacteria, five leaves were placed in 500 ml of Erlenmeyer flasks containing 0.85% of autoclaved saline water. These material were kept at 135 RPM (rotations per minute) for a period of 24 hours. After this period serial dilution of this solution was then performed, and dilutions 10-1 to 10-4 were plated with a Drigalski handle in Petri dishes of 9 cm of diameter. The culture media used were 100% Nutrient Agar (NA) and 5% Sodium Tryptone Agar Medium (TSA), both containing 0.05 g/ml of the Nystatin fungicide.

The plates were incubated for two weeks at room temperature and colonies with distinct growth and morphology were selected during this period. These colonies were then transferred to plates with 100% TSA medium, purified and preserved in 20% Glycerol at 80 °C for use in subsequent assays.

4.2.3 Genotypic characterization of the bacterial isolates from *Laguncularia racemosa* phylloplane by means of BOX-PCR technique

One hundred phylloplane isolates were subjected to genotypic screening by the BOX-PCR technique, according methodology developed by Rademaker et al (1997). For this, four days after growth in TSA culture medium (trypticase soy agar; 39 g L-1 BDA; pH 6.8) in BOD at 28 ° C, small growth masses of isolates were used to prepare bacterial suspensions. With the tip of an autoclaved toothpick a little biomass was collected and mixed in 100 µl of

autoclaved Milli-Q water into 2 mL capacity microcentrifuge tubes until a cloudy suspension was obtained.

To generate the BOX-PCR profiles, the polymerase chain reaction (PCR) was performed with the BOX 1AR primer (5'-CTACGGCAAGGCGACGCTGACG-3 '). DNA amplification reactions were prepared to a final volume of 25 µl containing: 13.45 µl autoclaved Milli-Q water, 1.0 µl BOX A1R primer (10 µM), 1.25 µl dNTPs (25 mM) each), 0.40 µl BSA (10 mg / ml), 2.5 µl 100% DSMO, 5 µl Gistchier 5X buffer, 0.4 µl Taq polymerase (5 U / µl) and 1 µl of the sample (bacterial suspension). Amplification was conducted in a thermal cycler programmed for 7 minutes at 95 ° C initial denaturation (one cycle), denaturation at 95 ° C for 1 minute, annealing at 40 ° C for 1 minute and extension at 65 ° C for 8 minutes (35 cycles). A final extension cycle was programmed at 65 ° C for 16 minutes with maintenance of 4 ° C.

Bands were visualized with 30 cm long 1.5% (w / v) agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA) stained with Red Gel (Biotium®) and Loading Buffer 1X (Invitrogen®); The electrophoresis was run at 60 V for 4 h at room temperature. The 1 kb Plus DNA Ladder (BioLabs) molecular marker was used for normalization, and the bands were visualized on photocumenter. Gel analysis was performed using Bionumerics software (Apllied Maths version 5.1). The dissimilarity cutoff chosen to select the isolates to be used in the work collection was 50% using Pearson's index.

4.2.4 Identification of ten bacteria isolates from the *Laguncularia racemosa* phylloplane by means of the 16S rRNA partial gene sequencing

To perform molecular identification of the epiphytic isolates, PCR reactions were performed using the primers: 27F (AGAGTTTGATCM TGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991). To obtain the Mix with a final volume of 25 µL, were added: 18.25 µL of Ultrapure Water (Milliq), 2.5 µL of 10X PCR Buffer, 2.5 µL of MgCl2 (25 mM), 0.5 µL of dNTP's (25 mM each), 0.50 µL of each primer (10 µM), 0.50 µL of Taq DNA polymerase (5 U/µL) and lastly 1 µL of sample DNA. Amplification cycles were performed on a 96 Well Thermal Cycler (Applied Biosystems) under the following conditions: 94 °C for 5 minutes (initial denaturation), 30 cycles of 94 °C for 30 seconds (denaturation), 55 ° C for 30 seconds (annealing), 72 ° C for 1 minute and 30 seconds (extension) and finally a final extension of 72 ° C for 5 minutes. After amplification, the reaction containing the PCR product was run on 1.5% agarose gel and analyzed by photocumenter.

The purification of the DNA product of the PCR of each bacterial isolate was performed using the Promega kit (Wizard SV Gel and PCR Clean-up System - Ref A9282). Sequencing reactions were then prepared with: 1 µL of DNA, 2 µL of Buffer 5X ABI, 4 µL of Autoclaved Milliq Water, 1 µL of Big Dye (Applied Biosystems, Forter City, CA) and 1 µL of each Primer (5 pmol) to a final volume of 10 µL. The primers that were used are: 27F, 357F, 357R, 704F, 704R, 1114F and 1114R.

The reactions were subjected to cycles of 96 ° C for 1 minute, 96 ° C for 15 seconds, 50 ° C for 15 seconds, 60 ° C for 4 minutes (35 cycles) and finally maintained at 4 ° C. Then 2 µL of Sodium Acetate: EDTA (1: 1) buffer (3M Sodium Acetate, pH 9.0 and 0.5M EDTA, pH 8.0) and 60 µL of absolute ethanol were added. The samples were centrifuged for 45 minutes at 3,000xg, and the supernatant was discarded. Then 150 µL of 70% (v / v) ethanol was added and the microtubes and they were centrifuged at 3,000 x g for 15 minutes. Then, the supernatant was removed and the samples were dried at room temperature in the dark. Samples were resuspended in 10 µL HiDi formamide (Applied Biosystems), denatured at 95 ° C for 5 minutes and injected into the 3500 Genetic Analyzer (Applied Biosystems).

The sequences were analyzed with the CLC Bio Genomic Workbench software v.5.0 software (http://www.clcbio.com) and compared against EzTaxon public database (https://www.ezbiocloud.ne) by the identify sequences tool.

The phylogenetic tree (Fig. 3) was created with the Mega program version 6.06 with the method Tamura Nei + G (gamma distribution) with 1000 bootstrap.

4.2.5 Bacterial multispecies' interaction assays

For the monoculture samples one single colony of each bacterial isolate was picked and inoculated in LB medium (Luria-Bertani g/l - Peptone 10.0, Yeast Extract 5.0, Sodium Chloride, pH=7.5; TermoFishing) and grown during 4 days at 28°C with agitation at 150 RPM (rotations per minute). After the incubation period the bacterial suspensions were adjusted to 0,1 at an optical density of OD550 in spectrophotometer, what correspond to 108 ufc/mL.

The multispecies interaction assays were done in Petri dishes of 90 × 15 mm filled with 20 mL of 20% TSA medium (trypticase soy agar - g/l. Tryptone 15.0, Digestion of Soybean Meal 5.0, sodium chloride 5.0, agar 15.0, pH= 7.3, Kasvi). The consortiums of three bacteria were done by inoculating 15 µL of the suspensions of each strain with a distance of 1cm between each other in a triangle conformation. For the pairwise combinations 15 µL of each bacteria suspensions were inoculated side by side also with 1 cm of distance between them. For the monocultures 15 µL of the suspensions of each strain were inoculated on the

middle of the plate (figure 1). The Petri dishes were incubated in BOD at 28 ºC during five days with photoperiod of 12 hours. Control plates with 15 µL of LB medium without the inoculation of any microorganism were maintained in the same conditions. After five days of incubation of the multispecies' interactions assays, they were evaluated according to the growth diameter of the bacteria. In the total, 36 consortiums with all the possible combinations between the ten bacteria was tested.

