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Original Article

Bacterial communities associated with a polydomous arboreal ant: inter-nest variation and interaction with the phyllosphere of a tropical tree

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Abstract

Arboreal ants, abundant and dominant insects in tropical forests, interact with the bacterial communities of the canopies, especially with the bacteria associated with leaf surfaces. In this study, we investigated what kind of interactions exist between the bacterial community associated with the cuticle of a polydomous arboreal ant and the bacterial community associated with the phyllosphere of a tropical tree, in a non-obligatory ant-plant mutualism in the Atlantic rainforest of Brazil. We collected ants of the species *Azteca chartifex* from main and satellite nests and leaves from *Byrsonima sericea* tree (Malpighiaceae), both from ant-colonized and ant-free trees. We used amplicon sequencing of the 16S rRNA gene to investigate the diversity and composition of bacterial communities associated with (i) ants from main and satellite nests, (ii) the phyllosphere of leaves with and without ants, and (iii) we investigated the similarity between the bacterial communities. The diversity and composition of bacterial communities on leaf phyllospheres from ant-colonized and ant-free trees were different as well. Ant presence can decrease bacterial richness and share some bacteria with the leaves they forage on. Our study shows that bacteria are components of tripartite interactions involving a polydomous ant and its facultative mutualistic host tree. Further investigation is needed to understand the role of these bacteria on ant-colony and plant health.

Key words: Hymenoptera, Formicidae, ant-plant mutualism, bacterial community, 16S rRNA gene amplicon sequencing.

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Introduction

Ants are an abundant and dominant insect group in tropical forests, and canopies have high ant abundance and species richness (WILSON 1987, LONGINO & COLWELL 2020). Arboreal ants nesting in the canopy forage extensively on foliage and can defend the host tree against herbivores to

such degree that the plant grows vigorously, and the inquiline colony can thrive (RIBEIRO & al. 2013, SOARES & al. 2022a). However, because of their high local density, eusocial mode of life, and genetic similarity among nestmates, the risk of spreading diseases within ant colonies exerts



great pressure on the defense strategies and behaviours of these insects (Bot & al. 2001, Fernández-Marín & al. 2006, HAMILTON 1996).

Several collective immunization strategies have evolved in large ant colonies, from induced antimicrobial defense produced in external glands (YEK & al. 2012, OFFENBERG & DAMGAARD 2019), and detection of infected individuals (LECLERC & DETRAIN 2016), to the interaction with symbiotic microorganisms (CURRIE & al. 1999, KALTEN-POTH 2009). The structure and composition of bacterial communities associated with social organisms and their environment are particularly important to understand their behavioural habits and the risk of spreading diseases (WILSON 1975). Bacteria associated with ant cuticle can play an important defensive role against pathogens (Cur-RIE & al. 1999, SAPOUNTZIS & al. 2019). Inside the nest, ants can influence the bacterial communities and decrease their richness in the "nursery" (LUCAS & al. 2019). Given that bacterial communities living on ant surfaces are in direct contact with the surrounding environment (LUCAS & al. 2017, BITAR & al. 2021), ants must be able to shape the species composition and density of associated bacteria (FERNÁNDEZ-MARÍN & al. 2009, KELLNER & al. 2015).

Arboreal ants interact with the bacterial communities of the forest canopy, especially with the bacteria associated with leaf surfaces (GONZÁLEZ-TEUBER & al. 2014, OFFENBERG & DAMGAARD 2019, BITAR & al. 2021). Phyllosphere is the microhabitat hosting a great diversity of microorganisms, mostly bacteria (LINDOW & BRANDL 2003). Epiphytic bacteria can either benefit (КЕМВЕL & al. 2014), induce susceptibility and pathogenicity (BAKER & al. 2010), or be neutral (also known as commensal) to the host (LINDOW & BRANDL 2003). Moreover, the diversity and abundance of bacterial communities in the phyllosphere can help to protect the plants exposed to natural enemies (SALEEM & al. 2017). Nonetheless, there is still little known about the interaction between the ant- and leaf-associated bacterial communities, as well as how the structures of these microbial communities interfere with each other.

