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MARILIA ROMÃO BITAR DE BRITO

ESPÉCIE DE FORMIGA DOMINANTE NO DOSSEL E MICROBIOTA ASSOCIADA: ADAPTAÇÕES COMENSAIS À AMBIENTES ADVERSOS

CANOPY DOMINANT ANT SPECIES AND ASSOCIATED MICROBIOTA: COMMENSAL ADAPTATIONS TO HARSH ENVIRONMENTS

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“Life on earth is more like a verb. It repairs, maintains, re-creates, and outdoes itself.”

Lynn Margulis

“A vida na Terra é mais como um verbo. Ela repara, mantém, recria e se supera”

Lynn Margulis

RESUMO

Organismos multicelulares compartilham uma longa história evolutiva com microrganismos. Os avanços modernos resultantes das pesquisas de metagenômica evidenciaram que as relações mutualísticas têm um papel central e determinante na existência de todas as formas vivas multicelulares. Conhecer a estrutura e diversidade das comunidades bacterianas é crucial para entender aspectos do funcionamento do ecossistema. Uma forma de entender a estrutura de uma comunidade bacteriana, além da composição, é quantificar as proporções de bactérias Gram-positivas e Gram-negativas. Nas formigas, a estrutura e diversidade das comunidades bacterianas associadas podem variar de acordo com sua dieta, casta, estágio de desenvolvimento, espécie e habitat. Assim, o ambiente ao redor da colônia tem grande influência em suas comunidades bacterianas. Através de uma revisão sistemática da literatura, de coleta de dados de campo e de técnicas de sequenciamento de nova geração (Next Generation Sequencing - NGS), o presente estudo teve como objetivo geral analisar as comunidades bacterianas associadas às formigas em diferentes ambientes. No primeiro capítulo, nós realizamos uma revisão sistemática em que buscamos estudos que analisaram as comunidades bacterianas associadas ao intestino e aos corpos de formigas de diferentes espécies, habitats e regiões climáticas. Calculamos as proporções de bactérias Gram-negativas destes microbiomas em diferentes ambientes, por serem mais resistentes à condições extremas. Logo, vimos que formigas que habitam ambientes imprevisíveis que produzem condições extremas para a sobrevivência dos insetos, como regiões temperadas e dosséis (formigas arborícolas), têm maior proporção de bactérias Gram-negativas nos seus microbiomas comparadas às formigas das regiões tropicais e que habitatm os solos. No segundo capítulo, investigamos as comunidades bacterianas associadas à uma formiga dominante do dossel, *Azteca chartifex* (Dolichoderinea), e as comunidades bacterianas associadas à filosfera das folhas de sua planta hospedeira, *Byrsonima sericea* (Malpighiaceae). Analisamos e comparamos a diversidade e composição das comunidades bacterianas dos corpos das formigas de ninhos polidômicos e das superfícies das folhas de plantas colonizadas e não-colonizadas pelas formigas. Os resultados revelaram significante variação nas comunidades bacterianas dos corpos das formigas de ninhos matriz e satélites. As bactérias generalistas compartilhadas entre formigas de ambos os ninhos podem ter sido adquiridas diretamente do ambiente ao redor ou entre forrageiras dos diferentes ninhos. Além disso, a presença das formigas nas árvores influenciam a composição das comunidades bacterianas das superfícies das folhas, diminuindo a diversidade de bactérias e

compartilhando bactérias entre formigas e folhas. No terceiro e último capítulo, investigamos as comunidades bacterianas da mesma espécie de formiga arborícola, porém de um ambiente contaminado por metais pesados devido ao rompimento de uma barragem de mineração. Comparamos as comunidades bacterianas dos corpos das formigas em ambiente afetado e de um ambiente protegido (dados do segundo capítulo). Vimos que as formigas dos ambientes contaminados exibiram maior alpha diversidade nas comunidades bacterianas associados aos seus corpos, e também apresentaram diferente composição bacteriana comparadas às formigas das áreas protegidas. A presença de bactérias bioindicadoras específicas das áreas contaminadas sugere o potencial destas bactérias em moldar as comunidades bacterianas associadas às formigas.

ABSTRACT

Multicellular organisms share a long evolutionary history with microorganisms. Modern advances resulting from metagenomic research have highlighted that mutualistic relationships play central and determinant role in the existence of all multicellular life forms. Understanding the structure and diversity of bacterial communities is crucial for understanding aspects of ecosystem functioning. In addition to the composition, one way to understand the structure of a bacterial community is to quantify the proportions of Gram-positive and Gram-negative bacteria. In ants, the structure and diversity of associated bacterial communities can vary according to their diet, caste, developmental stage, species, and habitat. Thus, the environment surrounding the colony has a significant influence on their bacterial communities. Through a systematic literature review, field data collection, and next-generation sequencing (NGS) techniques, this dissertation aimed to analyze the bacterial communities associated with ants in different environments. In the first chapter, we conducted a systematic review in which we looked for studies that analyzed the bacterial communities associated with the gut and bodies of ants from different species, habitats, and climatic regions. We calculated the proportions of Gram-negative bacteria in these microbiomes in different environments, as they are more resistant to extreme conditions. We found that ants inhabiting unpredictable environments that produce extreme conditions for insect survival, such as temperate regions and canopies (arboreal ants), have a higher proportion of Gram-negative bacteria in their microbiomes compared to ants from tropical regions and ground habitats. In the second chapter, we investigated the bacterial communities associated with a canopy dominant ant, *Azteca chartifex* (Dolichoderinea), and the bacterial communities associated with the phyllosphere of its host plant, *Byrsonima sericea* (Malpighiaceae). We analyzed and compared the diversity and composition of the bacterial communities from the bodies of ants from polydomous nests and the leaf surfaces of colonized and non-colonized plants. The results revealed significant variation in the bacterial communities of the ants' bodies from both the main and satellite nests. The generalist bacteria shared between ants from both nests may have been acquired directly from the surrounding environment or between foragers from different nests. Additionally, the presence of ants on trees influences the composition of bacterial communities on the leaf surfaces, decreasing bacterial diversity and sharing bacteria between ants and leaves. In the third and final chapter, we investigated the bacterial communities of the same species of arboreal ant, but from an environment contaminated by heavy metals due to the rupture of a mining dam. We compared the

bacterial communities on the ants' bodies in the affected environment with those from a protected environment (data from the second chapter). We found that ants from contaminated environments exhibited higher alpha diversity in the bacterial communities associated with their bodies and also had a different bacterial composition compared to ants from protected areas. The presence of specific bioindicator bacteria in the contaminated areas suggests the potential of these bacteria to shape the bacterial communities associated with ants.

SUMÁRIO

INTRODUÇÃO

Organismos multicelulares compartilham uma longa história evolutiva com microrganismos. Para os animais, as bactérias desenvolveram um papel importante para a sua nutrição, fisiologia (Mcfall-Ngai *et al*., 2012), resposta imune e equilíbrio neurológico (Tizard & Jones, 2018; Zheng *et al*., 2020; Ma *et al*., 2019). Para alguns insetos, o mutualismo com bactérias se tornou altamente especializado, com espécies bacterianas endêmicas ao microbioma originado no corpo desses insetos (Hongoh, 2010; Suenami *et al*., 2023). Os avanços modernos resultantes das pesquisas de metagenômica evidenciaram que as relações mutualísticas têm um papel central e determinante na existência de todas as formas vivas multicelulares (Yun *et al*., 2015; Liu *et al*., 2021; Perreau & Moran, 2022). Interações mutualísticas têm grande importância para a ecologia e evolução da vida na Terra, sendo essenciais para a manutenção dos ecossistemas e da biodiversidade. Segundo Lynn Margulis (1991),

> *"todos organismos evoluíram como um complexo simbiótico envolvendo inúmeras entidades vivas, integradas de diversas maneiras."*

As formigas são insetos eussociais que desenvolveram importantes associações com bactérias ao logo do seu processo evolutivo (Moreau, 2006). As bactérias presentes nos microbiomas das formigas ajudam na sua nutrição (Russell *et al*., 2017), proteção ao produzir antibióticos (Currie *et al*., 1999) e no domínio de novos ambientes (Pringle & Moreau, 2017). Pringle (2019), em uma revisão, concluiu que a associação com bactérias que ajudam na nutrição das formigas possibilitou a colonização de novos habitats. Um exemplo é o das formigas arborícolas herbívoras, do gênero *Cephalotes*, que obtém nutrientes necessários pela ciclagem de nitrogênio realizada pelo seu microbioma intestinal (Hu *et al*., 2018). Sendo assim, para habitar os dosseis florestais, os quais apresentam diferentes disponibilidade de recursos e interações ecológicas (Ribeiro *et al*., 2013), as formigas podem ter desenvolvido importantes associações com bactérias em todo o seu corpo.

As diversas espécies de formigas arborícolas variam em comportamento, estrutura de colônias e densidades populacionais (Rico-Gray & Oliveira, 2007). No entanto, as espécies de maior relevância ecossistêmica são as dominantes de dosséis tropicais (Hölldobler & Wilson, 1990,

Ribeiro *et al*. 2013, Soares *et al*., 2022). Essas formigas desenvolveram diversas estratégias de defesa, como o comportamento agressivo (Beattie, 1985), associado a patrulhamento de território rico em trofobiontes, com alta densidade de operárias (Dejean *et al*., 2007; Adams, 1994), além de produção de secreções antibióticas por glândulas especializadas (Yek & Mueller, 2010). Alguns estudos investigaram o papel das bactérias envolvidas no mutualismo formiga-planta (González-Teuber *et al*., 2014; Lucas *et al*., 2017; Bitar *et al*., 2021; Nepel *et al*., 2023). Contudo, ainda há lacunas no entendimento de como as comunidades bacterianas de formigas arborícolas dominantes se estruturam, e como o ambiente ao redor pode afetar esta configuração.

Conhecer estrutura e diversidade das comunidades bacterianas é crucial para entender aspectos do funcionamento do ecossistema (Zorz *et al*., 2019; Chua *et al*., 2018; Shi *et al*., 2021). Uma forma de entender a estrutura, além da composição, de uma comunidade bacteriana é quantificar as proporções de bactérias Gram-positivas e Gram-negativas. As bactérias Gram-positivas são limitadas por uma única membrana e geralmente possuem uma grossa camada de peptidoglicano. As bactérias Gram-negativas são envoltas por duas membranas diferentes, sendo uma delas externa e constituída de lipopolisacarídeo, que protege a célula contra antibióticos enquanto produzem suas próprias enzimas antimicrobianas, como as lisozimas (Gupta, 2011). Compreender estes dois grupos bacterianos como diferentes guildas ecológicas, devido à composição das membranas que as envolvem, abre janelas para investigar a utilização diferenciada de recursos por distintas comunidades bacterianas (Fanin *et al*., 2019). Do ponto de vista de resistência e competitividade, as bactérias Gram-negativas possuem mais mecanismos para resistir à ambientes extremos, em comparação às Gram-positivas (Ramos *et al*., 2001; Silhavy *et al*., 2010). Sendo assim, as proporções destes dois tipos de bactérias em um microbioma pode elucidar características do hospedeiro e do ambiente ao seu redor.

Portanto, o microbioma associado a um hospedeiro pode ser influeciado por inúmeros fatores. Nas formigas, a estrutura e diversidade das comunidades bacterianas associadas podem variar de acordo com sua dieta (Barcoto *et al*., 2020; deOliveira *et al*., 2016), casta (Koto *et al*., 2020), estágio de desenvolvimento (Ramalho *et al*., 2020), espécie (Ronque *et al*., 2020) e habitat (Rocha *et al*., 2023). Assim, o ambiente ao redor da colônia tem grande influência em suas comunidades bacterianas (Lucas *et al*., 2017). Em um contexto de grandes impactos ambientais causados pela atividade humana, pouco se sabe de como a poluição pode afetar estes microbiomas.

Sabe-se que poluição ambiental, como a presença de metais pesados no ambiente, podem impactar as comunidades bacterianas associadas aos insetos (Rothman *et al*., 2019; Li *et al*., 2021, Wu *et al*., 2022). Contudo, alguns estudo revelam que as formigas podem diminuir a contaminação de metais pesados nos solos (Shi *et al*., 2023) e ter na sua microbiota associada, bactérias com potencial de biorremediação (González-Escobar *et al*., 2020).

Nesta tese foram desenvolvidos 3 estudos que investigam a interação formiga-bactéria. Através de uma revisão sistemática da literatura, de coleta de dados de campo e de técnicas de sequenciamento de nova geração (HTS), estes estudos tiveram como objetivo geral analisar as comunidades bacterianas associadas às formigas em diferentes ambientes. Assim, investigamos quais os fatores ambientais podem afetar a diversidade e estrutura destas comunidades bacterianas.

No primeiro capítulo, nós realizamos uma revisão sistemática em que buscamos estudos que analisaram as comunidades bacterianas associadas ao intestino e aos corpos de formigas de diferentes espécies, habitats e regiões climáticas. Calculando as proporções de bactérias Gram-positivas e Gram-negativas destas microbiotas, nós buscamos testar a hipótese de que existe uma maior proporção de bactérias Gram-negativas nas comunidades bacterianas de formigas que habitam ambientes mais imprevisíveis, variáveis e que são ambientes extremos, dada as condições desafiantes do habitat criado pelo corpo desses insetos e sua vida social.

No segundo capítulo, investigamos as comunidades bacterianas associadas à uma formiga arborícola, *Azteca chartifex* Emery, 1896 (Dolichoderinea), e as comunidades bacterianas associadas à filosfera das folhas de sua planta hospedeira, *Byrsonima sericea* DC. (Malpighiaceae). Neste sistema existe um mutualismo facultativo entre a espécie de formiga arborícola neotropical, que domina o dossel florestal com seus ninhos polidômicos (ninho matriz e vários ninhos satélites) e uma planta nativa da Mata Atlântica, que ocorre ao longo de um ecótono lago-floresta. Assim, analisamos e comparamos a diversidade e composição das comunidades bacterianas dos corpos das formigas e das superfícies das folhas de plantas colonizadas e não-colonizadas pelas formigas. Desse modo, testamos a hipótese de que as comunidades bacterianas associadas às formigas, dos ninhos matriz e satélites, moldam as comunidades bacterianas das folhas que elas forrageam.

No terceiro e último capítulo, investigamos as comunidades bacterianas da mesma espécie de formiga arborícola, porém de um ambiente contaminado por metais pesados devido ao rompimento de uma barragem de mineração. Neste estudo, analisamos a diversidade e composição

das comunidades bacterianas associadas aos corpos das formigas, de um ambiente contaminado pelos rejeitos da mineração da empresa Samarco, resultante do desastre de rompimento de uma grande barragem na bacia do rio Doce, MG, Brasil. Comparamos as comunidades bacterianas dos corpos das formigas em ambiente afetado e de um ambiente protegido (dados do segundo capítulo). Assim, testamos a hipótese de que a presença dos rejeitos da mineração no ambiente em que as formigas arborícolas habitam podem afetar a diversidade e composição das comunidades bacterianas associadas aos seus corpos.