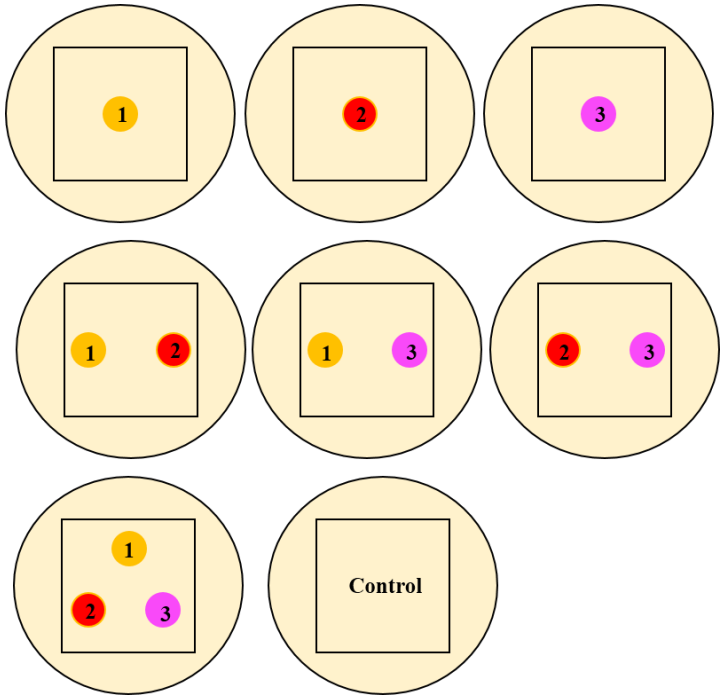


Figure 1: Schematic representation of the multispecies' interactions assays

4.2.6 Data analysis and statistics

The growth diameter of the bacteria in cm in the trio consortium and in the pairwise interactions were compared with the growth of the microorganisms when alone. All the tests were performed with repetitions and the values were expressed as the mean between them. A parametric variance test (ANOVA) was used to evaluate for significant differences in the growth diameter of the isolates while growing alone and within the interactions. After considering the assumptions of normality tested by the Shapiro-Wilk and equality of variance by bartlett test. The significant data were compared using the Tukey test ($p<0.05$).

4.3 Results:

4.3.1 Bacteria Isolation

Two hundred and forty-three bacteria were isolated from *Laguncularia racemosa* phylloplane, purified and stored from both sampling sites. The microorganisms were selected based on their morphological characteristics, where colonies with distinct appearances were selected. Therefore, the colony forming unit was not accounted for. In TSA 5% medium, 152 bacteria were isolated, while in NA medium 91 bacteria were isolated.

Table 1: Number of isolates of *L. racemosa* phylloplane at the three sampling sites

Sampling sites	NA medium	TSA 5% medium	Total
1	23	40	63
2	37	62	99
3	31	50	81

4.3.2 Box PCR technique

One hundred bacterial isolates from the *Laguncularia racemosa* phylloplane, with representatives of all different morphotypes were submitted to the BOX-PCR technique. The analysis of the band patterns captured on the gel was performed using the Bionumerics software (Apllied Maths version 5.1), and the dissimilarity cutoff was 50% using the Pearson's index. From the dendrogram generated from the DNA fragment band profiles (Figure 2) it was possible to observe the diversity in the bacterial groups from the *L. racemosa* phylloplane, as was confirmed by the independent culture method (Chapter 2). From this result ten isolates were selected to have 16S rRNA partial gene sequenced and to be tested in the interspecific interaction assays.

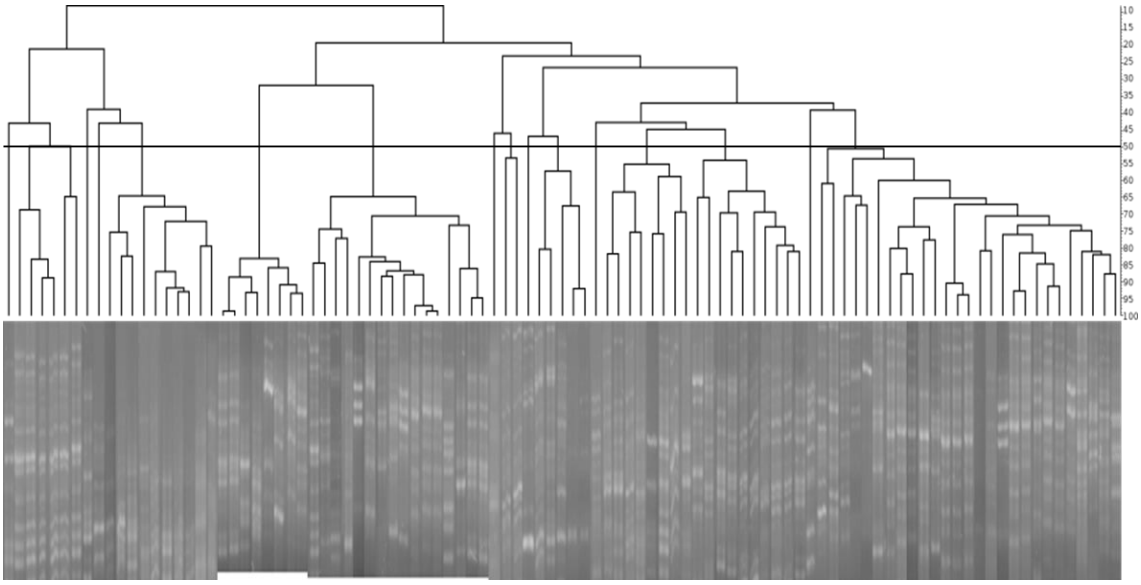


Figure 2: Dendogram of the 100 bacterial isolates of the *L. racemosa* phylloplane generated with the Pearson index. Vertical line represents the cutoff percentage of 50% chosen.

4.3.3 Bacteria identification

The phylogenetic analysis of the ten epiphytic isolates showed the selection of ten strains represented by 9 bacterial families of the following 3 phyla: Proteobacteria, Firmicutes and Actinobacteria (Table 2). The phylogenetic three showed the grouping of three different clades (Figure 3).

Table 2: Identification of *L. racemosa* phylloplane isolates by partial sequencing of the gene 16S rRNA gene

Code	Phylum	Class	Order	Family	Genus	Species	Code	Similarity %
bac. 1	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas* sp.	CMAA1768	98.84
bac. 7		Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	*Aureimonas*	*Aureimonas* sp.	CMAA1774	100
bac. 8						*Aureimonas* sp.	CMAA1771	99.85
bac. 4	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Microbacterium*	*Microbacterium* sp.	CMAA1767	100
bac. 5				Promicromonosporaceae	*Isoptericola*	*Isoptericola* sp.	CMAA1769	99.71
bac. 6				Micrococcaceae	*Micrococcus*	*Micrococcus* sp.	CMAA1770	99.70
bac. 10				Dermabacteraceae	*Brachybacterium*	*Brachybacterium* sp.	CMAA1775	99.70
bac. 9			Propionibacteriales	Nocardioidaceae	*Marmoricola*	*Marmoricola* sp.	CMAA1776	99.80
bac. 2	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* sp.	CMAA1773	100
bac. 3				Exiguobacteriaceae	*Exiguobacterium*	*Exiguobacterium* sp.	CMAA1772	99.73

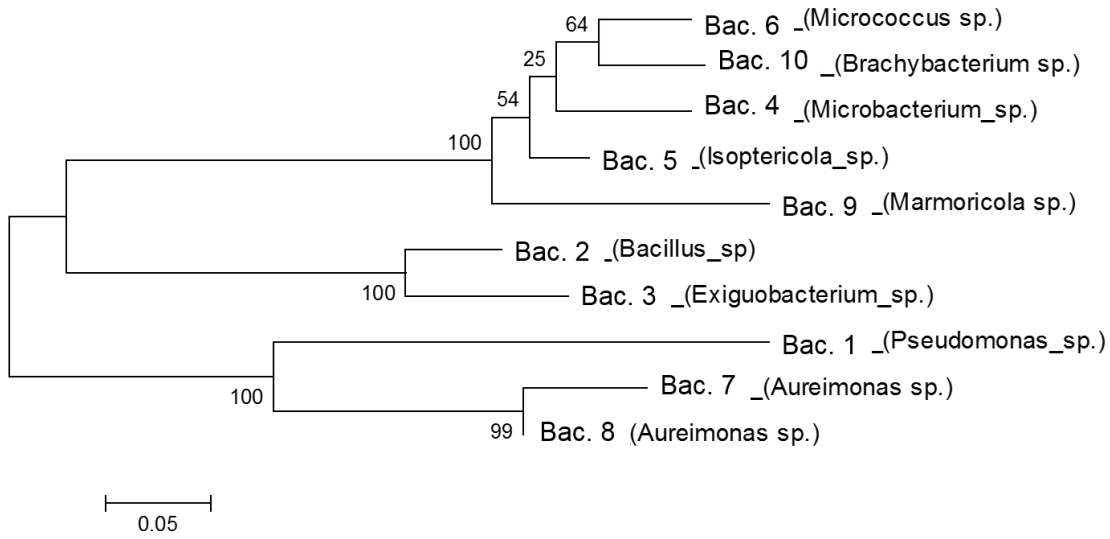


Figure 3: The phylogenetic tree showing the phyllogenetic relashioships between the ten strains

4.3.4 Bacteria interaction assays

Table 3: Multispecies interactions of the ten epiphytic bacteria from *Laguncularia racemosa* phyloplane. (+) increased growth and (-) decreased growth of the microorganism in the first column in relation to the second column. Regarding the phylogeny: DPC (Different phylogenetic clade) and SPC (Same phylogentic clade).