Azteca chartifex is a dominant ant in the mosaic of species in tropical canopies due to its aggressive territorial behaviour (RIBEIRO & al. 2013, SOARES & al. 2022b). They build multiple "carton" nests with cellulose and processed fibers, and the main nest hosting the queen (length > 2m) can harbor thousands of individuals (BACCARO & al. 2016). Queens and workers of this species are small (2 to 3 mm long), and their polydomous colonies (LONGINO 2007) consist of a main nest and several smaller "satellite nests", or socially connected nest units. Main and satellite nests harbor workers of different sizes (MIRANDA & al. 2021), and the main nest is stable in space and time since it is constructed on the principal tree trunk (SOARES & al. 2022b). Studies involving the genus Azteca and their obligate mutualistic Cecropia trees have shown that diversity and composition of bacterial communities inside the nests vary among nest galleries (LUCAS & al. 2029, NEPEL & al. 2023). In our study system, A. chartifex ants construct their carton nests on *Byrsonima sericea* trees, a non-obligatory association, in a forest-lake ecotone area in southeast Brazil. *Byrsonima sericea* is a native Brazilian tree, commonly occurring in forest-water transition areas (SACRAMENTO & al. 2007). In polydomous *A. chartifex*, the bacterial communities associated with the cuticle of ants from main and satellite nests have remained unknown.

Here, we tested the hypothesis that bacterial communities associated with the cuticle of *Azteca chartifex* workers, from main and satellite nests, shape the bacterial communities on leaf surfaces of *Byrsonima sericea*. Using 16S rRNA gene amplicon sequencing, we identified and analyzed the diversity and composition of bacterial communities of both ants and leaves. Specifically, we addressed the following questions: i) Do bacterial diversity and composition differ between ants from main and satellite nests of polydomous colonies? ii) Do bacterial diversity and composition differ between phyllospheres of trees with and without *Azteca chartifex* nests? iii) How similar is the bacterial community composition of ants and the leaves on which they forage?

Material and methods

Study area

Sampling was carried out in the Atlantic Forest reserve of the Parque Estadual do Rio Doce (hereafter PERD), 35,970 ha, in the state of Minas Gerais, southeast Brazil (19° 45' S 42° 38' W) (Fig. 1). The PERD contains nearly 40 natural lakes that occupy 11% of its area and is the third largest lacustrine system in the Neotropical region (LOURENÇO & al. 2019).

Sampling design

During the rainy season (November) 2020, *Byrsonima sericea* trees with *Azteca chartifex* nests and trees without nests were selected in three different ant populations located in two ecotones of distinct lakes within the park: Bonita (P1 and P2), and Dom Helvécio (P3). Ants from main and satellite nests were sampled from the three locations / populations. Leaves from ant-colonized and non-colonized trees were sampled from the P2 ecotone (Fig. 1). The ant specimens were identified using the key in BACCARO & al. (2016) and subsequent assistance by Rodrigo M. Feitosa, from the Universidade Federal do Paraná. *Byrsonima sericea* is a dominant and pioneer tree species that defines most of ecotone vegetation in PERD, forming a long-lived and complex canopy architecture (DE CARVALHO BARBOSA 2014).

In P1, pieces of two main nests and four satellite nests were sampled from four trees. In P2, leaves and nests (a total of two main and four satellites) from six trees of *Byrsonima sericea* were sampled, as well leaves from trees without ants. In P3, a total of one main and five satellites, distant 700 m from P2, were sampled. Pieces of each carton nest contained on average 50 to 70 ants.