CAPÍTULO 1

INSIGHTS INTO THE ROLE OF GRAM-NEGATIVE BACTERIA IN HOSTS UNDER VARIABLE ENVIRONMENTS: A SYSTEMATIC REVIEW OF ANT BACTERIAL COMMUNITIES

Marilia Romão Bitar, Marianne Azevedo-Silva, Gustavo Romero, Sérvio Pontes Ribeiro

Abstract

The proportion of Gram-positive and Gram-negative (GP-GN) bacteria can provide valuable information on bacterial communities' diversity and composition, being also associated to environmental conditions, and may affect quality of host-microbiota interaction and adaptation. Gram-negative bacteria are more likely to thrive under unpredictable and harsh environments and acquire competitive advantages to occupy ecological habitats with extreme conditions. Based on a systematic review approach, including data from 27 published works, summing up 193 microbiome data outputs, we analyzed the GP-GN bacteria proportion in ant microbiota (both from gut and whole body) and its potential association to environmental conditions at macro and microscales. We hypothesize that, regardless of microbiota type (gut *vs* whole body), the proportion of GN should be higher in environments with higher unpredictability and producing extreme harsh conditions for the insect´s survival. We observed a higher proportion of Gram-negative bacteria in ants from temperate regions worldwide and in the gut bacterial communities of ants from arboreal habitats, compared to tropical regions and ground habitats. These findings underscore the importance of the bacterial communities' structure in ants living in extreme environments and the role of Gram-negative bacteria in dominating and resisting environment variability at both macro and microscale.

Introduction

Bacterial communities or microbiota are largely recognized by their roles in insect behavior, ecology and evolution (Mondal *et al*., 2023; Zhang & Xu, 2023). For instance, the interaction between the hosts and their associated microorganisms impact insect survival under extreme environmental conditions (Gupta & Nair, 2020). Moreover, bacteria present in the insect's gut can provide different metabolic pathways adapted to diverse ecological niches, impacting the host nutrition, development, and defense against pathogens (Chen *et al*., 2016).

Insect's bacterial community is diverse and can be species-specific (Mondal *et al*., 2023). Geographic gradients (latitude and altitude), and local environmental factors (such as mean annual temperature and soil properties) can impact the global patterns of insect microbiota distribution (Lange *et al*., 2023; Magoga *et al*., 2023). These factors can shape the environmental microbiota that insects are exposed to, thus impacting the structure of their own associated microbiota (Hannula *et al*., 2019; Harvey *et al*., 2022). Additionally, temperature, precipitation, latitude, and longitude were found to be good predictors of symbiont abundance associated to insecticides resistance in the brown planthopper, *Nilaparvata lugens* (Zhang *et al*., 2023), suggesting that climate factors may shape microbiota and, in turn, influence the host interaction with the environment.

Moreover, quantifying Gram-positive and Gram-negative bacteria can provide valuable information on microbiota diversity and composition, being also associated to environmental conditions (Cao *et al*., 2021; Fanin *et al*., 2019;). Gram-positive and Gram-negative bacteria have distinct physiological and metabolic characteristics due to their different cell-membrane (Silhavy *et al*., 2010). Characterized by an outer membrane containing lipopolysaccharides and an inner membrane of peptidoglycan, the structure of Gram-negative bacteria provides protection against antibiotics and allows the production of antimicrobial enzymes (Gupta, 2011). Thus, Gram-negative bacteria are more likely to thrive under diverse and harsh environments and acquire competitive advantages (Atanaskovic *et al*., 2022; Schwechheimer *et al*., 2013), which could be extended to their hosts.

Ants, one of the most diverse and dominant groups among terrestrial insects, have had their evolutionary history and success influenced by interactions with microorganisms (Boursaux-Eude

& Gross, 2000; Moreau *et al*., 2020). This symbiotic relationship has shaped the evolutionary trajectory and ecological success of ants (Russell *et al*., 2009; Pringle & Moreau, 2017). Several factors can influence ants' microbiota including diet (Hu *et al*., 2014), social interactions (Ivens *et al*., 2018), the environment (Lucas *et al*., 2017), colony structure (Green & Klassen, 2022), invasiveness (Hu *et al*., 2016), vertical transmission (Zhukova *et al*., 2017), pathogen pressure (Sapountzis *et al*., 2018) and genetics (Segers *et al*., 2019). Indeed, the diversity and composition of ant-associated bacterial community can reflect on various aspects of ant biology and ecology (Lucas *et al*., 2019; Ronque *et al*., 2020; Rocha *et al*., 2023). Therefore, understanding the structure and composition of ant bacterial communities is essential for comprehending how these symbiotic relationships contribute to ant fitness, ecological success, and adaptation to different environmental conditions.

There is evidence that temperature increments can change the abundance and composition of ant-associated bacteria in both field and laboratory experiments (McMunn *et al*., 2022). Additionally, the number of Gram-negative bacteria is suggested to be important, for example, for arboreal ants as a strategy to outcompete Gram-positive leaf bacteria through overgrowth (Bitar *et al*., 2021). Despite the importance and potential to understand ant adaptation, no previous study investigated the GP-GN bacteria proportion in hosts' microbiota. From an ecological point of view, quantifying the ratio of these two bacterial groups would shed light on how this bacterial community structure can benefit host adaptation to distinct environmental conditions.

Based on a systematic review, including data from 27 published works, we analyzed the GP-GN bacteria proportion in ant microbiota (both from gut and whole body) and its potential association to environmental conditions at macro and microscales. Given Gram-negative bacteria are more likely to thrive under variable environmental conditions, we hypothesize that, regardless of microbiota type (gut *vs* whole body), the proportion of GN should be higher in environments under higher unpredictability and producing extreme harsh conditions for the insect´s survival. Thus, we predicted that GN proportion would be higher in ant microbiota from temperate zones compared to the tropical ones, because temperate climate greatly varies over the year. Moreover, we predicted that GN bacteria proportion would be higher in microbiota of arboreal, mainly forest canopy, ants than ground ants, as ground microclimate tends to be more stable than microclimate on the canopy surface. Finally, we predicted that invasive ants would be associated with more GN

bacteria than native ants, given that unknown environmental conditions are constantly experienced by invasive species (Fig1). This study is the first systematic review that shows that the proportion of Gram-negative bacteria in ant's microbiome is indeed associated to more variable environmental conditions for both macro and microscale, shedding light on microbiome contribution to ant radiation, adaptation, and evolution.

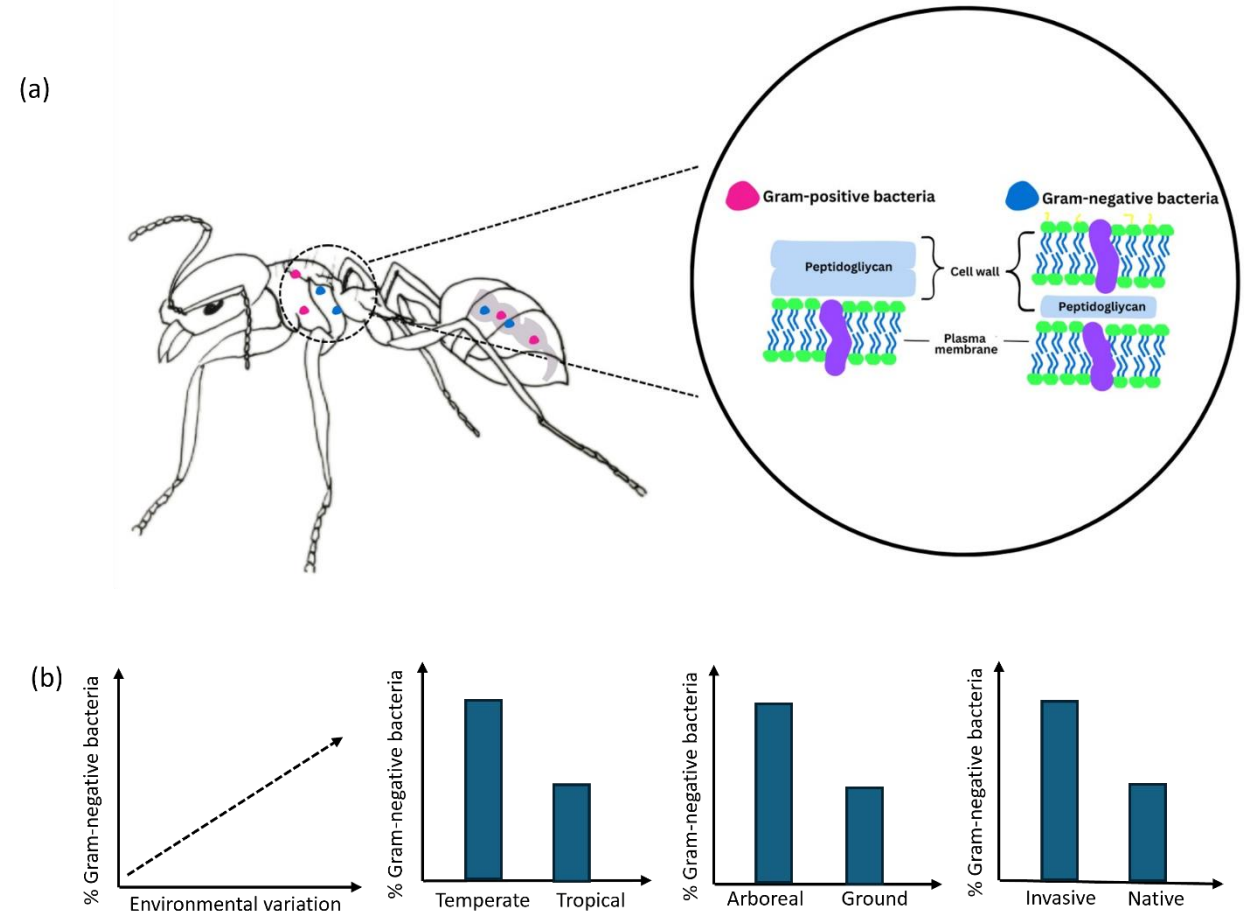

Fig 1 – (a) Scheme showing ant's microbiota (gut and whole body) and the difference in membrane composition of Gram-positive and Gram-negative bacteria. (b) Graphs of the hypothesis tested in the present review.

Material and methods

Systematic literature review and exclusion criteria

To assess the proportion of Gram-positive and Gram-negative bacteria in ants' microbiomes, a comprehensive search was conducted for peer-reviewed studies that analyzed the bacterial communities of ants. Databases such as Scopus and Web of Science (title, abstract and keywords= (ant AND 16S*) OR (ant AND bacterial communities*) were used to search for articles from 2000-2021. Only articles published in English were included. Initial searches yielded a total of 231 papers from Web of Science and 285 papers from Scopus. Exclusion criteria were applied to filter out studies not directly relevant to the review's objectives. Articles without ants or lacking 16S rRNA amplicon sequencing, as well as those focusing solely on the microbiota of ants' surrounding environments (nest, fungus garden, dump or soil surrounded or employing culture-dependent methods), were excluded. Additionally, studies investigating specific symbionts or lacking essential data such as total sequence numbers and relative abundance of bacterial phylum were also excluded. From each study, we collected information on ant species, caste, microbiota type (whole body or gut), ant habitat type (ground or arboreal), diet, geographic regions, and total number of reads. The proportion of Gram-negative and Gram-positive bacteria was determined based on the relative abundance of phyla within each sample. Phylum abundances of less than 1% were classified as "others". When available, this information was obtained using the number of reads; otherwise, information was extracted from relative abundance bar plots using ImageJ software (Schneider *et al*., 2012). Finally, 27 studies were evaluated. Given a single study can contain more than one bacterial community information, from those 27 studies, we analyzed 193 bacterial communities' data outputs. This study followed the instructions of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Page *et al*., 2020) (Fig 2).

A hierarchical key scheme was constructed to assess the distribution of the studies across distinct categories (Figure S1). The studies were classified for: Microbiota (whole body or gut), Macroscale environment (temperate or tropical), invasiveness (native or invasive), microscale environment (arboreal or ground), and diet (omnivorous, herbivore, predator or fungivore). However, as ant diet is correlated to habitat and macroscale environment this category was excluded from the analyses but discussed based on ecological traits of each guild. For instance, herbivore ants were all arboreal while fungivore ants were only present at ground in neotropical regions (Fig S2).

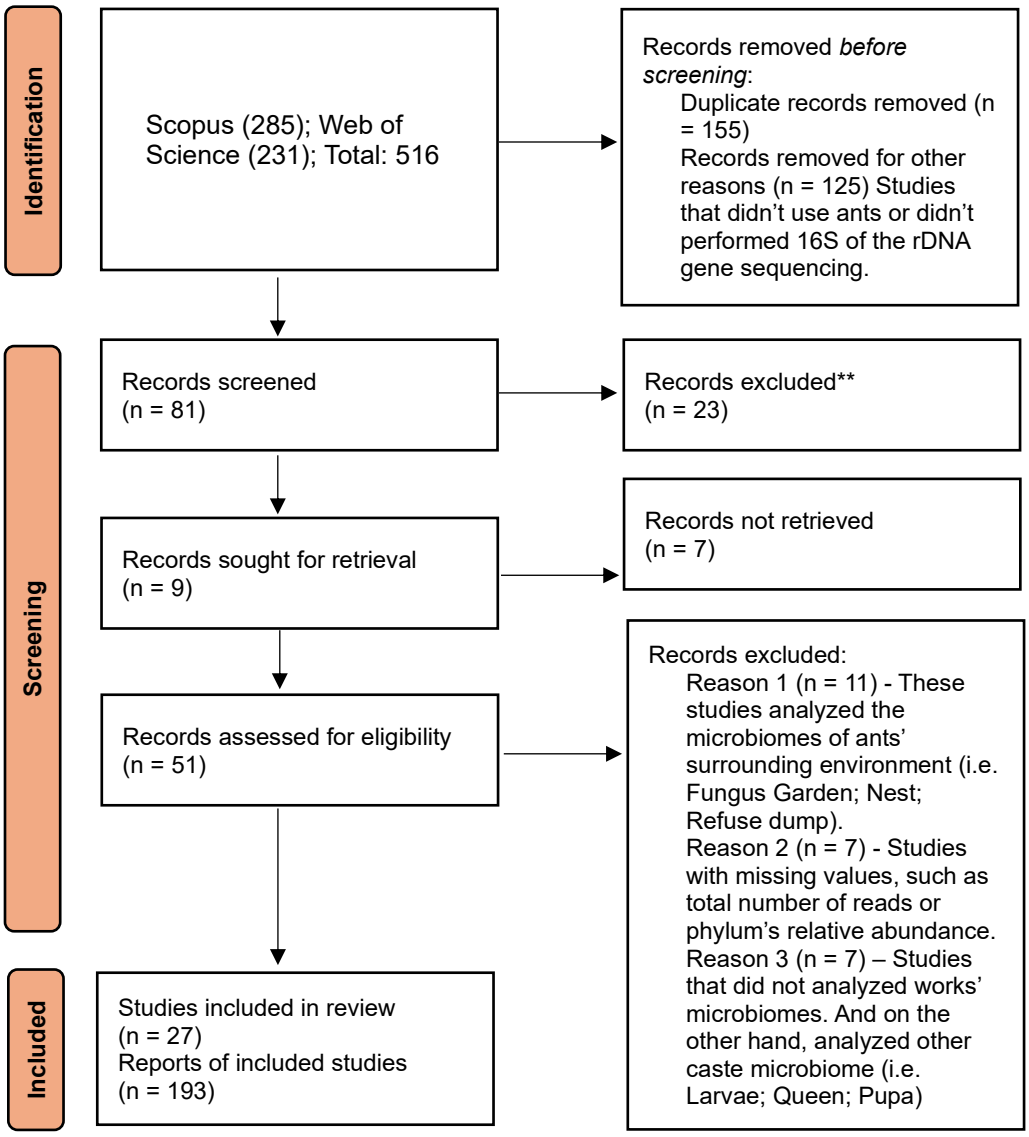

Fig 2 – Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) filtration of journal articles.