Multi-species interactions			Growth	Phylogeny
1 - *Pseudomonas* sp. CMAA1768	growing with	2 - *Bacillus* sp. CMAA1773	+	DPC
3 - *Exiguobacterium* sp. CMAA1772	growing with	1 - *Pseudomonas* sp. CMAA1768	-	DPC
2 - *Bacillus* sp. CMAA1773	growing with	4 - *Microbacterium* sp. CMAA1767	-	DPC
3 - *Exiguobacterium* sp. CMAA1772	growing with	2 - *Bacillus* sp. CMAA1773	-	SPC and DPC Respectevely
4 - *Microbacterium* sp. CMAA1767	growing with	2 - *Bacillus* sp. CMAA1773	-	DPC
4 - *Microbacterium* sp. CMAA1767	growing with	3 - *Exiguobacterium* sp. CMAA1772	-	DPC
2 - *Bacillus* sp. CMAA1773	growing with	3 - *Exiguobacterium* sp. CMAA1772	-	SPC
6 - *Micrococcus* sp. CMAA1770	growing with	2 - *Bacillus* sp. CMAA1773	-	DPC
2 - *Bacillus* sp. CMAA1773	growing with	9 - *Marmoricola* sp. CMAA1776	+	DPC
4 - *Microbacterium* sp. CMAA1767	growing with	5 - *Isoptericola* sp. CMAA1769	-	SPC
4 - *Microbacterium* sp. CMAA1767	growing with	9 - *Marmoricola* sp. CMAA1776	+	SPC
4 - *Microbacterium* sp. CMAA1767	growing with	10 - *Brachybacterium* sp. CMAA1775	+	SPC
10 - *Brachybacterium* sp. CMAA1775	growing with	4 - *Microbacterium* sp. CMAA1767	+	SPC
6 - *Micrococcus* sp. CMAA1770	growing with	5 - *Isoptericola* sp. CMAA1769	-	SPC
8 - *Aureimonas* sp. CMAA1771	growing with	4 - *Microbacterium* sp. CMAA1767	-	DPC
8 - *Aureimonas* sp. CMAA1771	growing with	5 - *Isoptericola* sp. CMAA1769	-	DPC
8 - *Aureimonas* sp. CMAA1771	growing with	7 - *Aureimonas* sp. CMAA1774	-	SPC
3 - *Exiguobacterium* sp. CMAA1772	growing with	2 - *Bacillus* sp. CMAA1773 and 4 - *Microbacterium* sp. CMAA1767	-	SPC and DPC Respectevely
4 - *Microbacterium* sp. CMAA1767	growing with	2 - *Bacillus* sp. CMAA1773 and 3 - *Exiguobacterium* sp. CMAA1772	-	DPC
3 - *Exiguobacterium* sp. CMAA1772	growing with	2 - *Bacillus* sp. CMAA1773 and 5 *Isoptericola* sp. CMAA1769	-	SPC and DPC Respectevely
3 - *Exiguobacterium* sp. CMAA1772	growing with	2 - *Bacillus* sp. CMAA1773 and 6 - *Micrococcus* sp. CMAA1770	-	SPC and DPC Respectevely
6 - *Micrococcus* sp. CMAA1770	growing with	2 - *Bacillus* sp. CMAA1773 and 3 - *Exiguobacterium* sp. CMAA1772	-	DPC
9 - *Marmoricola* sp. CMAA1776	growing with	2 - *Bacillus* sp. CMAA1773 and 3 - *Exiguobacterium* sp. CMAA1772	-	DPC
6 - *Micrococcus* sp. CMAA1770	growing with	3 - *Exiguobacterium* sp. CMAA1772 and 4 - *Microbacterium* sp. CMAA1767	+	DPC and SPC Respectevely
4 - *Microbacterium* sp. CMAA1767	growing with	3 - CMAA1772 *Exiguobacterium* sp. and 7 - CMAA1774 *Aureimonas* sp.	+	DPC
7 - *Aureimonas* sp. CMAA1774	growing with	3 - CMAA1772 *Exiguobacterium* sp. and 4 - CMAA1767 *Microbacterium* sp.	+	DPC
4 - *Microbacterium* sp. CMAA1767	growing with	5 - *Isoptericola* sp. CMAA1769 and 8 - *Aureimonas* sp. CMAA1771	-	SPC and DPC Respectevely
8 - *Aureimonas* sp. CMAA1771	growing with	4 - *Microbacterium* sp. CMAA1767 and 5 - *Isoptericola sp.CMAA1769*	-	DPC
10 - *Brachybacterium* sp. CMAA1775	growing with	8 - *Aureimonas* sp. CMAA1771 and 9 - *Marmoricola* sp. CMAA1776	+	DPC

Ten strains were used to perform assays of multi-species interactions in all possible combinations (36 consortiums X 8 combinations X 4 repetitions). All the strains had their growth diameter measured when growing alone compared against their own growth in the pairwise interactions and in the consortium of three bacteria. In the total, fifteen consortia showed significant differences in the growth diameter in at least one combination (Figure 4 A) ($P < 0.05$, Tukey exact test). twenty-one consortia combinations did not show significant differences in their growth while interacting (Figure 4 B).

In the total, 9 interactions where positive and the strains had their growth enhanced in the interactions against 20 negative interactions, where the bacteria growth was lower when interacting in the pairwise and in the consortium (table 2). Most of the interactions occurred between bacteria from different phylogenetic clades (table 2). The majority of the interactions were observed between the duo of bacteria, which totalized seventeen interactions, five increasing the growth rates and twelve decreasing the growth rates. In the consortium of the three bacteria occurred twelve interactions, in which four increased the growth rates and eight decreased the growth rates (Table 2).

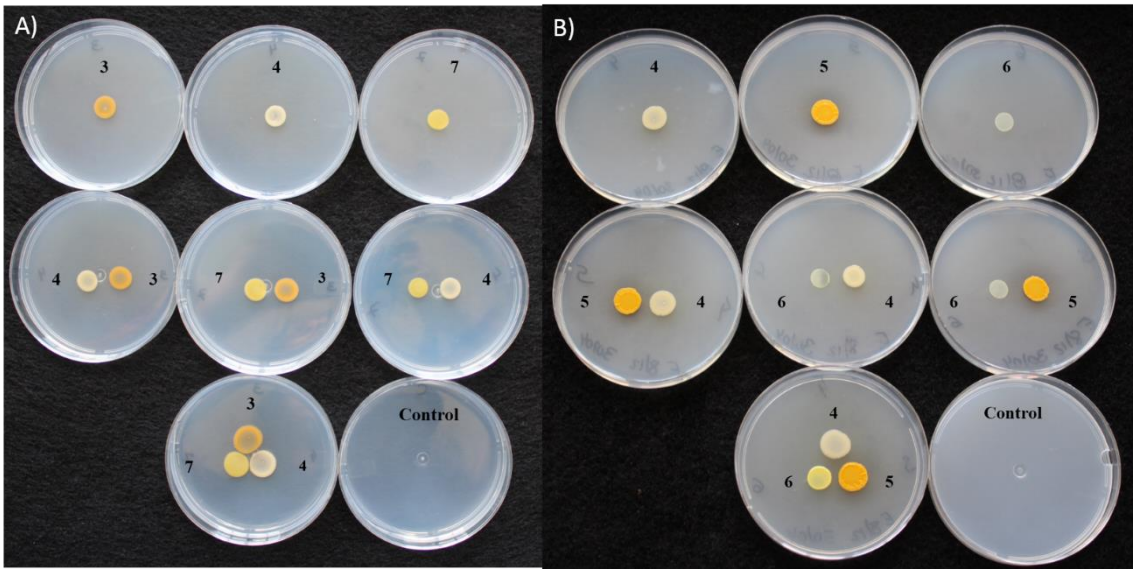
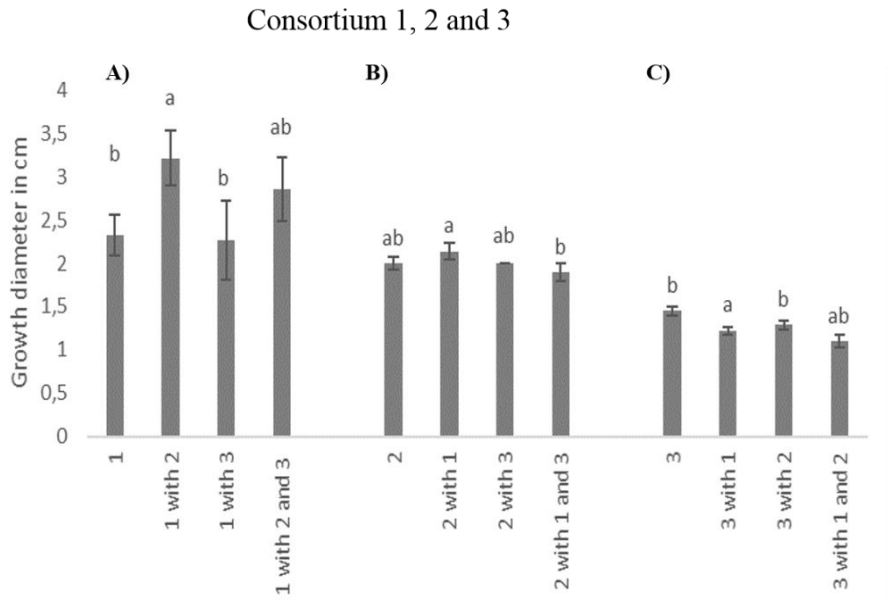