Nests were sampled using a sterilized machete and bucket. All leaf samples (20 per tree) were sampled using



Fig. 1: Map of Brazil and Rio Doce State Park, showing the *Byrsonima sericea* tree and *Azteca chartifex* ant populations (P1, P2, and P3) across the study areas, located at two distinct forest-lake ecotones. Sampling design across the studied populations, showing trees with main and satellite nests and without nests.

gloves and sterile plastic bags. Samples were taken to the Laboratory of Molecular and Computational Biology of Fungi (LBMCF), at the Federal University of Minas Gerais (UFMG) and stored in the freezer at -20 °C until DNA extraction.

Extraction, 16S rRNA amplification, and sequencing

The DNA extraction from bacteria associated with ant cuticles and leaf phyllosphere was performed in conditions as sterile as possible, following the protocol (with some modifications) of the Quick-DNA[™] Miniprep Kit (Zymo Research No. D3024, Irvine, CA, USA). Thirty Azteca chartifex individuals of each nest were placed in 2 ml tubes and washed with the extraction kit buffer. The samples were gently shaken (not vortexed) six times, with 10 shakes each time, at 5 min intervals, for a total duration of 30 min, such that all DNA of cuticle bacteria was extracted. Furthermore, five leaves from each tree were sampled and saved in Falcon tubes. By using an extraction kit buffer, so that the adhered DNA of bacteria on the surface of the leaves could be extracted, leaves were washed and vortexed for 5 s at a 15 min interval for one hour. DNA was extracted and analyzed in agarose gel for a total of 28 samples of ants (n = 18) and leaves (n = 10), from the three populations.

Bacterial identification and relative quantification were done using high-throughput amplicon sequencing of the 16S rRNA gene. Library preparation followed proprietaries protocol (see Appendix S1, as digital supplementary material to this article, at the journal's web pages). The primers 341F (CCTACGGGRSGCAGCAG; 5'-3') and 806R (GGACTACHVGGGTWTCTAAT; 5'-3') were used to amplify the V3-V4 regions (WANG & QIAN 2009). Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., San Diego, California, USA). Paired-end runs of 500 cycles were performed using V2x500 or V3x600 sequencing kits (Illumina, USA) on average > 100,000 reads coverage per sample. It is noteworthy that all samples were subjected to uniform wet lab and sequencing conditions to ensure methodological consistency and minimize the potential impact of contamination.

Bioinformatic and Statistical analyses

Output files (in *fastq* format) resulting from the 16S rRNA gene amplicon sequencing of all the samples comprise the raw primary data. These raw data were imported to Qiime2-2023.9 (BOYLEN & al. 2019) using the Casava 1.8 paired-end demultiplexed fastq protocol. Subsequently, sequence reads were trimmed, removing reads smaller than 300 bp to maintain read quality regions, a process carried out using DADA2 (CALLAHAN & al. 2026). Taxonomic identification of Amplicon Sequence Variants (ASVs) was performed using the SILVA 132 QIIME database (GLÖCKNER 2019) with a 99% similarity threshold. The resulting ASV table, including taxonomic assignments, was then utilized for the statistical analyses in R Software.

All analyses were performed using R environment (version 4.3.0) (R CORE TEAM 2021). Sequence reads were rarefied to the lowest sample size depth (2,494 reads), a normalization step in data analysis. The phyloseq package (MCMURDIE & HOLMES 2013) was used to create the phyloseq object. For the visualization of rarefaction curves, the ranacapa package (KANDIKLAR & al. 2018) was utilized. To represent the taxonomic diversity of each sample, the phylum relative abundance matrix was used to create a barplot using the ggplot2 package (WICKHAM 2009).