Statistical analysis

The proportion of Gram-negative bacteria was analyzed in response to macroscale environment for whole body and gut microbiota, separately. However, given there were few records on whole body microbiomes of arboreal ants in the tropics, for microscale analyses, we only analyzed gut microbiota. Generalized linear mixed effect models (GLMM) were performed, using binomial distribution. We used the GlmmTMB package (Brooks *et al*., 2017), which accounts for

zero-inflated data. Studies were included as random effect. To test significant effects of climate and habitat on the GN proportion in ant's microbiota, the Analysis of variance was used. We used ggplot2 and sciplot packages to construct the graphs. All statistical analyses were made using R software (version 4.3.0) (R Core Team, 2021).

Results

We analyzed ant bacterial communities from 53 species and 29 genera, from 27 studies (Supplementary Material). When analyzing both macro and microscale at the same time, we found that ants from ground habitats in temperate regions had a higher proportion of Gram-negative bacteria in their gut microbiota, while arboreal ants had high proportion of those bacteria on both climate regions. On the other hand, there was a greater proportion of GN bacteria in ant gut microbiota in arboreal than ground ants in tropical regions (Habitat: Chisq = 2633.3, Df=1, p< 2.2e-16; Climate: Chisq = 824.57, Df = 1, p< 2.2e-16; Fig 3a). The proportion of Gram-negative bacteria for whole body, analyzing the invasiveness and climate, was higher for native ants from temperate regions than any other combination (Invasivenesse: Chisq = 5141.8, Df = 1, p< 2.2e-16; Climate: Chisq = 10449.8, Df = 1, p< 2.2e-16; Fig 3b), thus, not supporting our hypothesis prediction.

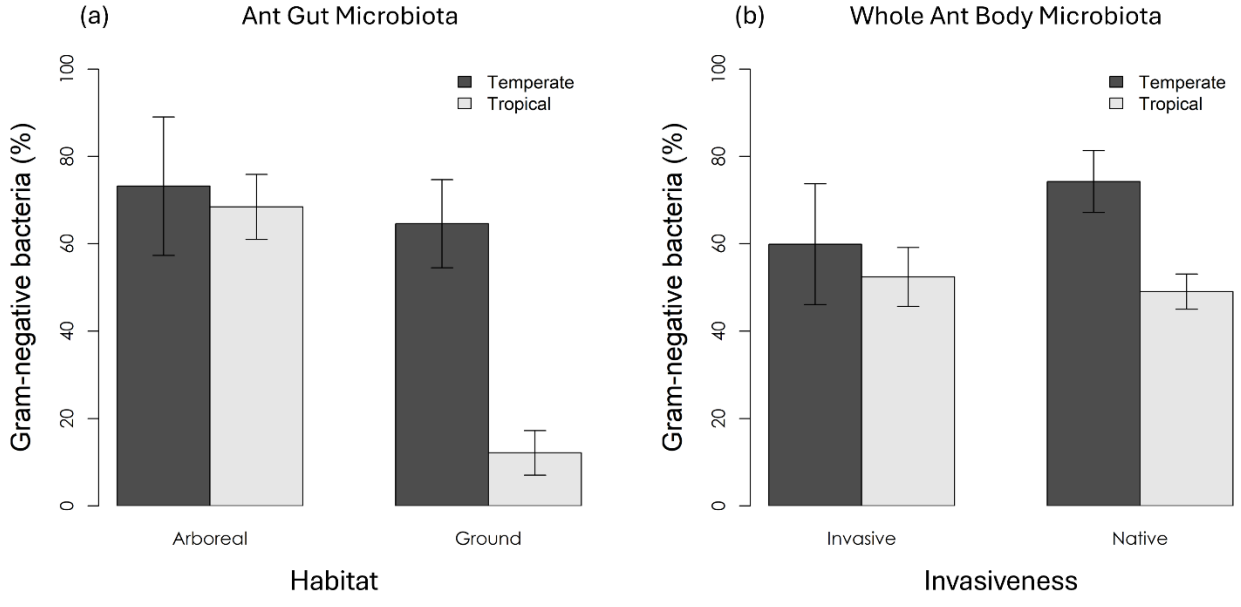

Fig. 3 Proportion of Gram-negative bacteria in ant's gut microbiota from different microscale habitats and macroscale environments. (a) arboreal *versus* ground habitats from temperate *versus* tropical regions. (b) invasive *versus* native ants from temperate *versus* tropical regions.

When analyzing the microscale (arboreal and ground habitats) and macroscale (temperate and tropical regions) separated, the proportion of Gram-negative bacteria in ant gut microbiome of arboreal ants was higher than the ground ones (Fig S2), and ants from temperate regions presented higher proportions of Gram-negative bacteria in both whole body and gut microbiomes (Fig S3).

Discussion

In this systematic review, which includes microbiomes of 53 ant species across different climate regions and habitats, we found greater proportion of Gram-negative bacteria in both ants' whole body and gut microbiota from environments under higher unpredictability and producing extreme harsh conditions for the insect´s survival. Namely, we observed a higher proportion of Gram-negative bacteria in ants from temperate regions worldwide, and in the gut microbiota of ants from arboreal habitats.

External host-associated bacterial communities are influenced by climate, while internal bacterial communitiess are shaped by immunity complexity, trophic level, and climate (Woodhams *et al*., 2020). Insects' microbiotas adapt to daily and seasonal temperature fluctuations, enhancing resistance to abiotic stress (Ferguson *et al*., 2018; Ren *et al*., 2023). Therefore, the higher proportion of Gram-negative (GN) bacteria in ants' microbiota may provide resistance to greater temperature variability in temperate regions. GN bacteria exhibit evolutionary responses to temperature variation, such as the synthesis of specialized proteins (Ramos *et al*., 2001). Consequently, understanding the dynamics of these insect-bacteria associations in the context of climate change, which exhibits complex variability at spatial and temporal scales, is essential (Iltis et al., 2021).

Ants living in arboreal habitats are also exposed to greater temperature variability than ants from ground habitats. Forest canopies often have vertical stratification, where the upper canopy can receive more sunlight directly and be warmer than the lower canopy and the ground (Didham & Ewers, 2014; Vinod *et al*., 2022). Studies had already shown that arboreal ants have a higher

thermal tolerance and resilience to climatic variance compared to ground ants (Leahy *et al*., 2020; Leong *et al*., 2020). Hence, ant-associated bacteria can confer host tolerance facing environmental variability. This information has been shown for other insects (Grutenko *et al*., 2017; Fergurson et al., 2018; Lemoine *et al*., 2020). The hypothesis that invasive ants would be associated with more GN bacteria was refuted. Indeed, native ants from temperate regions presented higher proportions of GN bacteria, that can be explained by host adaptation to temperate climate. The microbiota of invasive insects can have lower diversity and different composition compared to native ones (Li *et al*., 2021). Overall, the differences in microbiotas between invasive and native ants is primary associated to the gut and related with trophic relations (Hu *et al*., 2016).

Ants' species have a wide dietary niche, they can be predator, herbivore, fungivore, detritivore or omnivore. The availability of food resources in their habitats shapes their foraging pattern and influences their trophic interactions within the ecosystem. In our data, specialized diets such as herbivory are related to arboreal ants, and most of predator ants were found to live on the ground. The fungivore were restricted to ants of the Attine Tribe, which make their nests below ground. Moreover, mutualistic microbes have benefitted the ant's dietary specialization and, consequently, the ecological dominance (Pringle, 2019). For example, bacteria of the genus *Blochmania* provide essential amino acids to carpenter ants (Feldhaar *et al*., 2007). Bacteria in this genus inhabits specialized cells and has evolved with the highly diverse and cosmopolitan Camponotini groups for over 40 million years (Wernegreen *et al*., 2009). In a tropical forest canopy, *Cephalotes setulifer* ants and their scale insect partners harbor microbial symbionts that help optimize the nutritional quality of the phloem sap they consume (Pringle & Moreau, 2017). The Attini ants have a symbiotic interaction with fungi, which they cultivate for food, and their gut microbiota are dominated by Mollicutes, Proteobacteria and Actinobacteria (Sapountzis *et al*., 2019). Mollicutes is a bacteria class that belongs to Firmicutes phylum. This bacteria class is characterized by the absence of cell wall, although they originated by gram-positive bacteria (Tully, 1993). Hence, the data analyzed by this systematic review shows that ants from the ground are mostly fungivore and predators, and their associated-gut microbiota is dominated by bacteria that lack a cell wall and gram-positive bacteria, respectively. On the other hand, the arboreal ants, mostly herbivore, are dominated by gram-negative bacteria in their gut microbiota.

Here, we analyzed the proportion of Gram-negative bacteria in ants' microbiota and found variations related to micro and macroscale environments. We have corroborated the first two hypotheses that there is a higher proportion of Gram-negative bacteria in more variable environments. Then, ants that live in temperate regions and arboreal habitats have more Gram-negative bacteria in their bacterial communities than ants living in tropical regions and ground habitats. These findings underscore the importance of the bacterial community' structure in ants living in extreme environments and the role of Gram-negative bacteria in dominating and resisting environment variability at both macro and microscale.

Supplementary Information

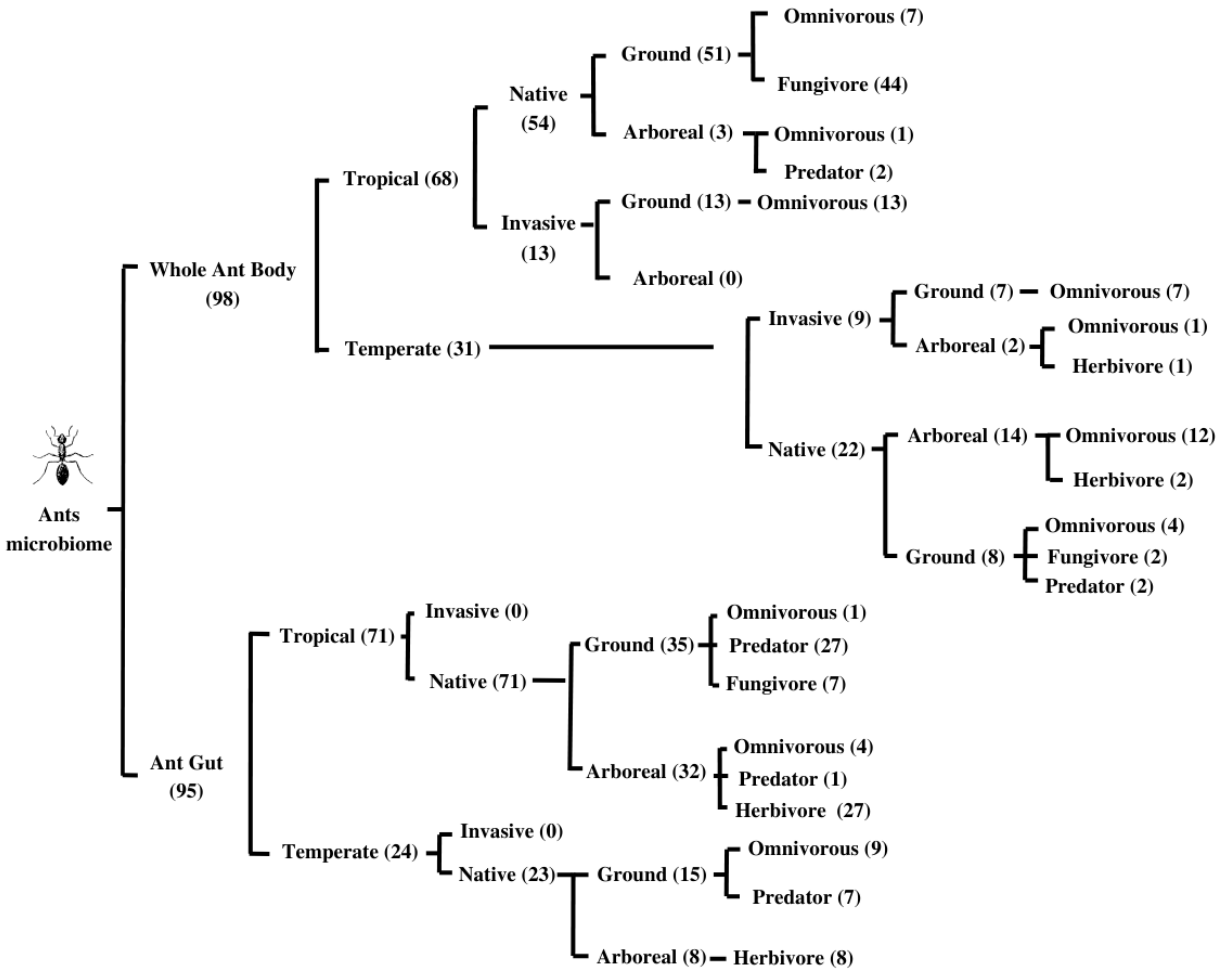

Fig S1 - Hierarchical organization of predictors tested and the number of outputs in each category in parentheses.

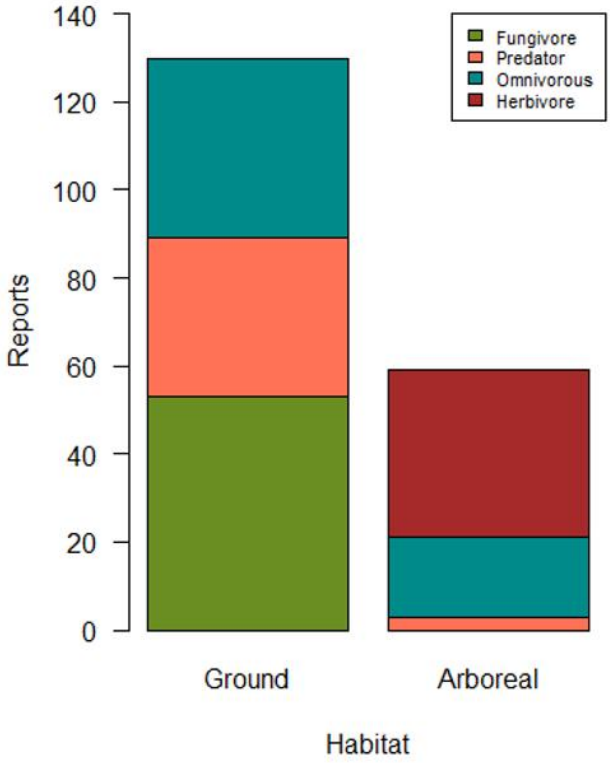

Fig S2 – Barplot showing the number of reports analyzing ants' microbiota from Ground and Arboreal habitats. Different colors represent the ants' diets corresponding to their habitats.

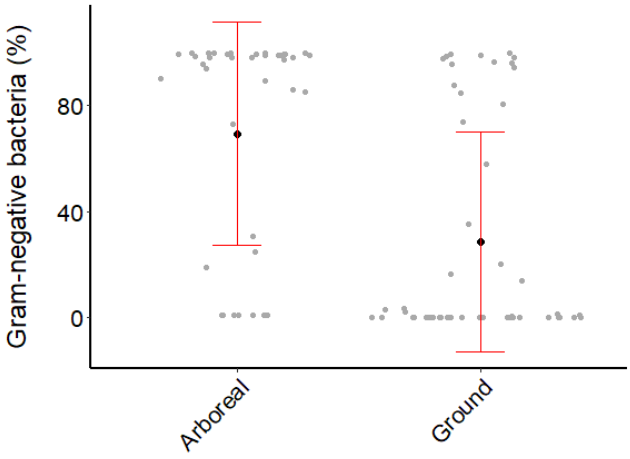

Fig S3 - Comparison of the proportion of Gram-negative bacteria in ant's gut microbiota between microscale environments (arboreal *versus* ground habitats).