Figure 4: Bacterial interactions. A) *Exigobacterium* sp., *Microbcterium* sp. and *Aureimonas* sp. B) *Microbacterium* sp., *Isoptericola* sp. and *Micrococcus* sp.

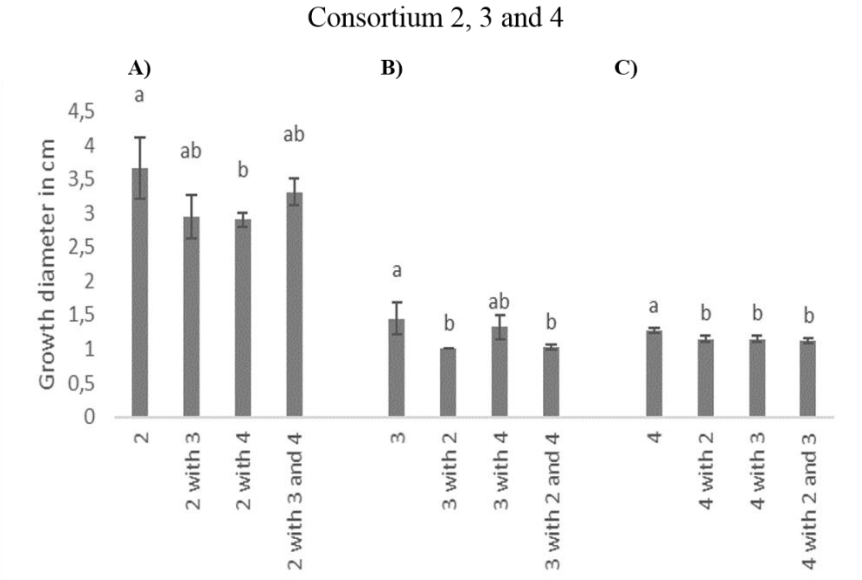
For the consortium 1, 2 and 3 Pseudomas sp. CMAA1768 grew more while in the pairwise interaction with the Bacillus sp. CMAA1773 (Fig. 5 a). Exiguobacterium sp. CMAA1772 grew less while interacting with the Pseudomonas sp. CMAA1768 (Fig. 5 c).



1- *Pseudomonas* sp. CMAA1768 2- *Bacillus* sp. CMAA1773 3 - *Exiguobacterium* sp. CMAA1772

Figure 5: Growth diameter in cm of the consortium: Strain 1- *Pseudomonas* sp. CMAA1768, strain 2- *Bacillus* sp. CMAA1773 and strain 3- *Exiguobacterium* sp. CMAA1772. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 2, 3 and 4 *Bacillus* sp. CMAA1768 grew less with the *Microbacterium* sp. CMAA1767 (Figure 6 a). *Exiguobacterium* sp. CMAA1772 grew less with the *Bacillus* sp. CMAA1773 (Figure 6 b) and *Microbacterium* sp. CMAA1767 grew less in the pairwise and in the consortium interactions (Figure 6 c).



2- *Bacillus* sp. CMAA1773 3- *Exiguobacterium* sp. CMAA1773 4- *Microbacterium* sp. CMAA1767

Figure 6: Growth diameter in cm of the consortium: strain 2 - *Bacillus* sp. CMAA1773, strain 3 - *Exiguobacterium* sp. CMAA1772 and strain 4 - *Microbacterium* sp. CMAA1767. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 2, 3 and 5 *Exiguobacterium* sp. CMAA1772 grew less with the *Bacillus* sp. CMAA1773 (Figure 7 b).

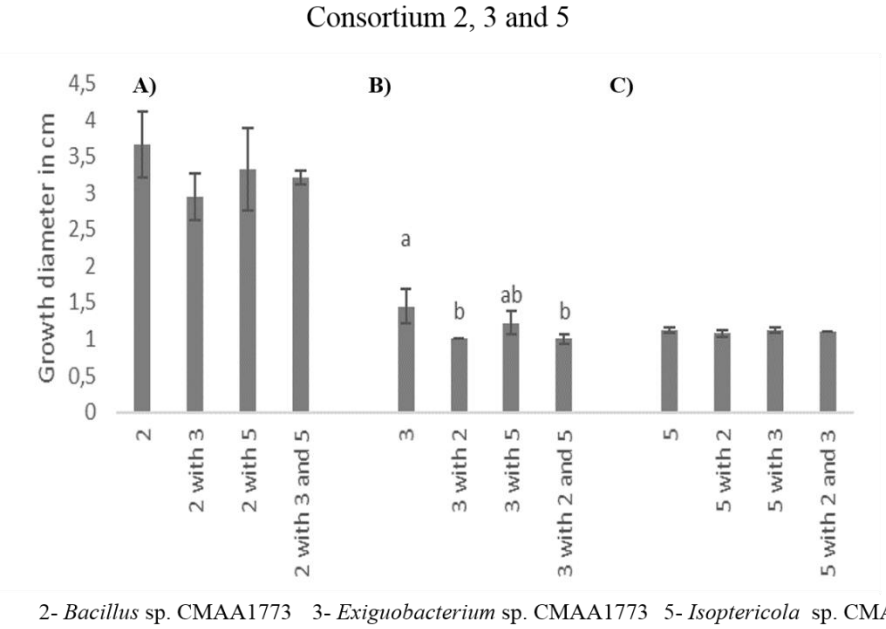


Figure 7: Growth diameter in cm of the consortium: strain 2 - *Bacillus* sp. CMAA1773, strain 3 - *Exiguobacterium* sp. CMAA1772 and strain 5 - *Isoptericola* sp. CMAA1769. Different letters are significant according to the Tukey test (p<0.05).

In the consortium 2, 3 and 6 *Bacillus* sp. CMAA1773 grew less with *Exiguobacterium* sp. CMAA1772 (Figure 8 a) and Exiguobacterium sp. CMAA1772 grew less in the pairwise interactions with the *Bacillus* sp. CMAA1773 and in the consortium (Figure 8 b). *Micrococcus* sp. CMAA1770 grew less with the *Bacillus* sp. CMAA1773 and in the consortium of three bacteria (figure 8 c).