To answer whether there is a difference of ant-associated bacterial communities from main and satellite nests in the different locations / populations, alpha and beta diversity were calculated using vegan package (OKSANEN & al. 2005). From the dataset, samples from 5 main and 8 satellite nests, coming from all three populations, were selected for analysis. The Kruskal-Wallis test was used to evaluate dissimilarities between alpha diversity associated



Fig. 2: Phylum variety analysis barplot of bacterial communities from ecotones samples: Samples of *Azteca chartifex* (main and satellite nests) from three locations (P1, P2 and P3) and samples of leaves of *Byrsonima sericea* trees with and without ant nests (location P2). Bars show the relative abundance of the most abundant bacterial phyla of ant cuticle and of leaves phyllosphere.

with ants from main and satellite nests. To examine differences in beta diversity and composition in ants' bacterial communities among nest types (main and satellite) and populations, a Permanova analysis (using "adonis" function) based on the "Bray-Curtis" dissimilarity method was performed. Non-metric multidimensional scaling (NMDS) was produced to illustrate the composition of bacterial communities across samples and locations / populations. Furthermore, a CLAM test (CHAZDON & al. 2011) was conducted to classify species into generalist, specialist, and rare taxa between two groups of samples (i.e., types of nests). This multinomial species classification method, based on relative abundances, provides insights into the distribution patterns of taxa within and between sample groups.

To address the following two questions, only the P2 samples dataset was used for analysis. First, to investigate potential differences in taxonomic diversity and composition between bacterial communities associated with leaves with and without ants, the same analyses as described for the ants from main and satellite nests were performed. Finally, to assess the similarity in the taxonomic composition between bacterial communities of ants and the leaves they forage on, Permanova and CLAM analyses were performed. For all statistical tests involving the calculation of a p-value (p), an alpha of 0.05 was used to assess statistical significance.

Results

Bacterial community diversity of ant cuticles and leaf phyllosphere

The 16S rRNA gene amplicon sequencing of bacterial communities generated a total of 6,015,549 raw reads in 28 samples and a total of 472 ASVs. In general, ant cuticles and leaf surfaces were dominated by the phyla Proteobacteria, Bacteroidota, and Actinobacteria (Fig. 2).

The phyla Proteobacteria and Bacteroidota, consisting of gram-negative bacteria, represented the highest proportion on the ant cuticle of P1 (36.68% and 41.02%, respectively), P2 (37.77% and 27.53%, respectively), and P3 (34.83% and 38.7%, respectively) areas. At P2, bacterial communities of the leaf phyllosphere had a high proportion of gram-negative Proteobacteria in ant-colonized trees (60.82%) and non-colonized trees (69.22%). At P2, we found a higher phylum diversity on leaves foraged by the ants compared with ant-free leaves.

Bacterial community diversity and composition from main and satellite ant nests in different locations / populations

The observed alpha diversity of bacterial communities of ants from main and satellite nests didn't show differences (Kruskal-Wallis: X^2 (1) = 0.343, p = 0.558). In the analysis of bacterial taxa composition between main and



Fig. 3: (A) Non-metric multidimensional scaling (NMDS), with Bray-Curtis dissimilarity index, shows bacterial community composition of ants from different populations and nest types (main and satellite). (B) Observed alpha diversity of bacterial communities associated with ants, leaves with ants, and leaves without ants. (C) Non-metric multidimensional scaling (NMDS), with Bray-Curtis dissimilarity index, shows bacterial community composition of ants, leaves with ants, and leaves without ants.

satellite ant nests (NMDS), there was significant variation between the types of nests (Permanova: F = 1.81, $R^2 = 0.14$, p = 0.022; Fig. 3A), however, there was no variation between populations (Permanova: F = 1.17, $R^2 = 0.18$, p = 0.230). Ant bacterial communities from each population were compared pair-to-pair, and the analysis showed no difference in their composition (Table S1).



Leaves with ants (abundance +1)

Fig. 4: CLAM test: (A) showing the specialist bacteria in ants' cuticles from satellite nests, specialist bacteria in ants' cuticles from main nests, and the generalist bacteria shared between the two sample types; (B) showing the specialist bacteria in leaves without ants, leaves with ants, and the generalist bacteria shared between the two sample types; (C) showing the specialist bacteria shared between the two sample types.