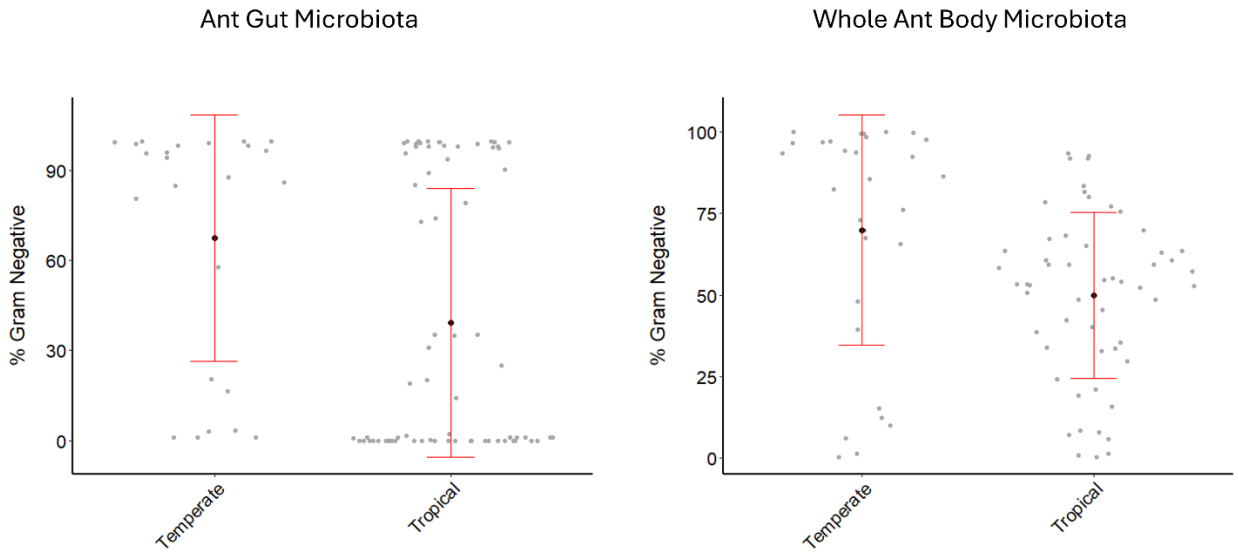

Fig S4 - Comparison of the proportion of Gram-negative bacteria in microbiota of ant's (a) gut and (b) whole body between macroscale environments (temperate *versus* tropical regions).

Table S1 – Summary of studies included in the systematic review of ant bacterial communities. This table lists the studies analyzed, including the ant species, subfamily/tribe, caste, diet, habitat, biogeographic region, climatic zone, invasiveness, microbiota type studied, proportion of Gram-negative bacteria, proportion of Gram-positive bacteria, proportion of other bacterial types (Mollicutes class), total number of reads per sample, and the number of Gram-negative bacteria detected.

AuthorYear	Ant	Subfamily/Tribe	Castes	Diet	Habitat	Biogeographic Region	Climate Region	Invasiveness	Microbiota	G- (%)	G+(%)	Others	Reads	GramNeg
Ashigar2021	*Pheidole rugaticeps (Hospitals)*	Attini	Worker	Omnivorous	Ground	Afrotropical(Ethiopian)	Tropical	Native	Whole_Ant_Body	42.38	57.03	8109	14073	5964
Ashigar2021	*Pheidole rugaticeps (Adm areas)*	Attini	Worker	Omnivorous	Ground	Afrotropical(Ethiopian)	Tropical	Native	Whole_Ant_Body	54.07	44.44	6565	14293	7728
Ashigar2021	*Pheidole rugaticeps (Residencial)*	Attini	Worker	Omnivorous	Ground	Afrotropical(Ethiopian)	Tropical	Native	Whole_Ant_Body	81.67	18.26	3352	18285	14933
Brown-Wernegreen2016	*Camponotus chromaiodes (DF 798)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	1	0	78	79	1
Brown-Wernegreen2016	*Camponotus chromaiodes (DNP 789)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	84.89	15.11	52	344	292
Brown-Wernegreen2016	*Camponotus chromaiodes (DF 799)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	96.6	3.33	5	150	145
Brown-Wernegreen2016	*Camponotus chromaiodes (DF 800)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	98.17	1.82	4	219	215
Brown-Wernegreen2016	*Camponotus chromaiodes (DNP 791)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	98.3	1.7	6	353	347
Brown-Wernegreen2016	*Camponotus chromaiodes (DNP 793)*	Camponotini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Ant_Gut	99.03	0.97	4	412	408
Chua2018	*Oecophylla smaragdina (U2)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	39.3	60.7	100004	164752	64748
Chua2018	*Oecophylla smaragdina (U4)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	65.71	33.8	92729	270427	177698
Chua2018	*Oecophylla smaragdina (PGA1)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	67.45	21.5	45117	138607	93490
Chua2018	*Oecophylla smaragdina (U3)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	69.9	30.1	75240	249966	174726
Chua2018	*Oecophylla smaragdina (PGA4)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	76.1	23.6	48344	202278	153934
Chua2018	*Oecophylla smaragdina (F4)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	82.37	3	39913	226395	186482
Chua2018	*Oecophylla smaragdina (F1)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	85.61	14.39	46775	325050	278275
Chua2018	*Oecophylla smaragdina (U1)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	93.54	0.59	11742	181763	170021
Chua2018	*Oecophylla smaragdina (PGA2)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	94.22	5.78	12931	223719	210788
Chua2018	*Oecophylla smaragdina (F3)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	97.12	1.66	6318	219361	213043

Chua2018	*Oecophylla smaragdina (F2)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	97.7	2.26	5148	223814	218666
Chua2018	*Oecophylla smaragdina (PGA3)*	Oecophyllini	Worker	Omnivorous	Arboreal	Oriental	Temperate	Native	Whole_Ant_Body	99.53	0.25	1118	237945	236827
Cooling2017	*Anoplolepis gracilipes (Low abundance)*	Plagiolepidini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	0.77	15.6	47832	48203	371
Cooling2017	*Anoplolepis gracilipes (High abundance)*	Plagiolepidini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	40.07	23.3	37469	62522	25053
Cooling2017	*Anoplolepis gracilipes (Medium abundance)*	Plagiolepidini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	83.4	9.1	13327	80282	66955
González-Escobar2018	*Liometopum apiculatum (2)*	Tapinomini	Worker	Omnivorous	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	91.8	8.2	1229	14990	13761
González-Escobar2018	*Liometopum apiculatum (3)*	Tapinomini	Worker	Omnivorous	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	91.9	8.1	412	5082	4670
González-Escobar2018	*Liometopum apiculatum (1)*	Tapinomini	Worker	Omnivorous	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	92.7	7.3	350	4795	4445
Hernández2017	*Allomerus octoarticulatus*	Attini	Worker	Omnivorous	Arboreal	Neotropical	Tropical	Native	Ant_Gut	30.9	48.6	13820	20000	6180
Hosmath2019	*Camponotus*	Camponotini	Worker	Omnivorous	Ground	Oriental	Tropical	Native	Ant_Gut	1.56	97.56	18135	18422	287
Hosmath2019	*Oecophylla*	Oecophyllini	Worker	Predator	Arboreal	Oriental	Tropical	Native	Ant_Gut	25.09	73.7	116906	156062	39156
Hu2014	*Cephalotes varians (YH064)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	1	0	12910	13040	130
Hu2014	*Cephalotes varians (YH075)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	1	0	11139	11252	113
Hu2014	*Cephalotes varians (CSM2037)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	85.95	11.95	624	4442	3818
Hu2014	*Cephalotes varians (CSM1980)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	98.89	0.88	88	7926	7838
Hu2014	*Cephalotes varians (CSM1957)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	99.49	0	94	18412	18318
Hu2014	*Cephalotes varians (CSM1973)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	99.78	0.15	31	14209	14178
Hu2014	*Cephalotes varians (CSM1884)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	99.81	0	36	18822	18786
Hu2016	*Linepithema humile (Large)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	12.43	85.09	313160	357611	44451
Hu2016	*Linepithema humile (Argentina)*	Leptomyrmecini	Worker	Omnivorous	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	32.8	58.71	101854	151568	49714
Hu2016	*Linepithema humile (Lake Skinner)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	92.38	0.07	12557	164786	152229
Hu2016	*Linepithema humile (Lake Hodges)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	98.35	0.11	1606	97328	95722
Ishak2011a	*Solenopsis germinata*	Solenopsidini	Worker	Omnivorous	Ground	Nearctic	Temperate	Native	Whole_Ant_Body	1.37	1.55	18322	18576	254
Ishak2011a	*Solenopsis invicta*	Solenopsidini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	15.41	59.71	11614	13730	2116
Ishak2011b	*Trachymyrmex septentrionalis*	Attini	Worker	Fungivore	Ground	Nearctic	Temperate	Native	Whole_Ant_Body	10.15	74.3	2617	2913	296
Ishak2011b	*Trachymyrmex septentrionalis*	Attini	Worker	Fungivore	Ground	Nearctic	Temperate	Native	Whole_Ant_Body	6.15	82.41	6859	7308	449
Kaczmarczyk-Ziemba2020a	*Formica polyctena (Dlugie Lake)*	Formicini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Whole_Ant_Body	93.55	5.99	23671	366988	343317
Kaczmarczyk-Ziemba2020a	*Formica polyctena (Lipniak village)*	Formicini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Whole_Ant_Body	96.62	2.9	10166	300783	290617
Kellner2015	*Mycocepurus smithii*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	21.16	74.19	20901	26511	5610
Lester2017	*Linepithema humile (Davis)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	0.22	0.78	5958	5971	13

Lester2017	*Linepithema humile (Entre Rios)*	Leptomyrmecini	Worker	Omnivorous	Ground	Neotropical	Tropical	Invasive	Whole_Ant_Body	24.14	75.19	1376	1814	438
Lester2017	*Linepithema humile (Buenos Aires)*	Leptomyrmecini	Worker	Omnivorous	Ground	Neotropical	Tropical	Invasive	Whole_Ant_Body	35.34	64.4	2605	4029	1424
Lester2017	*Linepithema humile (Auckland)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	38.52	22.77	3726	6061	2335
Lester2017	*Linepithema humile (Hastings)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	45.37	20.46	4517	8269	3752
Lester2017	*Linepithema humile (Hawaii)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	48.06	51.93	982	1891	909
Lester2017	*Linepithema humile (Melbourne)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	60.53	39.47	2285	5789	3504
Lester2017	*Linepithema humile (Corrientes)*	Leptomyrmecini	Worker	Omnivorous	Ground	Neotropical	Tropical	Invasive	Whole_Ant_Body	60.58	30.41	2096	5317	3221
Lester2017	*Linepithema humile (Missiones)*	Leptomyrmecini	Worker	Omnivorous	Ground	Neotropical	Tropical	Invasive	Whole_Ant_Body	67.13	32.72	2359	7178	4819
Lester2017	*Linepithema humile (Nelson)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	68.35	7.77	2468	7797	5329
Lester2017	*Linepithema humile (Santa Clara)*	Leptomyrmecini	Worker	Omnivorous	Ground	Nearctic	Temperate	Invasive	Whole_Ant_Body	72.91	27.02	1347	4972	3625
Lester2017	*Linepithema humile (Wellington)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	77.26	16.53	1756	7720	5964
Lester2017	*Linepithema humile (Christchurch)*	Leptomyrmecini	Worker	Omnivorous	Ground	Australian	Tropical	Invasive	Whole_Ant_Body	79.95	13.55	1702	8490	6788
Lucas2017	*Azteca trigona*	Leptomyrmecini	Worker	Omnivorous	Arboreal	Neotropical	Tropical	Native	Whole_Ant_Body	53.32	42.79	92038	197168	105130
Lukasik2017	*Eciton burchellii (6)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	85.2	67567	67567	0
Lukasik2017	*Eciton burchellii (C1)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	56.4	81846	81846	0
Lukasik2017	*Eciton burchellii (C3)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	70.55	102871	102871	0
Lukasik2017	*Eciton burchellii (CSM2407)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	79.3	86313	86313	0
Lukasik2017	*Eciton burchellii (CSM2482)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	79.5	97993	97993	0
Lukasik2017	*Eciton burchellii (PL022)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	81.45	91228	91228	0
Lukasik2017	*Eciton burchellii (SOD013)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	96.16	72928	72928	0
Lukasik2017	*Eciton burchellii (SOD015)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	99.55	88314	88314	0
Lukasik2017	*Eciton burchellii (SOD016)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	83.27	93742	93742	0
Lukasik2017	*Eciton burchellii (05/37)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	84.8	66296	66296	0
Lukasik2017	*Eciton burchellii (05/53)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	70.3	46246	46246	0
Lukasik2017	*Eciton burchellii (HP047)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	25.2	83409	83409	0
Lukasik2017	*Eciton burchellii (HP092)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	58.23	50116	50116	0
Lukasik2017	*Labidus predaetor (PL034)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	55.82	60699	60699	0
Lukasik2017	*Labidus predaetor (PL039)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	44.71	24225	24225	0
Lukasik2017	*Labidus predaetor (PL037)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	0.97	55427	55427	0