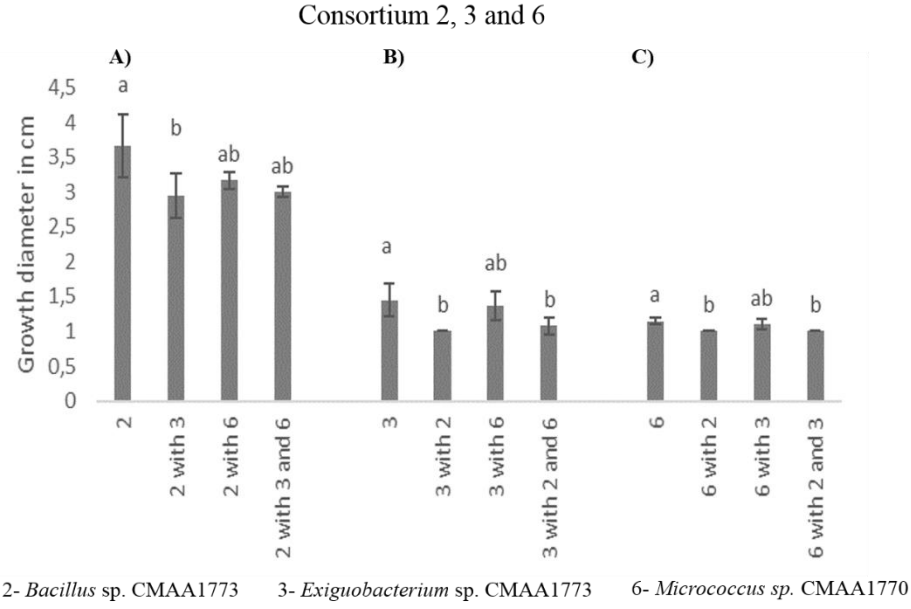


Figure 8: Growth diameter in cm of the consortium: strain 2 - *Bacillus* sp. CMAA1773, strain 3 - *Exiguobacterium* sp. CMAA1772 and strain 6 - *Micrococcus* sp. CMAA1770. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 2, 3 and 9 the *Bacillus* sp. CMAA1773 had their higher growth while interacting with the *Marmoricola* sp. CMAA1776 (Figure 9 a) And the strain *Marmoricola* sp. CMAA1776 grew less in the trio consortium. (Figure 9 c).

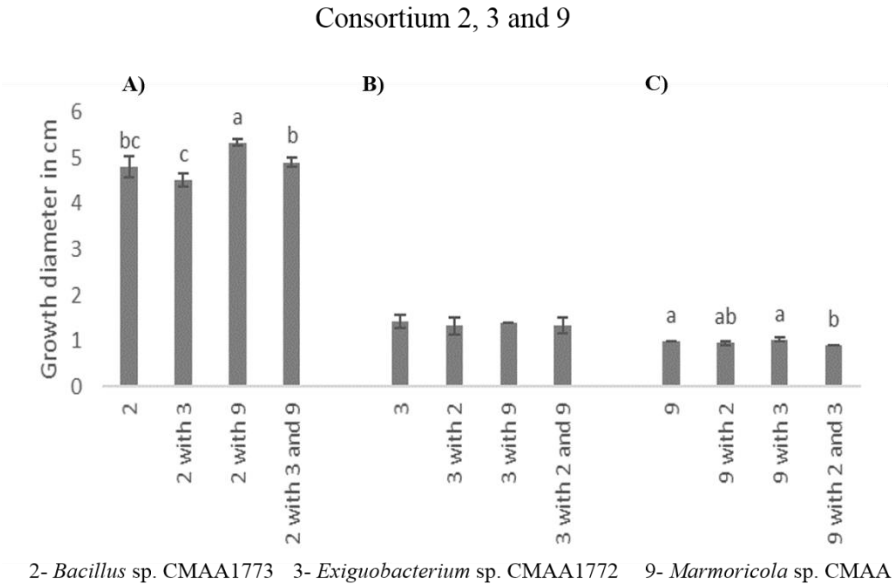


Figure 9: Growth diameter in cm of the consortium: strain 2 - *Bacillus* sp. CMAA1773, strain 3 - *Exiguobacterium* sp. CMAA1772 and strain 9- *Marmoricola* sp. CMAA1776. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 3, 4 and 5 *Microbacterium* CMAA1767 grew less with *Isoptericola* sp. CMAA1769 (Figure 10 b).

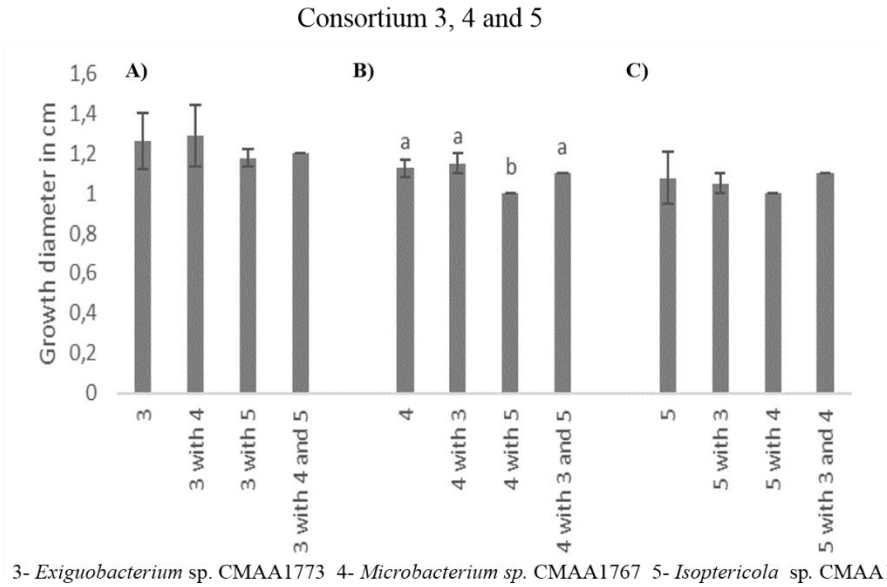


Figure 10: Growth diameter in cm of the consortium: strain 3- *Exiguobacterium* sp. CMAA1773, strain 4- *Microbacterium* sp. CMAA1767 and strain 5 –*Isoptericola* sp. CMAA1769. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 3, 4 and 6 *Micrococcus* CMAA1770 grew more while interacting in the consortium (Figure 11 c).

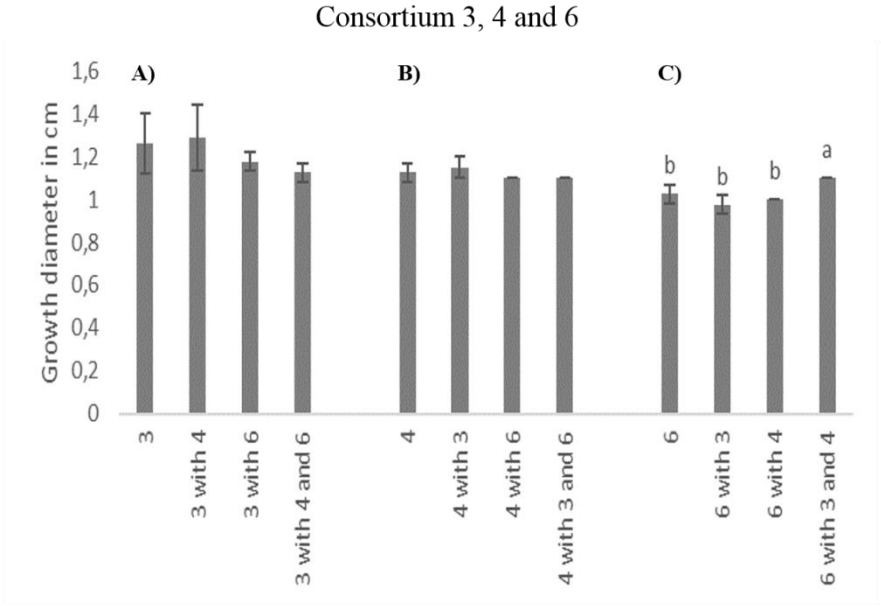


Figure 11: Growth diameter in cm of the consortium: strain 3- *Exiguobacterium* sp. CMAA1773, strain 4 – *Microbacterium* sp. CMAA1767 and strain 6- *Micrococcus* sp. CMAA1770. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 3, 4 and 7 *Microbacterium* sp. CMAA1767 and *Aureimonas* sp. CMAA1774 grew more while interacting in the consortium of three bacteria (figure 12 a and Figure 12 b).