The CLAM test showed that abundant bacteria *Staphylococcus, Flavobacterium* sp. 2, and *Weissella* sp. 2 were specialists in main ant nests. *Mucilaginibacter* sp. 1 and *Massilia* sp. 1 were specialists in satellite nests. *Lactobacillus, Aliihoeflea, Weissella* sp. 1, and *Brevundimonas* were the most abundant ant-associated bacteria occurring both in main and satellite nests. Among the classified bacteria taxa, 51.6% were satellite nest specialists, 30.9% were main nest specialists, and 14.1% were generalists in both types of nests. Also, 3.4% of the taxa were too rare to be classified with confidence (Fig. 4A).

Bacterial community diversity and composition in ants and phyllosphere with and without ants

The alpha diversity measure of the leaves' bacterial communities varied between trees with and without ants. Hence, the alpha diversity between ant-associated bacteria and bacteria associated with leaves foraged by ants was different as well (Kruskal-Wallis: X^2 (2) = 13.346, p = 0.001; Fig. 3B). Bacterial communities from leaves with ants presented lower diversity when compared with communities from leaves without ants. However, the bacterial taxa composition between leaves with ants and leaves without ants (NMDS) did not differ significantly (Permanova: F = 1.63, R² = 0.37, p = 0.122; Fig. 3C). Finally, we found a significant difference between the bacterial taxa composition between ants and leaves with ants (Permanova: F = 0.29; R² = 1.00; p = 0.003; Fig. 3C).

The CLAM test showed that *Aureimonas* sp. 1, *Methylocella* sp. 1, and *Weissella* sp. 1 were found exclusively and abundantly on leaves foraged by ants (Fig. 4B). On the other hand, *Sphingomonas* sp. 2 and *Byssovorax* were exclusive and most abundant on leaves not foraged by ants. *Methylobacterium* sp. 1 was the most abundant generalist bacterium in both types of leaf samples, with and without ants. Generalists comprised 28.01% of the sampled bacteria taxa, whereas 12.14% were classified as specialists on leaves not foraged by ants. Moreover, 4.53% of the sampled bacteria were too rare to be classified.

In the comparison between ants and leaves with ants, the CLAM test showed that *Lactobacillus* was an ant-associated specialist. *Mucilaginibacter* sp. 1, *Massilia* sp. 1, and *Devosia* sp. 1 were classified as generalists associated with ants and with leaves foraged by them. In this analysis, 49.1% of the bacteria taxa were classified as ant specialists, 40.3% were classified as phyllosphere specialists, and 8.4% of the bacteria as shared generalists. Finally, 2.2% of the taxa were too rare to be classified (Fig. 4C).

Discussion

This study shows that the composition of bacterial communities differs between *Azteca chartifex* workers from main and satellite nests, with some shared bacterial taxa among colonies from three locations / populations. The bacterial community associated with the cuticle of an arboreal dominant ant can affect the bacterial communities of a tropical tree phyllosphere in a non-obligatory ant-plant association, especially concerning the bacterial richness. The cuticles of *A. chartifex* and the phyllospheres of *Byr-sonima sericea* leaves have distinct bacterial communities, showing the specificity of each organism's association with bacteria. The phyllosphere's bacterial community of trees with and without ants differed in diversity, although no difference was found in community composition.

Main and satellite nests harbor ants with different bacterial community composition. This may be due to effects from the queen and the brood in the main nest, which have different microbiomes depending on the stage of development (RAMALHO & al. 2017; NEPEL & al. 2023), colony productivity (SEGERS & al. 2019), and investment in defense strategies (BITAR & al. 2021). In addition, the substantially large size of the main nest may produce a much more buffered environment, likely to keep a constant and more predictable environment than the small satellite nests, which includes better defensive conditions against potential pathogens (WILSON & al. 2002, TURNBULL & al. 2011).