Lukasik2017	*Labidus predaetor (PL040)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	91.87	60070	60070	0
Lukasik2017	*Labidus predaetor (SOD001)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	77.3	48609	48609	0
Lukasik2017	*Labidus predaetor (SOD010)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	99.1	64218	64218	0
Lukasik2017	*Labidus predaetor (HP003)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	99.68	37374	37374	0
Lukasik2017	*Labidus predaetor (HP060)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	99.68	33765	33765	0
Lukasik2017	*Labidus predaetor (HP094)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	99.75	37938	37938	0
Lukasik2017	*Labidus predaetor (HP116)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	83.79	23765	23765	0
Lukasik2017	*Labidus predaetor (CS386)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	93.71	51654	51654	0
Lukasik2017	*Labidus predaetor (CS491)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0	89.8	5496	5496	0
Lukasik2017	*Eciton burchellii (PL032)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	0.8	40.3	47063	47443	380
Lukasik2017	*Eciton burchellii (PL028)*	Dorylinae	Worker	Predator	Ground	Neotropical	Tropical	Native	Ant_Gut	2.3	52.7	91450	93603	2153
Moreau2017	*Paraponera clavata*	Paraponerinae	Worker	Omnivorous	Ground_Arboreal	Neotropical	Tropical	Native	Ant_Gut	20.13	78.35	4863	6089	1226
Moreau2017	*Paraponera clavata*	Paraponerinae	Worker	Omnivorous	Ground_Arboreal	Neotropical	Tropical	Native	Ant_Gut	35.1	64.9	2072	3193	1121
Moreau2017	*Paraponera clavata*	Paraponerinae	Worker	Omnivorous	Ground_Arboreal	Neotropical	Tropical	Native	Ant_Gut	35.23	48.11	29330	45283	15953
Moreau2017	*Paraponera clavata*	Paraponerinae	Worker	Omnivorous	Ground_Arboreal	Neotropical	Tropical	Native	Ant_Gut	79.14	18.71	5764	27630	21866
Pringle2017	*Azteca ssp.*	Leptomyrmecini	Worker	Omnivorous	Arboreal	Neotropical	Tropical	Native	Ant_Gut	89.18	7.85	90	833	743
Pringle2017	*Cephalotes setulifer*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	98.8	0	6	461	455
Ramalho2017	*Camponotus planatus (MOR#69)*	Camponotini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Invasive	Whole_Ant_Body	100	0	0	2932951	2932951
Ramalho2017	*Colobopsis riehlii (MOR#62)*	Camponotini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Whole_Ant_Body	100	0	0	4679415	4679415
Ramalho2017	*Camponotus planatus (MOR#69)*	Camponotini	Worker	Omnivorous	Arboreal	Nearctic	Temperate	Invasive	Whole_Ant_Body	99.34	0	32412	4910883	4878471
Ramalho2017	*Camponotus floridanus (MOR#59)*	Camponotini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Whole_Ant_Body	99.71	0	1599	551343	549744
Ramalho2020a	*Atta sexdens (Atlantic Forest 2)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	1.49	0.83	172928	175544	2616
Ramalho2020a	*Atta sexdens (Sugar Cane 4)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	15.74	82.5	32453	38515	6062
Ramalho2020a	*Atta sexdens (Citrus 5)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	19.16	79.7	113807	140781	26974
Ramalho2020a	*Atta sexdens (Atlantic Forest 5)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	29.69	61.11	261452	371856	110404
Ramalho2020a	*Atta sexdens (Citrus 3)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	33.72	64.65	79830	120444	40614
Ramalho2020a	*Atta sexdens (Cerrado 5)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	34.02	63.41	37831	57337	19506
Ramalho2020a	*Atta sexdens (Sugar Cane 5)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	48.66	49.44	10782	21001	10219
Ramalho2020a	*Atta sexdens (Sugar Cane 3)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	48.7	45.93	52650	102631	49981
Ramalho2020a	*Atta sexdens (Sugar Cane 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	5.83	93.77	19645	20861	1216
Ramalho2020a	*Atta sexdens (Cerrado 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	50.65	45.33	143993	291780	147787

Ramalho2020a	*Atta sexdens (Atlantic Forest 4)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	52.2	36.7	83696	175096	91400
Ramalho2020a	*Atta sexdens (Atlantic Forest 3)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	52.9	44.4	34893	74083	39190
Ramalho2020a	*Atta sexdens (Citrus 4)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	52.95	46.33	55495	117949	62454
Ramalho2020a	*Atta sexdens (Eucalyptus 2)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	53.39	41.7	95574	205050	109476
Ramalho2020a	*Atta sexdens (Cerrado 6)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	54.7	39.2	62375	137694	75319
Ramalho2020a	*Atta sexdens (Sugar Cane 2)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	55.13	41.67	64820	144462	79642
Ramalho2020a	*Atta sexdens (Atlantic Forest 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	57.13	41.36	36136	84293	48157
Ramalho2020a	*Atta sexdens (Cerrado 4)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	58.23	36.22	37076	88763	51687
Ramalho2020a	*Atta sexdens (Cerrado 3)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	59.2	28.4	137117	336072	198955
Ramalho2020a	*Atta sexdens (Eucalyptus 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	59.2	25.25	49793	122041	72248
Ramalho2020a	*Atta sexdens (Eucalyptus 6)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	59.33	30.66	889	2185	1296
Ramalho2020a	*Atta sexdens (Eucalyptus 4)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	63.06	33.82	11789	31915	20126
Ramalho2020a	*Atta sexdens (Eucalyptus 3)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	63.49	27.2	15816	43321	27505
Ramalho2020a	*Atta sexdens (Cerrado 2)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	63.6	35.2	46343	127315	80972
Ramalho2020a	*Atta sexdens (Eucalyptus 5)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	65.06	31.22	36142	103441	67299
Ramalho2020a	*Atta sexdens (Citrus 6)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	69.76	26.55	35676	117977	82301
Ramalho2020a	*Atta sexdens (Citrus 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	7.14	92.17	123429	132919	9490
Ramalho2020a	*Atta sexdens (Atlantic Forest 6)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	7.96	65.84	27778	30180	2402
Ramalho2020a	*Atta sexdens (Atlantic Forest 8)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	75.64	18.95	15062	61830	46768
Ramalho2020a	*Atta sexdens (Horto 1)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	78.46	21.54	1314	6099	4785
Ramalho2020a	*Atta sexdens (Citrus 2)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	8.42	90.8	131110	143164	12054
Ramalho2020a	*Atta sexdens (Atlantic Forest 7)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	93.51	5.74	3297	50801	47504
Ramalho2020b	*Daceton armigerum (3520)*	Attini	Worker	Predator	Arboreal	Neotropical	Tropical	Native	Whole_Ant_Body	64.77	0	350586	995135	644549
Ramalho2020b	*Daceton armigerum (3518)*	Attini	Worker	Predator	Arboreal	Neotropical	Tropical	Native	Whole_Ant_Body	66.77	4.66	66750	200873	134123
Ronque2020	*Mycetophylax morschi (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	0.11	88.41	317719	318069	350
Ronque2020	*Mycocepurus smithii (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	0.45	53.11	668864	671887	3023
Ronque2020	*Mycetophylax morschi (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	28.21	70.29	79631	110922	31291
Ronque2020	*Sericomyrmex parvulus (Forest)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	43.19	17.2	314619	553810	239191
Ronque2020	*Sericomyrmex saussurei (Forest)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	52.87	38.13	301256	639202	337946
Ronque2020	*Mycetarotes parallelus (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	7.7	74.5	763386	827070	63684
Ronque2020	*Mycocepurus smithii (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	73.23	26.11	266680	996189	729509

Ronque2020	*Mycetarotes parallelus (Restinga)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	8.93	78.3	330458	362862	32404
Ronque2020	*Mycetophylax morschi (Dune)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	82.95	0.16	28377	166434	138057
Ronque2020	*Mycetophylax morschi (Dune)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	84.06	0.15	38556	241881	203325
Salvo2019	*Myrmica scabrinodis*	Myrmicini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Whole_Ant_Body	43.3	6.55	334248	589503	255255
Sanders2014	*Cephalotes clypeatus (11)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	16341	16506	165
Sanders2014	*Cephalotes targionii (53)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	10389	10494	105
Sanders2014	*Cephalotes minutos (51)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	5800	5859	59
Sanders2014	*Cephalotes persimilis (23)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	1713	1730	17
Sanders2014	*Cephalotes cordatus (48)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	9185	9278	93
Sanders2014	*Cephalotes eduarduli (20)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	1	0	9371	9466	95
Sanders2014	*Cephalotes spinosus (47)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	0.98	0	10282	10384	102
Sanders2014	*Pseudomyrmex sp.*	Pseudomyrmecini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	19.14	24.08	5105	6313	1208
Sanders2014	*Azteca sp.*	Leptomyrmecini	Worker	Omnivorous	Arboreal	Neotropical	Tropical	Native	Ant_Gut	72.86	21.59	3303	12170	8867
Sanders2014	*Crematogaster sp.*	Crematogastrini	Worker	Omnivorous	Arboreal	Neotropical	Tropical	Native	Ant_Gut	85.09	12.58	286	1918	1632
Sanders2014	*Cephalotes atratus (46)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	90.26	1.27	220	2160	1940
Sanders2014	*Cephalotes atratus(15)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	93.84	0.5	616	9988	9372
Sanders2014	*Cephalotes pusillus (14)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	95.65	0	200	4598	4398
Sanders2014	*Cephalotes umbraculatus (50)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	97.37	0.19	278	10564	10286
Sanders2014	*Cephalotes clypeatus (lmah1)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	97.89	0.16	41	1963	1922
Sanders2014	*Cephalotes grandinosus (21)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	97.95	0.18	185	9015	8830
Sanders2014	*Cephalotes maculatus (lmah2)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	98.03	0.48	121	6137	6016
Sanders2014	*Cephalotes pusillus (12)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	98.12	0	338	17979	17641
Sanders2014	*Cephalotes maculatus (19)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	98.44	0.16	97	6248	6151
Sanders2014	*Cephalotes pusillus (27)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	98.93	0	59	5550	5491
Sanders2014	*Cephalotes persimilis (26)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.05	0	13	1378	1365
Sanders2014	*Cephalotes persimilis (18)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.21	0.16	28	3531	3503
Sanders2014	*Cephalotes minutos (32)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.33	0	59	8776	8717
Sanders2014	*Cephalotes pallidoide (49)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.33	0	66	9842	9776
Sanders2014	*Cephalotes borgmeieri (24)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.5	0	30	5964	5934
Sanders2014	*Cephalotes pellans (10)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.6	0	32	7897	7865
Sanders2014	*Cephalotes rohweri (55)*	Attini	Worker	Herbivore	Arboreal	Nearctic	Temperate	Native	Ant_Gut	99.64	0	31	8457	8426
Sanders2014	*Cephalotes pallens (25)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.7	0	25	8192	8167
Sanders2014	*Cephalotes simillimus (45)*	Attini	Worker	Herbivore	Arboreal	Neotropical	Tropical	Native	Ant_Gut	99.8	0.2	-70564	7858	78422

Vieira2017	*Atta sexdens rubropilosa*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Whole_Ant_Body	0.24	0.57	182293	182732	439
Zheng2021	*Ectomomyrmex javanus Mayr (E2)*	Ponerini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Ant_Gut	16.42	7.56	50040	59871	9831
Zheng2021	*Odontomachus monticola Emery (O1)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	20.44	0.11	46987	59059	12072
Zheng2021	*Ectomomyrmex javanus Mayr (E2)*	Ponerini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Ant_Gut	3.02	4.92	54744	56449	1705
Zheng2021	*Odontomachus monticola Emery (O1)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	3.36	0	57882	59894	2012
Zheng2021	*Ectomomyrmex javanus Mayr (E1)*	Ponerini	Worker	Omnivorous	Ground	Palearctic	Temperate	Native	Ant_Gut	57.92	25.74	24162	57419	33257
Zheng2021	*Odontomachus monticola Emery (O2)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	80.6	10.19	10014	51622	41608
Zheng2021	*Odontomachus monticola Emery (O2)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Whole_Ant_Body	86.29	13.54	8333	60782	52449
Zheng2021	*Ectomomyrmex javanus Mayr (E1)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	87.66	10.35	6685	54170	47485
Zheng2021	*Odontomachus monticola Emery (O3)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	94.25	4.1	3662	63690	60028
Zheng2021	*Odontomachus monticola Emery (O2)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	95.68	2.36	2357	54558	52201
Zheng2021	*Odontomachus monticola Emery (O3)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Ant_Gut	96.04	2.66	2659	67134	64475
Zheng2021	*Odontomachus monticola Emery (O1)*	Ponerini	Worker	Predator	Ground	Palearctic	Temperate	Native	Whole_Ant_Body	96.74	2.65	1802	55288	53486
Zhukova2017	*Atta cephalotes (W)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	0.16	99.84	300	300	0
Zhukova2017	*Acromyrmex echinatior (715)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	14.18	85.82	257	300	43
Zhukova2017	*Atta cephalotes (Ca)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	35.2	64.78	194	300	106
Zhukova2017	*Atta cephalotes (Cr)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	74.01	12.71	78	300	222
Zhukova2017	*Acromyrmex echinatior (711)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	97.6	2.31	7	300	293
Zhukova2017	*Acromyrmex echinatior (712)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	99.38	0.61	2	300	298
Zhukova2017	*Acromyrmex echinatior (717)*	Attini	Worker	Fungivore	Ground	Neotropical	Tropical	Native	Ant_Gut	99.61	0.39	1	200	199

CAPÍTULO 2

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 ant species *Azteca chartifex* Forel, 1896 from main and satellite nests, and leaves from *Byrsonima sericeae* (Malpighiaceae) tree, both in ant-colonized and ant-free trees. We used amplicon sequencing of the 16S rRNA gene to investigate (i) the diversity and composition of bacterial communities associated with ants from main and satellite nests; (ii) the phyllosphere of leaves with and without ants; (iii) the similarity between the bacterial communities associated with ants and the leaves they forage on. We found that ants from main and satellite nests have different bacterial communities. The diversity and composition of bacterial communities on leaf phyllospheres from ant-colonized and ant-free trees are 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.

Introduction

Ants comprise 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 *et al*., 2013; Soares *et 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 behaviors of these insects (Bot *et al*., 2001; Fernández-Marín *et al*., 2006; Hamilton, 1996).

Several collective immunization strategies have evolved in large ant colonies, from induced antimicrobial defense produced in external glands (Yek *et al*., 2012; Offenberg & Damgaard, 2019), detection of infected individuals (Leclerc & Detran, 2016) and the interaction with symbiotic microorganisms (Currie *et al*., 1999; Kaltenpoth, 2009). The structure and composition of bacterial communities associated with social organisms and their environment are particularly important to understand their behavioral habits and the risk of spreading disease (Wilson, 1975). Bacteria associated with ant cuticles can play an important defensive role against pathogens (Currie *et al*., 1999; Sapountzis *et al*., 2019). Inside the nest, ants can influence the bacterial communities and decrease their richness in the "nursery" (Lucas *et al*., 2019). Given that bacterial communities living on ant surfaces are in direct contact with the surrounding environment (Lucas *et al*., 2017; Bitar *et al*., 2021), ants must be able to shape the species composition and density of associated bacteria (Fernández-Marín *et al*., 2009; Kellner *et al*., 2015).

Arboreal ants interact with the microbiomes of the forest canopy, especially with the microbiome associated with leaf surfaces (González-Teuber *et al*., 2014; Offenberg & Damgaard, 2019; Bitar *et al*., 2021). Phyllosphere is the microhabitat hosting a great diversity of microorganisms, mostly bacteria (Lindow & Brandl, 2003). Epiphytic bacteria can either benefit (Kembel *et al*., 2014), induce susceptibility and pathogenicity (Baker *et 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 *et al*., 2017). Nonetheless, little is still known about the interaction

between the ant- and leaf-associated bacterial communities, as well as how the structure of these microbial communities interferes with each other.

Azteca chartifex is a dominant ant in the mosaic of species in tropical canopies due to its aggressive territorial behavior (Ribeiro *et al*., 2013; Soares *et al*., 2022b). They build multiple "carton" nests with cellulose and processed fibers, and the main nest hosting the queen (length > 2 m) can harbor thousands of individuals (Baccaro *et 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 *et al*., 2021), and the main nest is stable in space and time since they are constructed on the principal tree trunk (Soares *et 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 *et al*., 2019; Nepel *et al*., 2023). In our study system, *A. chartifex* ants construct their carton nests on *B. 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 *et al*., 2007). In polydomous *A. chartifex*, the bacterial communities associated with the ants' cuticle from main and satellite nests remain unknown.

Here, we tested the hypothesis that bacterial communities associated with the cuticle of *A. chartifex* workers, from main and satellite nests, shape the bacterial communities on leaves surfaces of *B. sericea*. Using 16S rRNA gene amplicon sequencing of the samples, we identified and analyzed the diversity and composition of bacterial communities of both ants and leaves. Specifically, we addressed the following questions: 1) Do bacterial diversity and composition differ between ants from main and satellite nests of polydomous colonies? 2) Do the phyllosphere bacterial communities differ between trees with and without *A. chartifex* nests? 3) How similar is the taxa composition of bacterial communities between ants and the leaves on which they forage?