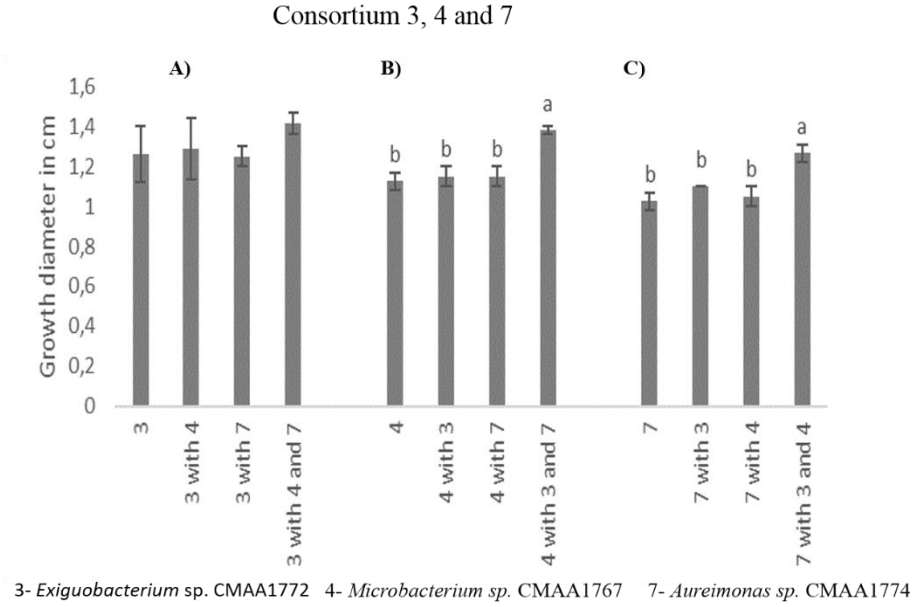
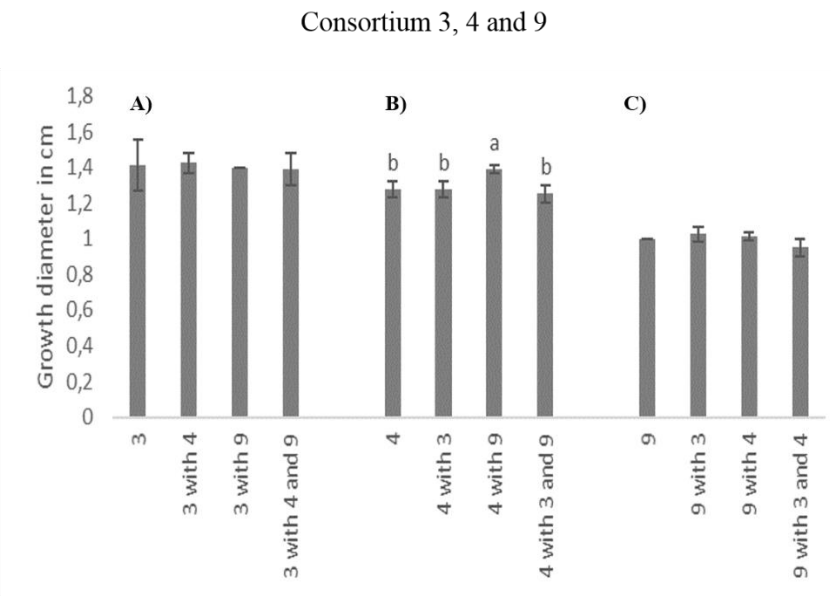


Figure 12: Growth diameter in cm of the consortium: strain 3 – CMAA *Exiguobacterium* sp., strain 4 – CMAA1767 *Microbacterium* sp. and strain 7 - CMAA1774 *Aureimonas* sp. Different letters are significant according to the Tukey test (p<0.05).

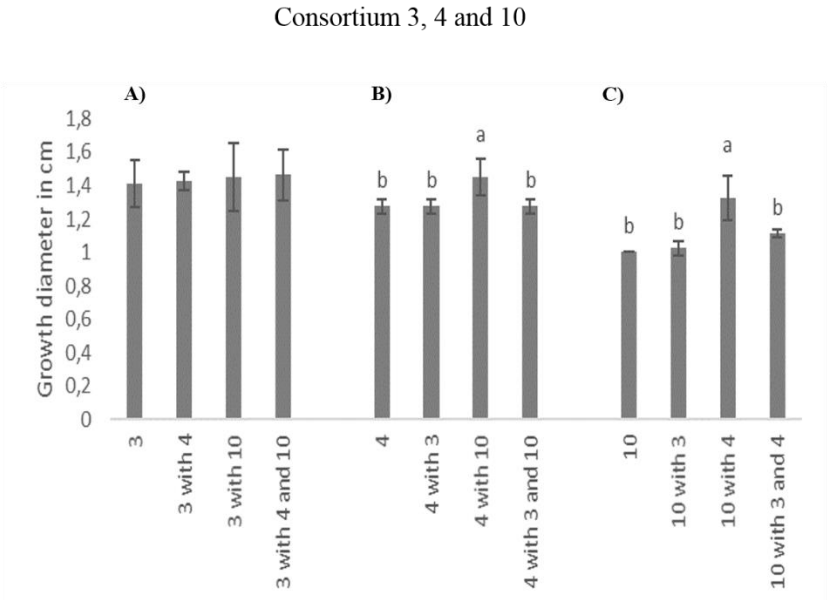
For the consortium 3, 4 and 9 *Microbacterium* sp. CMAA1767 grew more while interacting with *Marmoricola* sp. CMAA1776 (Figure 13 b).



3- *Exiguobacterium* sp. CMAA1772 4- *Microbacterium* sp. CMAA1767 9- *Marmoricola* sp. CMAA1776

Figure 13: Growth diameter in cm of the consortium: strain 3 – *Exiguobacterium* sp. CMAA1772, strain 4 – *Microbacterium* sp. CMAA1767 and strain 9 *Marmoricola* sp. CMAA1776 Different letters are significant according to the Tukey test (p<0.05).

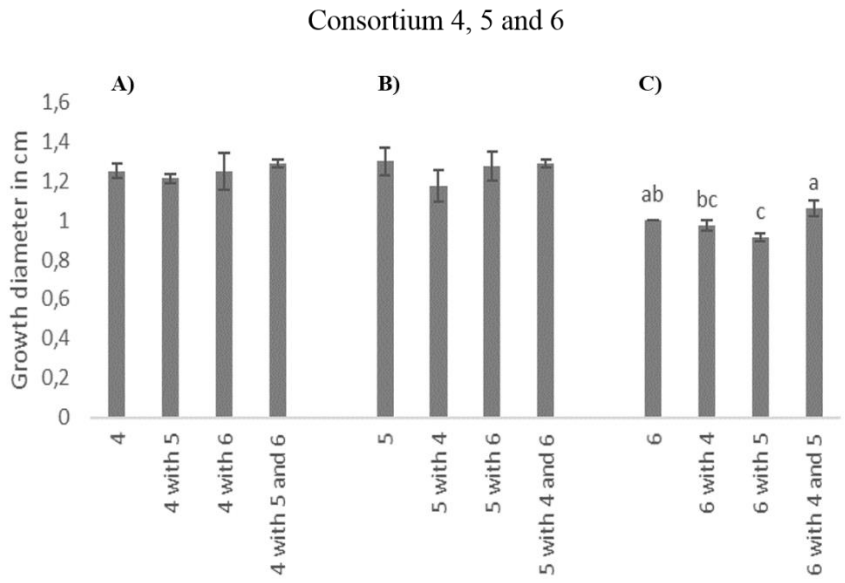
For the consortium 3, 4 and 10 *Microbacterium* sp. CMAA1767 and *Brachybacterium* sp. CMAA1775 grew more when growing together (Figure 14 b and Figure 14 c).



3- *Exiguobacterium* sp. CMAA1772 4- *Microbacterium* sp. CMAA1767 10- *Brachybacterium* sp. CMAA1775

Figure 14: Growth diameter in cm of the consortium: strain 3 – *Exiguobacterium* sp. CMAA1772, strain 4 – *Microbacterium* sp. CMAA1767 and strain 10 - *Brachybacterium* sp. CMAA1775. Different letters are significantly according to the Tukey test (p<0.05).