Furthermore, while comparing bacterial communities of ant's cuticles from the main nest and the satellite nests, the gram-positive genus Lactobacillus and the gram-negative genus Brevundimonas were present in great abundance in the ant cuticle from both types of nests. Species of the genus Brevundimonas are widely known as opportunistic pathogens causing human infections, but they have already been found in various environments (LIU & al. 2021), including the plant rhizosphere as a growth-promoting bacterium (KUMAR & GERA 2014). Thus, it is possible that foraging ants acquired these bacteria from the surrounding environment (ROCHA & al. 2023). Moreover, strains of Lactobacillus (Firmicutes) have antibiotic resistance (ANISIMOVA & YARULLINA 2019), providing greater protection for workers, consequently helping to optimize the traffic of the supercolony and foraging activity (LANDA & TULLOCK 2003).

Bacterial communities vary more within polydomous ant colonies than among plant individuals. Ant bacterial communities exhibit colony-specific signatures (CHUA & al. 2018, RONQUE & al. 2020). This phenomenon can be attributed to both genetic variation within the same ant species (HU & al. 2014) and the microbiome's production of odors in individuals from the same colony, which plays a vital role in nestmate recognition (DOSMANN & al. 2016). In contrast, bacterial communities in the phyllosphere show greater specificity within the same plant species (REDFORD & al. 2010). LAFOREST-LAPOINTE & al. (2016) showed that the identity of the plant species is what explains the variation in the structure of phyllosphere bacterial communities, more than individual identity or the location of leaves in the canopy.

When comparing trees with and without ant nests, we found lower alpha diversity in ant-foraged leaves, and more than half of the bacteria were classified as specialists. This suggests that ant presence may influence the phyllosphere bacterial community (NADARASAH & STAVRINIDES 2011). A species of the genus *Methylobacterium* was abundant on leaves with and without ants. It is known that this genus is commonly found in the phyllosphere (KUTSCHERA 2007, HOLLAND 1997), promoting plant growth (DOURADO & al. 2015). Lactobacillus can be considered as a specialist of the ant's cuticle, and it was not recorded on leaves foraged by ants. This genus was found to be dominant in the infrabuccal pockets and crops of ants that feed on aphid honeydew (ZHENG & al. 2022) and can be acquired from the environment rather than acquired vertically (KELLNER & al. 2015). Mucilaginibacter sp. 1 and Massilia sp. 1 were considered as specialists in ant cuticles from satellite nests, also occurring on leaves foraged by ants. These genera had already been found in plant rhizosphere (MADHAIYAN & al. 2010) and in the black ant Polyrhachis (OSIMANI & al. 2018). Indeed, insects are known to carry bacteria to leaf surfaces, facilitating colonization (WHIPPS & al. 2008). Therefore, further investigation is needed on the role of these species in tropical canopy phyllosphere and on how the presence of ants is related to low diversity and high specificity to some bacteria groups.

In conclusion, bacterial communities on ant cuticles show inter-nest variation across main and satellite nests of polydomous *Azteca chartifex*. Some generalist bacteria shared between nest types may have been acquired from the surrounding environment or from ant traffic among nest units. Bacterial communities' composition on leaf phyllospheres from ant-colonized and ant-free trees are different. Ant presence can decrease bacterial richness and share some bacteria with the leaves they forage on. Therefore, transient or even symbiotic bacteria are components of tripartite interactions involving ants and plants. Future investigations on the functional and ecological role of bacteria found in this system are essential to understand the interactive interface of the bacterial communities associated with ants and plants.

Author contributions

MRB, AGN and SPR designed the study. MRB and SPR contributed to fieldwork. MRB and LMT conducted all laboratory work. MRB, FVC, and RBK conducted statistical and bioinformatic analyses. MRB and SPR lead the manuscript preparation, and all authors read and approved the final manuscript.

Declaration on use of generative artificial intelligence tools

The authors declare that they did not utilize generative artificial intelligence tools in any part of the composition of this manuscript.

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Data availability

All raw sequence data were deposited in the NCBI Sequence Read Archive (accession number PRJNA1100516).

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