Material and methods

Study Area

Samples were 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 *et al*., 2019).

Sampling design

During the rainy season (November 2020), *B. sericea* trees with *A. chartifex* nests and trees without nests were selected in three different 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).

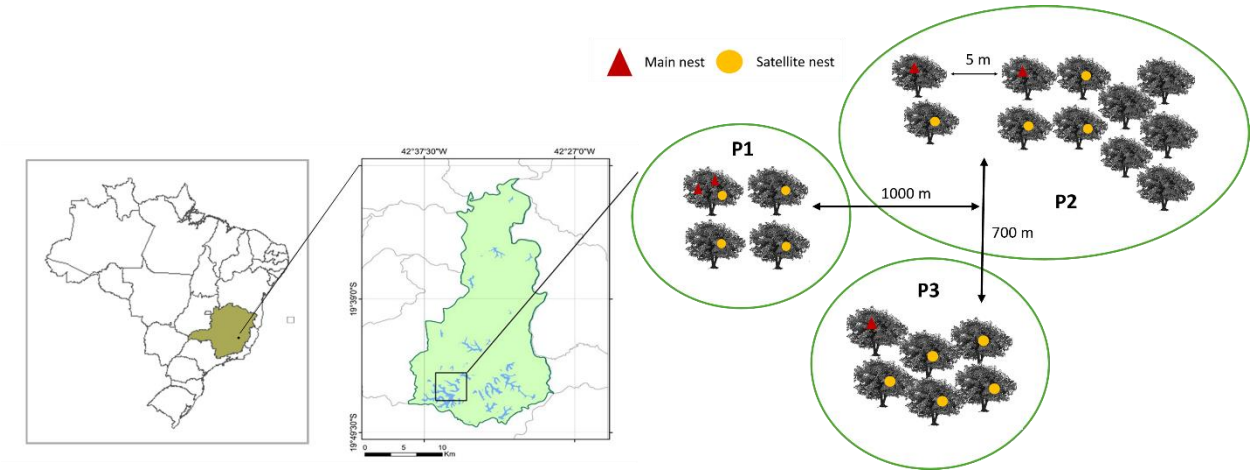

Fig. 1: Map of Brazil and Rio Doce State Park showing the *Byrsonima sericeae* tree and *Azteca chartifex* ant populations (P1, P2, and P3) across the study areas, located at two distinct forest-lake ecotones. Sampling design across the studied populations, showing trees with main and satellite nests,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

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 five trees of *B. 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.

Nest samples were sampled using a sterilized machete and bucket. All leaf samples (20 per tree) were sampled using 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 -20ºC until DNA extraction.

Extraction, 16S rRNA amplification and sequencing

The DNA extraction from bacteria associated with ant cuticles and leaf phyllosphere was performed in sterile conditions, following the protocol (with some modifications) of the Quick-DNA™ Miniprep Kit (Zymo Research No. D3024). Thirty individuals of *A. chartifex* of each nest were placed in 2 mL tubes and washed with the extraction kit buffer. We gently shook the samples (no vortex) 10 times at each 5 min interval for 30 min, such that all DNA of cuticle bacteria was extracted. Also, 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 5s at each 15 min interval for one hour. A total of 28 samples of ants (N=18) and leaves (N=10), from the three populations, had DNA extracted and analyzed in agarose gel. Bacterial identification and relative quantification were made using high-throughput sequencing of the 16S rRNA gene. The preparation of libraries for bacterial amplicon sequencing was carried out using the specific oligonucleotides 341F and 806R targeting the V3/V4 region of the 16S rRNA gene in a two-step PCR protocol (Wang & Qian, 2009; Caporaso *et al*., 2012). The primers used in the first PCR, in addition to containing a specific target region for V3/V4, also encompass a region corresponding to a partial Illumina adapter based on the TruSeq structure (Illumina, USA). The presence of this adapter allows for a second PCR that adds indexing sequences following established (Caporaso *et al*., 2011). Indexing is performed with unique dual indices for each sample in the second PCR. Two microliters of extracted DNA from each sample were used as a template in the first PCR reaction. PCR reactions were carried out using Platinum Taq (Invitrogen, USA) under the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45s, 55 °C for 30s, and 72°C for 45s, with a final extension of 72 °C for 2 min for PCR 1. For PCR 2, the conditions were 95 °C for 5 min, 10 cycles of 95 °C for 45s, 66 °C for 30s, and 72 °C for 45s, with a final extension of 72 °C for 2 min. All PCR reactions were performed in triplicate. The final PCR products were purified using Neobeads® (Sera-Mag™ magnetic beads), and an equivalent volume of each sample was added to the sequencing pool. In each round of PCR, a Negative

Reaction Control (NRC) was included. For each Receiving Order (RO), a Negative Extraction Control (NEC) was also included. The final DNA concentration of the library pool was estimated using Picogreen dsDNA (Invitrogen, USA), and then diluted for quantification by qPCR using the Collibri™ Library Quantification Kit (Invitrogen, USA), which had been optimized for Illumina libraries. The sequencing pool was adjusted to a final concentration of 11 pM (for V2 kits) or 17.5 pM (for V3 kits) and sequenced on the MiSeq system (Illumina, USA), using the Illumina sequencing primers provided with the manufacturer's kit. The paired-end 500-cycle runs were performed using V2x500 or V3x600 sequencing kits (Illumina, USA) with >100,000 reads coverage per sample.

Bioinformatic and Statistical analyses

Output files (in *fastq* format) resulting from the 16S rRNA gene sequencing of all the samples comprise our raw primary data. These raw data were imported to Qiime2-2023.9 (Boylen *et 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 *et al*., 2016). Taxonomic identification of Amplicon Sequence Variants (ASVs) was performed using the SILVA 132 QIIME database (Glöckner *et al*., 2019) with a 99% similarity threshold. The resulting ASV table, including taxonomic assignments, was then utilized 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. We used the phyloseq package (McMurdie & Holmes, 2013) to create the phyloseq object. For the visualization of rarefaction curves, the ranacapa package (Kandiklar *et 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 location/populations, were calculated the alpha and beta diversity using vegan package (Oksanen *et al*., 2005). From the dataset, samples from 5 main and 8 satellite nests from each of the three populations were selected for analysis. The Kruskal-Wallis test was used to evaluate dissimilarities between bacterial communities associated with ants from main and

satellite nests. To examine differences in beta diversity variation 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 (NDMS) was produced to illustrate the composition of bacterial communities across samples and locations/populations. Furthermore, a CLAM analysis (Chazdon *et 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 sequencing of the 16S rRNA region of bacterial communities generated a total of 6,015,549 raw reads in 28 samples. After rarefaction to 2,494 reads per sample, the analysis was carried out with all the samples (Ants P1 = 6; Ants P2 = 6; Leaves P2 = 5; Leaves with ants P2 = 5; Ants P3 = 6) and 401 ASVs (Fig S1). In general, ant cuticles and leaf surfaces were dominated by Proteobacteria, Bacteroidota and Actinobacteria (Fig. 2).

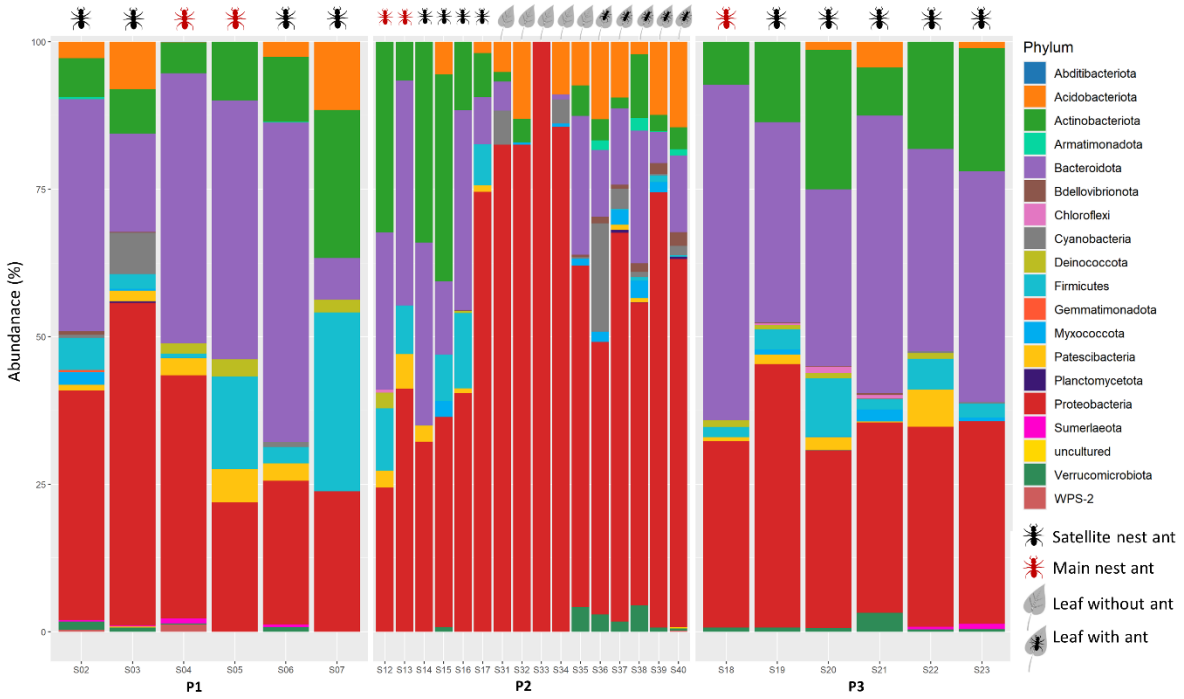

Fig. 2: Phylum variety analysis barplot of bacterial communities from ecotones samples: Samples of *Azteca chartifex* (main and satellite nests) from three locations (P1, P2 and P3), and of leaves of *Byrsonima sericeae* trees with and without ant nests (location P2). Bars show the relative abundance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

The phylum Proteobacteria and Bacteroidetes, consisting of Gram-negative bacteria, represented the highest proportion on the ant cuticle of the P1 (36.68% and 41.02%), P2 (37.77% and 27.53%), and P3 (34.83% and 38.7%) 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 to ant-free leaves.

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

The alpha diversity measure between bacterial communities of ants from main and satellite didn't show differences (Kruskal-Wallis: X^2 (1) = 0.343, p = 0.558). In the analysis of bacterial taxa composition between main and satellites nests ants, there was significant variation between the types of nests (Permanova: F=1.81; R^2=0.14; p=0.022; Fig. 3), 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).

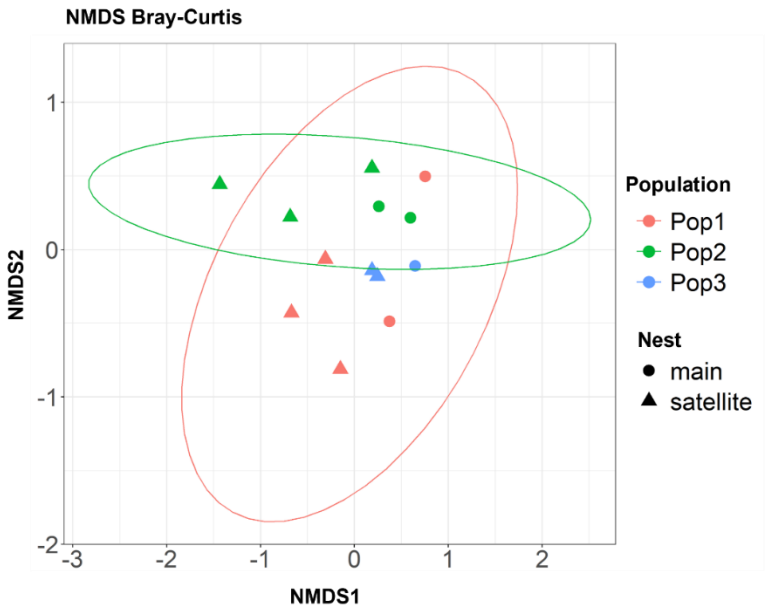

Fig. 3: Non-metric multidimensional scaling (NMDS), with Bray-Curtis dissimilarity index, shows bacterial community composition of ants from different populations and nest types (main and satellite).

The multinomial species classification method 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 satellite nest specialists. *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, 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 are too rare to be classified with confidence (Fig. 4).

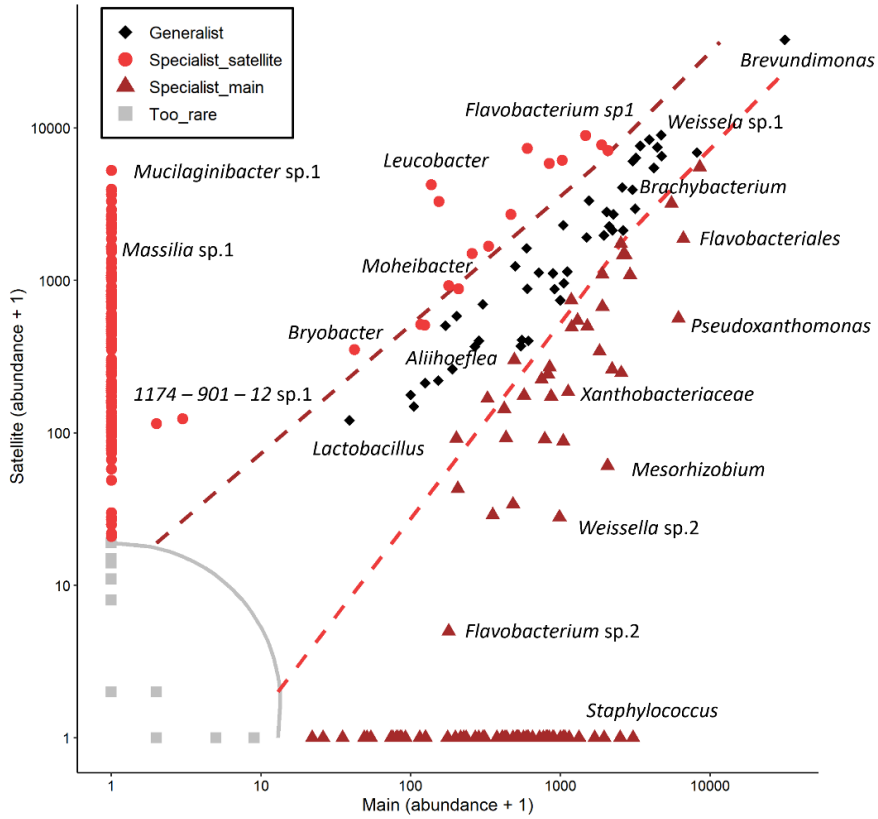

Fig. 4: Multinomial species classification method (CLAM), showing the specialist bacteria in ants' cuticles from satellite nest, specialist bacteria in ants' cuticle from main nest and the generalist bacteria shared between the two samples.

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

The alpha diversity measure of the leaves' bacterial communities varies 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 (Wilcoxon test: X^2 (2), p= 0.0012; Fig. 5a). 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 did not differ (Permanova: F=1.63; R^2=0.37; p=0.122; Fig. 5A). Finally, we found a significant difference between the bacterial taxa composition between ants and leaves with ants (Permanova: F=0.29; R^2=1.00; p=0.003; Fig. 5B).