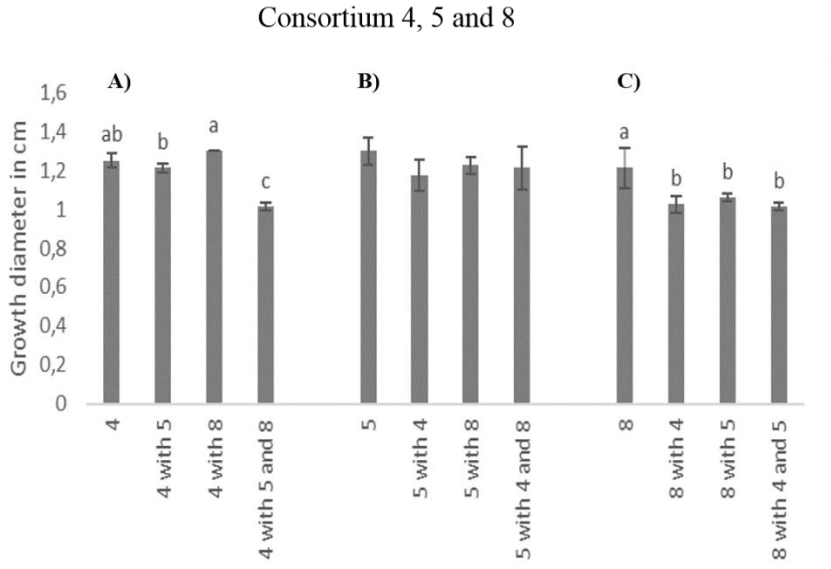
For the consortium 4, 5 and 6 *Micrococcus* sp. CMAA1770 grew less with *Isoptericola* sp. CMAA1769 (Figure 15 c).



4- *Microbacterium* sp. CMAA1767 5- *Isoptericola* sp. CMAA1769 6- *Micrococcus* sp. CMAA1770

Figure 15: Growth diameter in cm of the consortium: strain 4 - *Microbacterium* sp. CMAA1767, strain 5 - *Isoptericola* sp. CMAA1769 and strain 6 - *Micrococcus* sp. CMAA1770. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 4, 5 and 8 *Microbacterium* sp. CMAA1767 grew less in the consortium (Figure 16 a) and Aureimonas sp. CMAA1771 grew more alone then in the three possible combinations of the interactions (Figure 16 c).



4- *Microbacterium* sp. CMAA1767 5- *Isoptericola* sp. CMAA1769 8- *Aureimonas* sp. CMAA1771

Figure 16: Growth diameter in cm of the consortium: strain 4 - CMAA1767 *Microbacterium* sp., strain 5 - CMAA1769 *Isoptericola* sp and strain 8 - CMAA1771 *Aureimonas* sp. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 4, 5 and 10 *Brachybacterium* sp. CMAA1775 grew more while interacting with *Microbacterium* sp. CMAA1767 (Figure 17 c).

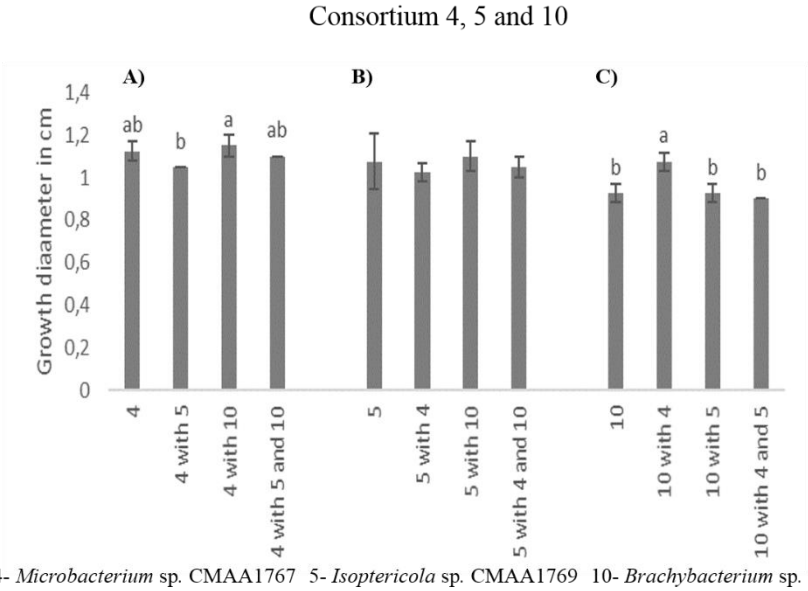


Figure 17: Growth diameter in cm of the consortium: strain 4 - *Microbacterium* sp. CMAA1767, strain 5 - *Isoptericola* sp. CMAA1769 and strain 10 - *Brachybacterium* sp. CMAA1775. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 6, 7 and 8 *Aureimonas* sp. CMAA1771 grew less with other strain of *Aureimonas* sp. CMAA1774 (Figure 18 c).

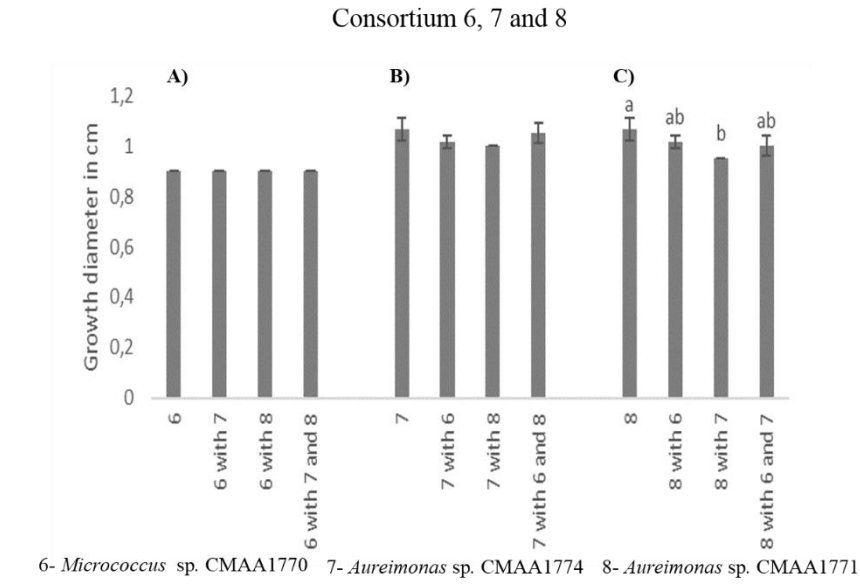


Figure 18: Growth diameter in cm of the consortium: strain 6 - *Micrococcus* sp. CMAA1770, strain 7 - *Aureimonas* sp. CMAA1774 and strain 8 - Aureimonas sp. CMAA1771. Different letters are significant according to the Tukey test (p<0.05).

For the consortium 8, 9 and 10 *Brachybacterium* sp. CMAA1775 grew more in the consortium interactions of three bacteria (Figure 19 c).

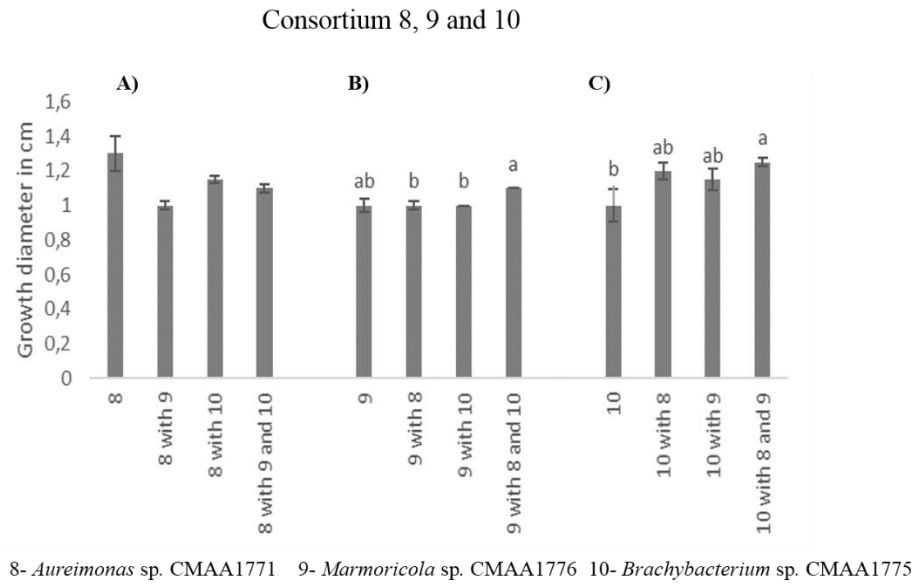


Figure 19: Growth diameter in cm of the consortium: strain 8 - *Aureimonas* sp. CMAA1771, strain 9: *Marmoricola* sp. CMAA1776 and strain 10: *Brachybacterium* sp. CMAA1775. Different letters are significant according to the Tukey test (p<0.05).

4.4 Discussion:

The number of organisms selected shows that the culture medium NA and TSA 5% were favorable for the isolation of the bacteria from *L. racemosa* phylloplane. The TSA culture medium is favorable for isolation of the heterotrophic bacteria (17) and also from organisms from the marine environment (17). The NA medium has already been favorable to the isolation of bacteria from the *L. racemosa* philloplane (18).

When compared to the sequencing of the 16S rRNA amplicon gene, the BOX-PCR technique has some limitations, especially when used for species characterization (19). However, when used as a screening method this technique has its value (20). In such cases, programs that use specialized algorithms are more accurate in observing multiple-input dissimilarity patterns and help to interpret these results (20).

The ten species utilized in the multispecies interactions are represent by nine diverse bacterial families. *Pseudomonas* spp., one of the bacteria isolated from *L racemosa* phylloplane thrive in a diverse set of ecological niches because they have a huge metabolic diversity, with the production of a large spectrum of secondary metabolites (21). They are one of the most ecologically important groups of bacteria and includes species that are plant commensals and pathogens (22).

Aureimonas spp., other strain isolated from this work, are gram-negative bacteria constantly recovered from marine environments (23). Members of the genus *Aureimonas* have

already been isolated from the phyllosphere of different plants, melted caves and lagoons of the Antarctic Sea (24).

The genus *Bacillus* that were found in the phylloplane of *L racemosa* consists of a large number of diverse, rod-shaped Gram-positive bacteria that are motile and aerobic. They are able to produce a wide range of secondary metabolites with different structures with a broad spectrum of activities (25). Also are good antibiotic producers with antagonistic activity against fungal and some bacterial pathogens (26). In these results we observed a negative relationship of *Bacillus* sp. with several other genera tested (table), however, a positive effect was observed when growing with *Pseudomonas* sp. CMAA1768 and *Marmoricola* sp. CMAA1776 This positive effect might have occurred due to the synergistic effect between these two genus, despite that the mechanisms involved in this effect still need to be elucidated.

Exiguobacterium spp. were found in the phylloplane of *L racemosa* and this group is composed by gram positive bacteria of biotechnological importance given its potential for antibiotic production (27). *Exiguobacterium* spp. possesses a variety of gene clusters involved in the synthesis of antimicrobial compounds (28) with activity against clinical pathogens (27). However, in this work, this genus was negative influenced by co-culture.

The phylum *Actinobacteria* was represented in this work by five strains. The genus *Brachybacterium* was first described in 1988 and harbor gram-positive and non-motile organisms (29). *Isoptericola* spp. are gram-positive, aerobic bacteria that have been isolated from the sand of Chinese beaches (30). *Marmoricola* spp. are marine actinobacteria that were isolated from sponges from São Paulo state (31). *Microbacterium* spp. are gram-positive, fermentative bacteria that were isolated from different environmental sources (32). *Micrococcus* sp. It is isolated from different environmental sources, from leaves to the oral cavity of monkeys (33). These observations show the great metabolic diversity of Phylum Actinobacteria, that allow them to colonize different niche and environment. In this study *Microbacterium* sp. inhibited *Bacillus* sp., but showed a synergic growth with *Brachybacterium* sp. and *Marmoricola* sp. that belong to the same Phylum.

Microbial interactions are really complex and has been poorly investigated in different environments (34), because they are difficult to measure and characterize (35). Most of the interactions studies has so far been focusing on the identification of antimicrobial substances (34). In this work we found that bacteria isolated from *L. racemosa* phylloplane can sense species nearby from a certain distance, and most of the interactions occurred between duo of

bacteria. In the majority of these interactions occurred the decrease of the rates of growth when interacting in the pairwise or in the consortia of three bacteria, when comparing with their own growth when growing alone. We expected to observe higher number of synergic relationships in the consortia of bacteria when compared to the pairwise interactions. A total of 29% of the pairwise interactions were positive while from the consortium a total of 33% were positive. However, it is not possible to extract statistical inference from these data. Environmental microorganisms typically live in multispecies communities and bacterial interactions like cooperation and competition are important for microbial communities' structuration (36). Species residing in these complex bacterial communities usually interact both intra- and interspecifically (16). In general, these interactions are mediated by the soluble and volatile secondary metabolites like antibiotic or growth factors that can affect the secondary metabolites production of other bacteria nearby (34,37). Different types of interactions can lead to distinct types of spatial organization (38), for this reason the phylloplane is a promising environment to explore bacterial relationships. The consortium composed by *Exiguobacterium* sp. CMAA1772 *Microbacterium* sp. CMAA1767 and *Aureimonas* sp. CMAA1774 showed an interesting pattern of synergism where all of them had promoted growth in coculture.

Previously studies with cocultures showed that synergic effects are positive to microbial communities. A study that investigated the biofilm formation among seven different soil isolates, cocultured in combinations of four species, observed greater biofilm biomass production in 63% of the four-species culture combinations tested than in the biofilm formed by single-species, demonstrating a high prevalence of synergism in multispecies biofilm formation (38).

A factor that must be considered in multispecies relationships is the growth rates (39). A cocultivation study with two ubiquitous and well-studied microbes *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* showed that *P. aeruginosa* had a distinct growth-rate advantage in cocultures, increasing its relative abundance in the biofilm growth. The authors concluded that quorum sensing and motility via pili and flagella as functions that contributed to its competitive prevalence in the binary coculture system (39).

Quorum sensing is a key process in multispecies interactions, which may confer advantage for some groups to the detriment of others. Several quorum-sensing-regulated secreted functions related to nutrient acquisition might explain that (39). Evidence indicates that epiphytic bacteria living in high cell density aggregates are subject to certain phenotype expression like quorum sensing communication between different species (7). This could

explain the interactions observed in this study, where the multispecies interactions were perfomed considering 1 cm far from each other. The molecules produced by one specie could be sensed by others in certain distances, causing prompt changes in the population morphology.

A study combined biofilm assays and molecular techniques to demonstrate that N. *europaea* makes very little biofilm on its own, and relies on the activity of associated heterotrophic bacteria to establish a biofilm. However, N. *europaea* has a vital role in the proliferation of mixed-species communities under carbon-limited conditions (40). A study with binary co-cultures of marine-derived microorganisms showed that five co-cultures were able to induce changes in the metabolic production due diffusible compounds (41). Members of multispecies communities may influence each other antagonistically through resource competition or production of inhibitory compounds (38). They also can act synergistically; via mechanisms such as cometabolism, biofilm induction or enhanced resistance (38).

A study to understand the interspecific interaction between the Gram-positive *Paenibacillus* sp. AD87 and the Gram-negative *Burkholderia* sp. revealed that the interaction between the two bacteria affected their fitness, gene expression and the production of secondary metabolites (42). A work with industrially relevant multispecies biofilm models, showed that the presence of *Raoultella* can also directly enhance the inherent tolerance of *Pseudomonas* to antimicrobial treatment, either because the species protect each other or because they induce specific tolerance phenotypes as a response to the competitors (43).

In conclusion this work has showed that bacteria from *L. racemosa* phylloplane can sense other strains nearby and alter their rates of growth in response to the co-cultures. We also showed that most interactions are negative where the bacteria decrease their rates of growth while interacting in the pairwise and in the consortia of multi species. More work is required to understand microbial interactions in different environments, mainly in the molecular levels. The approach used in this study may be very useful to elucidate molecules of recognition between microbial multispecies interactions. And It also can be useful to know about the compatibility of microrganisms from different species in a microbial consortium. Further studies are required to elucidate the chemical aspects involved in multispecies interactions, mainly regarding synergistic effects. This knowledgement is important to provide ecological insights in the bacterial community structure.

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Conclusions

The molecular strategies to survive in harsh environmental conditions are not fully comprehended, but it is known that microorganisms from severe habitats have developed interesting biomolecules and biochemical pathways for biotechnological purposes. The studies of bacterial populations from little explored habitats represent a promising path to identify new natural products and also to understand the role of the interactions in the social aspect.

The phyllosphere of *L. racemosa* from mangrove ecossystems stilll have unknown bacteria and it is a promising source for the discovery of new species. In this habitat epiphytic bacteria from a single host species present different patterns of assemble, with locations in the intermediate zone in a characteristic mangrove gradient, being more diverse than locations near de continent. Regarding the interactions, epiphytic bacteria from *L. racemosa* can sense other strains nearby and interact most in a non excludend way, responding with changes in their morphology to these interactions. Therefore, we conclude that mangrove habitats are a promising source for the studies of microbial diversity and synesgistic interactions between bacteria.