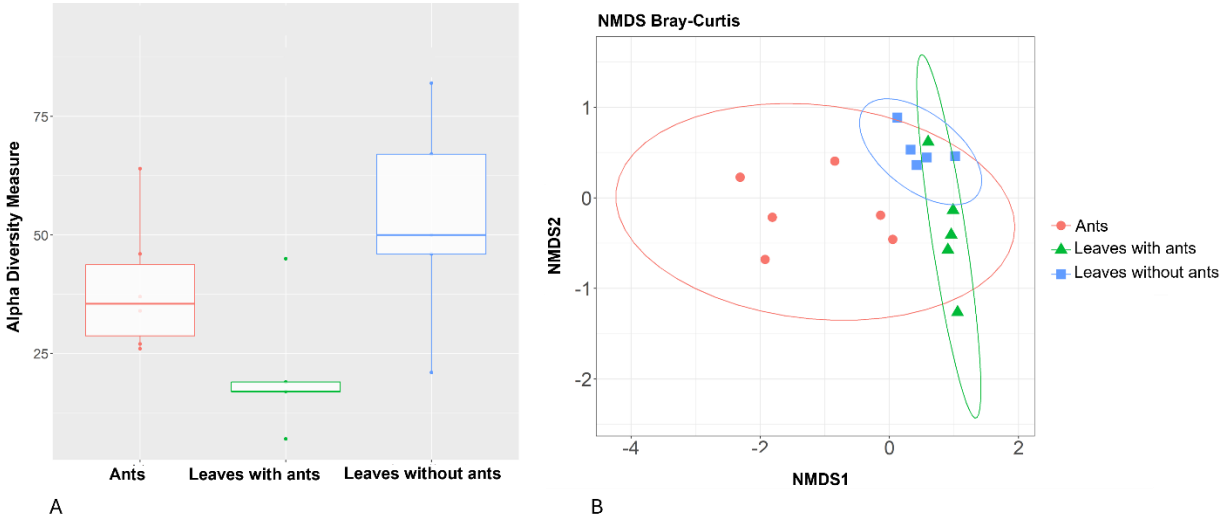

Fig. 5: Alpha and Beta diversities of bacterial communities associated with *Azteca chartifex* and *Byrsonima sericea* from one location (P2) in PERD. (A) Alpha diversity measure of bacterial communities associated with ants, leaves with ants, and leaves without ants. (B) Non-metric multidimensional scaling (NMDS), with Bray-Curtis's dissimilarity index, shows bacterial community composition of ants, leaves with ants, and leaves without ants.

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. 6). 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 leaf samples, with and without ants. Generalists comprised 28.0% of the sampled bacteria, whereas 12.1% were classified as specialists on leaves not foraged by ants, and 55.3% were classified as specialists on leaves foraged by ants. Moreover, 4.5% of the sampled bacteria were too rare to be classified (Fig. 6).

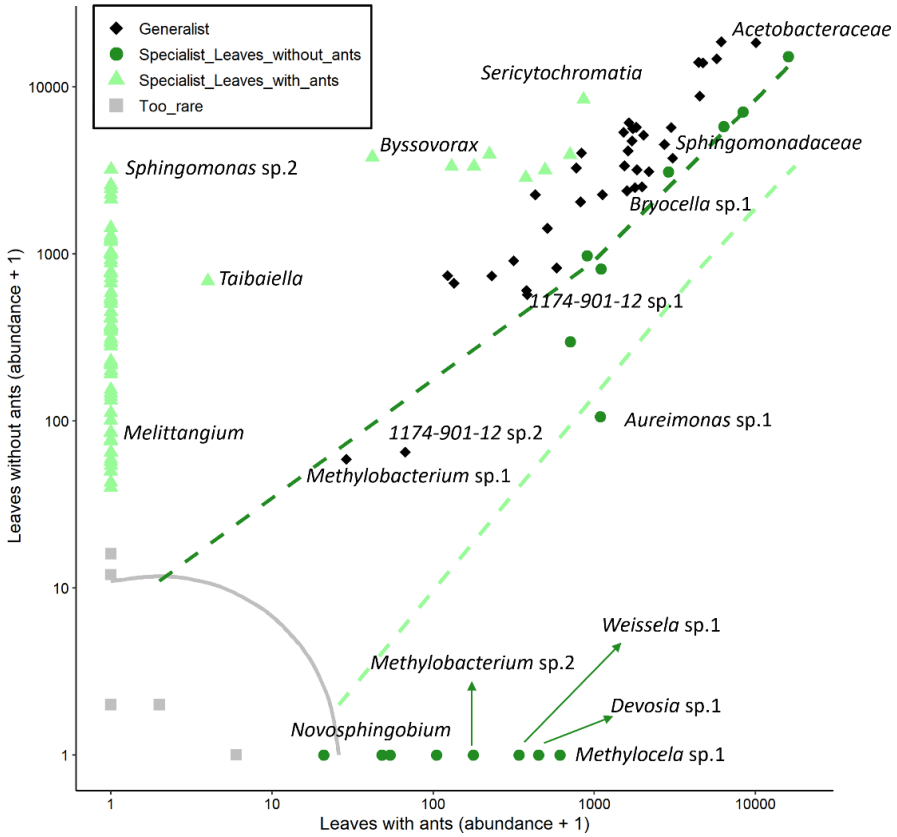

Fig. 6: Multinomial species classification method (CLAM), showing the specialist bacteria in leaves without ants, leaves with ants and the generalist bacteria between the two samples.

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 bacteria were classified as ants' specialists, 40.3% were classified as phyllosphere specialists, and shared 8.4% of bacteria (generalists). Finally, 2.2% of the taxa were too rare to be classified (Fig. 7).

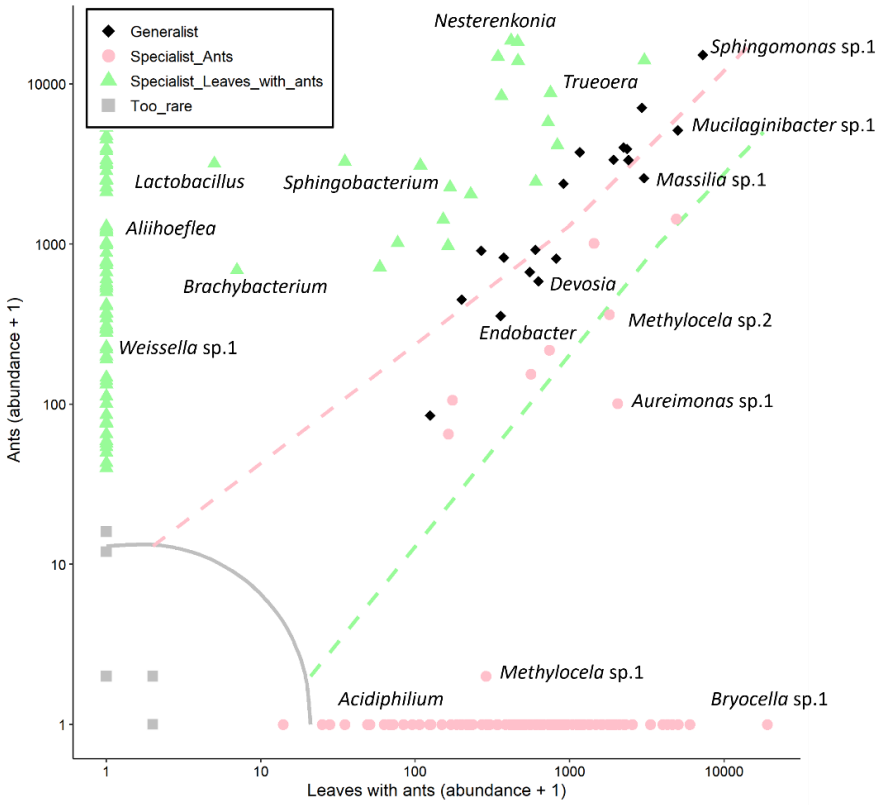

Fig. 7: Multinomial species classification method (CLAM), showing the specialist bacteria in ants, in leaves with ants and the generalist bacteria shared between the two samples.

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 *B. 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 *et al*., 2017; Nepel *et al*., 2023), colony productivity (Segers *et al*., 2019), and investment in defense strategies (Bitar et 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 *et al*., 2002; Turnbull *et al*., 2011).

Also, while comparing bacterial communities of ant's cuticles from the main nest and the satellite nests, the gram-positive genera *Lactobacillus* and the gram-negative *Brevundimonas* were present in greater 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 *et 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 *et 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 super colony and foraging activity (Landa & Tullock, 2003).

Bacterial communities vary more within ant polydomous colonies than among plant individuals. Ant bacterial communities exhibit colony-specific signatures (Chua *et al*., 2018; Ronque *et al*., 2020). This phenomenon can be attributed to both genetic variation within the same ant species (Hu *et al*., 2014) and the microbiome's production of odors in individuals from the same colony, which plays a vital role in nestmate recognition (Dosmann *et al*., 2016). On the other hand, bacterial communities in the phyllosphere show greater specificity within the same plant species (Redford *et al*., 2010). Laforest-Lapointe *et 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 phyllosphere (Kutschera, 2007; Holland, 2007),

promoting plant growth (Dourado *et al*., 2015). *Lactobacillus* can be considered as specialist of 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 *et al*., 2022) and can be acquired from the environment rather than acquired vertically (Kellner *et 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 *et al*., 2010) and in the black ant *Polyrhachis* (Osimani *et al*., 2018). Indeed, insects are known to carry bacteria to leaf surfaces, facilitating colonization (Whipps *et 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 specifity 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.

Supplementary Information

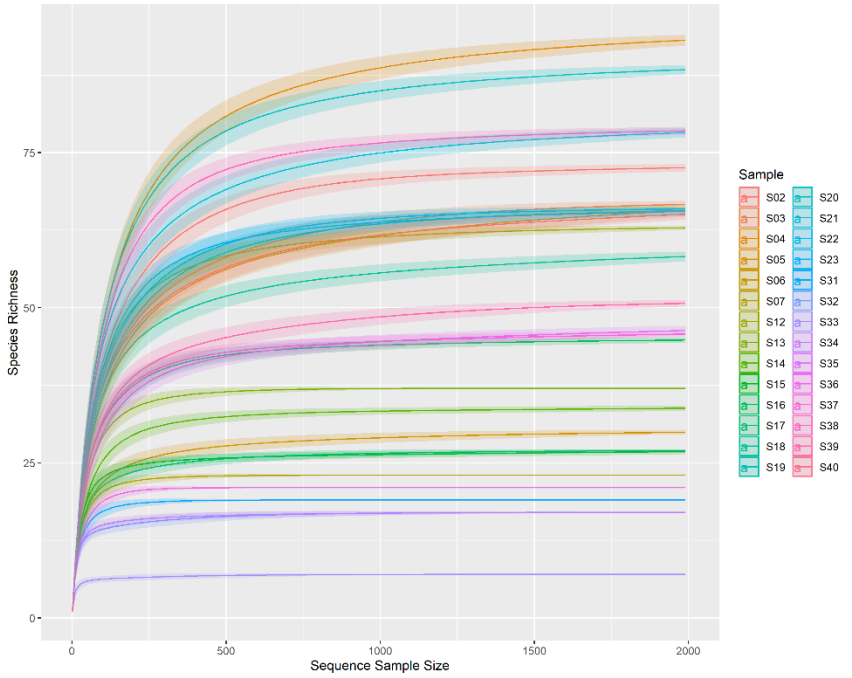

Fig S1 – Rarefaction curves of bacteria amplicon sequence variants, showing species richness in 28 samples (Ants = 18; Leaves without ants = 5, Leaves with ants = 5).

Table S1 - Permutational multivariate analysis of variance (PERMANOVA) of bacterial community composition using abundance data with Bray-Curtis distances. (a) Between ants from main and satellite nests from three locations/populations. (b) Pairwise Permanova among ants from main and satellite nests. (b) Pairwise Permanova among each location/population.

(a) Ants from main and satellite nests from three locations/populations					
	Df	SumOfSqs	R2	F	Pr(>F)
Nest	1	0.4806	0.14188	1.8141	**0.022 ***
Pop	2	0.6208	0.18325	1.1715	0.230
Nest:Pop	2	0.4316	0.12740	0.8144	0.762
Residual	7	1.8546	0.54747		
Total	12	3.3876	1.00000		
(b) Pairwise tests: main vs. satellite					
	Df	SumOfSqs	R2	F	Pr(>F)
Nest	1	0.4806	0.14188	1.8187	**0.021 ***
Residual	11	2.9070	0.85812		
Total	12	3.3876	1.00000		
(c) Pairwise tests: locations/populations					
Pop1 vs. Pop2					
	Df	SumOfSqs	R2	F	Pr(>F)
Pop	1	0.28366	0.10301	0.9187	0.556

Residual	8	2.46999	0.89699		
Total	9	2.75365	1.00000		
Pop1 vs. Pop3					
	Df	SumOfSqs	R2	F	Pr(>F)
Pop	1	0.27556	0.15249	1.0796	0.424
Residual	6	1.53146	0.84751		
Total	7	1.80702	1.00000		
Pop2 vs. Pop3					
	Df	SumOfSqs	R2	F	Pr(>F)
Pop	1	0.34367	0.1791	1.309	0.143
Residual	6	1.57522	0.8209		
Total	7	1.91889	1.0000		

CAPÍTULO 3

THE IMPACT OF TOXIC MINING CONTAMINATION ON THE BACTERIAL CUTICLE COMMUNITIES OF A DOMINANT NEOTROPICAL ARBOREAL ANT *AZTECA CHARTIFEX* (FORMICIDAE: DOLICHODERINAE)

Marilia Romão Bitar, Aristóteles Góes-Neto, Paulo S. Oliveira, Corrie S. Moreau & Sérvio Pontes Ribeiro

Abstract

Ants are diverse and abundant, playing a fundamental role in the functioning of terrestrial ecosystems. By maintaining symbiotic bacteria that may prevent pathogen infections, ants safeguard their colonies and enhance their resilience to stressful environments. Indeed, environmental stressors such as heavy metal contamination pose significant threats to insect populations, including social insects such as ants. Here, we tested the hypothesis that exposure to waste rich in heavy metals, from mining disaster in Brazil, would affect the diversity and species composition of bacterial communities associated with *Azteca chartifex* Forel, 1896. We analyzed and identified the ants' bacterial communities through 16S rRNA gene amplicon sequencing of samples from two natural environments (contaminated and control). We addressed the following specific questions: 1) Do ants from protected and contaminated areas differ in the diversity and composition of bacterial communities associated with their cuticles? 2) Does the presence of heavy metals favor more Gram-negative than Gram-positive bacteria on the ants' cuticle? 3) Do ants from contaminated area have specific bacteria, resistant to heavy metals, associated on their cuticle? Our results showed that the composition of the ants' bacterial communities differed between the protected and contaminated areas. We found that ants from the contaminated area exhibited higher bacterial alpha diversity on their cuticle, along with a lower abundance of bacterial taxa classified as bioindicators of contaminated areas. Gram-negative Proteobacteria were dominant regardless of contamination status, with distinct bioindicator bacteria present in each area. This study represents a first assessment of the impacts of mining tailings resulting from a dam disaster on the bacterial communities associated with a dominant arboreal ant species in the Atlantic forest ecosystem. Further investigation into the functional aspects of these bacteria is necessary to fully understand the interactions between ants, bacteria, and the environment.

Introduction

Ants are abundant and present across the planet, playing a fundamental role in the functioning of terrestrial ecosystems (Hölldobler & Wilson, 1998; Underwood & Fisher, 2006). Because most ants live in stationary nests and are easy to collect, they frequently serve as bioindicators of environmental contamination (Andersen 1997; Majer & Kaspari, 2000; Grześ 2010; Skaldina *et al*., 2018). However, the use of ants as bioindicators has never considered the interaction of their associated bacterial community with the surrounding environment and changes caused by human impacts.

Ants have diverse and, in some instances, functional host-associated microbiomes playing important roles for their hosts (de la Fuente & Marquis, 1999; Heil *et al*., 2002; Van Borm *et al*., 2002; González-Teuber *et al*., 2014; Russell *et al*., 2017; Moreau, 2020; Birer *et al*., 2020). These microbiomes are unique to each ant species and can even vary within castes and nest environments, with potential implications for understanding behavior and disease dynamics (Wilson, 1975; Ishak *et al*., 2011; Kellner *et al*., 2015; Lucas *et al*., 2017; Ronque *et al*., 2020; Rocha *et al*., 2023). By maintaining symbiotic bacteria that can prevent pathogen infections, ants safeguard their colonies and enhance their resilience to stressful environments (Currie *et al*., 1999; Fernández-Marín *et al*., 2009; Kaltenpoth *et al*., 2009; Wernegreen *et al*., 2009; Kellner *et al*., 2015; Li *et al*., 2018; Díez-Mendez *et al*., 2019; Ashigar *et al*., 2021).

The diversity of bacterial communities is influenced by the coexistence or competitive exclusion between Gram-positive (GP) and Gram-negative (GN) bacterial groups, due to their different metabolic capabilities (Schwechheimer *et al*., 2013). Studies have demonstrated the competitive advantage of GN bacteria over GP bacteria in certain ecological contexts, such as in associations with arboreal ants (Bitar *et al*., 2021). This interplay between different bacterial groups not only shapes the structure of microbiomes but also influences resource utilization by bacteria in the environment (Fanin *et al*., 2019).

In addition to biological factors, environmental stressors such as heavy metal contamination pose significant threats to insect populations, including social insects such as ants (Galloway & Depledge, 2001; Sorvari *et al*., 2006; Feldhaar & Otti, 2020). The vulnerability of ant colonies to diseases and parasites is increased by the density-dependent selective pressure stemming from

genetic similarity and eusociality (Van Meyel *et al*., 2018). Consequently, social insect colonies have evolved collective immunity behaviors to mitigate the risk of infections and diseases (Currie *et al*., 1999; Cremer *et al*., 2007; Van Meyel *et al*., 2018). Although exposure to heavy metals affects colony size and survival, as well as the impairment of the individual's immune system (Eeva *et al*., 2004; Grześ, 2010; Sorvari *et al*., 2007), ants have some tolerance to these contaminants (Rabitsch, 1995; Eeva *et al*., 2004; Grześ, 2009). In a study of the effect of heavy metals on the immune response of *Formica aquionia* (Formicinae), Sorvari *et al*., (2006) reported a mechanism of encapsulation of contaminants carried out by cells of the immune system, a response revealed to be higher in contaminated areas. Furthermore, Klimek *et al*. (2022) showed that the activity of the ant *Lasius niger* (Formicinae) in contaminated soil decreases the content of heavy metals at a microscale and favors the activity and microbiological biomass of the soil. However, to date no study has tested if heavy metals can affect the bacterial communities associated with the ant exoskeleton.

In 2015, the rupture of an iron mine tailing dam from a big mining industry (Samarco) resulted in the largest Brazilian, or global, environmental disaster (Garcia *et al*., 2017). The mining tailing waste was released along 600 km of the Doce River in Southeastern Brazil. Rich in heavy metals, the mud negatively impacted the ecosystems along the river basin, the socio-economy and human health at a large scale (Escobar, 2015; Fernandes *et al*., 2016). The 50 million m^3 of mining tailings affected parts of the Atlantic forest biome and impacted the soil invertebrates and plants (Alves *et al*., 2023), as well the benthic assemblages (de Oliveira Gomes *et al*., 2017; Gabriel *et al*., 2021). The iron-dominated tailings modified the riverbank soil in many areas along the Doce River, affecting the physical and chemical structure, as well as the biological properties of the soil (Segura *et al*., 2016; Couto *et al*., 2021; Araújo, 2022). So far, few independent studies have approached the impact of this disaster in fine-tuned ecological interactions in riparian Atlantic forests (Cruz *et al*., 2020; Omachi *et al*, 2018; Ribeiro *et al*., 2023). Consequently, investigations into ant species in this region affected by the mud remain limited (Fietto *et al*., 2024).

The Neotropical ant *Azteca chartifex* is an arboreal dominant species exhibiting aggressive territorial behavior (Longino, 2007). In the Atlantic forest reserve at the Parque Estadual do Rio Doce, *A. chartifex* builds arboreal “cartoon” nests, with cellulose and processed fibers, on trees of *Byrsonima sericea* (Malpighiaceae) (Bitar *et al*., 2021; Soares *et al*., 2022). The main nest, where

the queen lives, can reach more than 2 m in height and shelters thousands of individuals (Baccaro *et al*., 2015). Queens and workers of this species are 2-3 mm long, and their colonies are polydomous, with a main large nest and several smaller satellite nest units (Longino, 2007). Foraging activity by *A. chartifex* takes place on foliage and on the ground (Wheeler, 1986); ant foragers prey on a variety of arthropods, generating a trophic cascade in the host tree (Soares *et al*., 2022).

Here, we test the hypothesis that exposure to waste rich in heavy metals, from the Samarco mining disaster, would affect the diversity and species composition of bacterial communities associated with *Azteca chartifex*. We analyzed and identified the ants' bacterial communities through 16S rRNA gene amplicon sequencing of samples from two natural environments (contaminated and control). We addressed the following specific questions: 1) Do ants from protected (control) and contaminated areas differ in the diversity and composition of bacterial communities associated with their cuticles? 2) Does the presence of heavy metals select more Gram-negative than Gram-positive bacteria on the ants' cuticle? 3) Do ants from contaminated area have indicator bacteria associated to their cuticle?

Methods

Study Area

Field work was carried out in 2020 and 2022, in the protected Atlantic forest reserve of the Parque Estadual do Rio Doce (PERD; 19° 45' S 42° 38' W), and in an area contaminated by heavy metals from a dam collapse of a big mining company (Samarco) in 2015, near Mariana city (20° 12' S 43° 27' W), state of Minas Gerais, Southeast Brazil (Fig 1).

Sampling design

We compared the cuticle bacterial communities of *Azteca chartifex* ants on *Byrsonima sericea* trees from two study areas (Fig. 1): (a) Protected Area (PERD): three sites, and (b) Contaminated Area: two sites outside the forest reserve. In each area, ants from each polydomous nest of *A. chartifex* were sampled from the main nest and from respective satellite nest units (Fig. 1a,b,c).

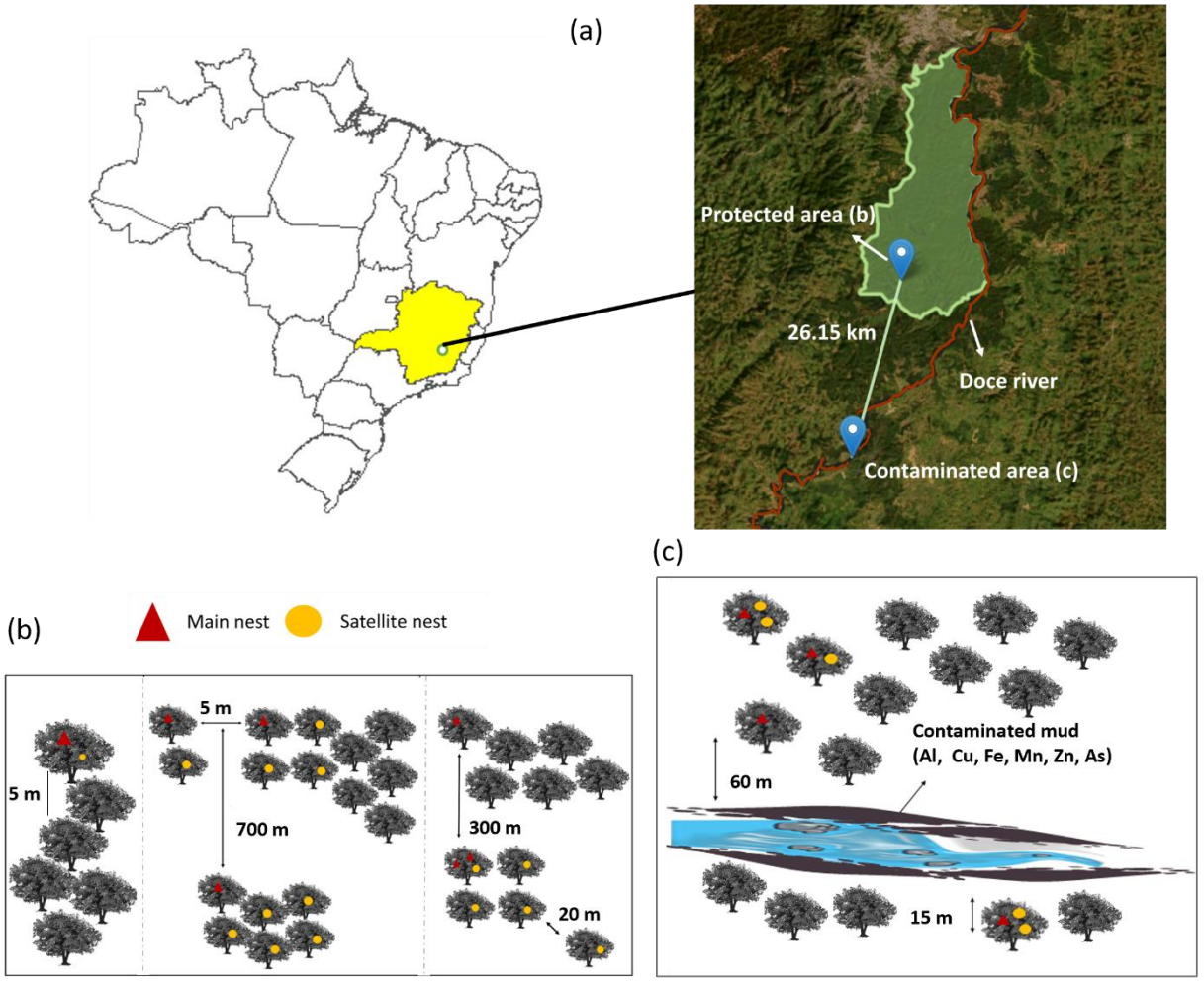

Fig 1. (a) Map of Brazil and aerial view of the Atlantic forest in Eastern Minas Gerais state (yellow); the area of the Parque Estadual do Rio Doce (PERD) is highlighted. Samplings of main and satellite nests were made in the Protected area inside the PERD (b), and in one Contaminated area outside the park along the Doce river (c). Inside the park, 7 main and 15 satellite nests of *A. chartifex* ant were collected (data from Bitar *et al*., 2024). In the Contaminated area, 4 main and 5 satellite nests of *A. chartifex* ant were sampled.

We sampled ants from 7 main and 15 satellite nests in the Protected area (Fig. 1a; Bitar *et al*., 2024). In the Contaminated area (Fig 1b), we collected ants from two sites at different distances from the contaminated river. In the first site (60 m from the river), we collected 3 main and 3 satellite nests. In the second site (15 m from the river) we collected 1 main and 2 satellite nests. From each nest we had three ants' samples. We processed a total of 49 samples from Protected area (n=22) and Contaminated area (n=27).

DNA Extraction and 16S sequencing

The DNA extraction of bacteria associated with the ant cuticle was performed in sterile conditions, following the protocols of the Quick-DNA™ Miniprep Kit (Zymo Research No. D3024) and QIAamp DNA Micro Kit (Qiagen Ltd.), with some modifications. Thirty individuals of *A. chartifex* of each nest (main and satellite) were placed in 2 mL tubes and washed with the extraction kit buffer. We gently shook the samples (no vortex) 10 times at 5 min intervals for 30 min, such that all DNA of cuticle-associated bacteria was extracted. A total of 49 samples of ants had DNA extracted and visualized in agarose gels.

Bacterial identification and relative abundance were made using high-throughput sequencing of the 16S rRNA gene. Library preparation followed proprietary protocol (Zymo Research, Irvine, CA). The oligonucleotides 341F (CCTACGGGRSGCAGCAG, CCTACGGGDGGCWGCAG, CCTAYGGGGYGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT, GACTACNVGGGTMTCTAATCC) were used to amplify the V3-V4 regions. Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA). Paired-end runs of 500 and 600 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

All the data were imported into Qiime2-2022.2 (Boylen *et al*., 2019) using the Casava 1.8 paired-end demultipexed fastq protocol. The sequence reads were trimmed (forward reads trunc 300, reverse reads trim 5 and trunc 200) for maintaining read quality regions, using DADA2 (Callahan *et al*., 2016). The SILVA 132 QIIME database (Glöckner, 2019) with 99% similarity was used to access ASVs (Amplicon Sequence Variants) with taxonomic identification. ASV table with taxonomic assignments were used for the statistical analyses.

Statistical analyses were performed using the R environment (version 4.3.0) (R Core Team, 2021). Samples were rarefied to the lowest sample size depth (4,201 reads) as a normalization step in data analysis. We used the phyloseq package to create the phyloseq object (McMurdie & Holmes,

2013), and the ranacapa package (Kandlikar *et al*., 2018) to generate the visualization of rarefaction curves (Figure S1).

We used the phyloseq object to create the phylum relative abundance graph. We calculated the ASV's richness to use in alpha diversity measures with the package vegan (Oksanen *et al*., 2019) and used the ggpubr package (Kassambara, 2020) to add Wilcoxon comparisons on the Alpha Diversity graphs. For alpha diversity indexes, we included community richness (Observed ASVs) and community diversity for the calculation of richness and evenness (Shannon and Simpson estimates). For beta diversity, we used the package vegan to calculate the Bray-Curtis distances, the Anosim and Permanova tests. We used PCoA and NMDs graphs to visualize the similarity of host-associated bacterial communities between the environments/treatments. All the graphs were made using the ggplot2 package (Wickham, 2009). We calculated the total number of ASVs, as presence and absence, to understand the bacteria diversity found in the ant samples from each environment/treatment and which are shared between them. We used the package indispecies as a Differential Abundance Analysis, to see which bacteria genera were associated with an environment and which were shared between them (De Cáceres, 2013). The analysis identified associations between ASVs and the protected and contaminated habitats, utilizing species occurrence and abundance values from both sampled sites (not independently). In this model, we used the IndVal index, which measures the association between a species and a site group, and the group-equalized index "r.g", which avoids the unbalanced samples found in our study sites (De Cáceres *et al*., 2010). Finally, we used the package eulerr (Chen & Boutros, 2011) to create a Venn Euler diagram with the values of specific and shared bacteria genera.

Results

Alpha and Beta Diversity

The sequencing of the 16S rRNA region of bacterial communities generated a total of 8,417,738 raw reads in 49 samples. After rarefaction to 4,201 reads per sample, the analysis was carried out with all the samples. The alpha diversity of the ants' bacterial communities differed between the contaminated and protected areas (Observed ASVs: $p=0.01$; Shannon: $p=0.05$;

Simpson: p=0.05). In general, ants from both environments presented highly diverse bacterial communities. Nevertheless, ants from the Contaminated area showed higher alpha diversity in all estimates (Fig 2).

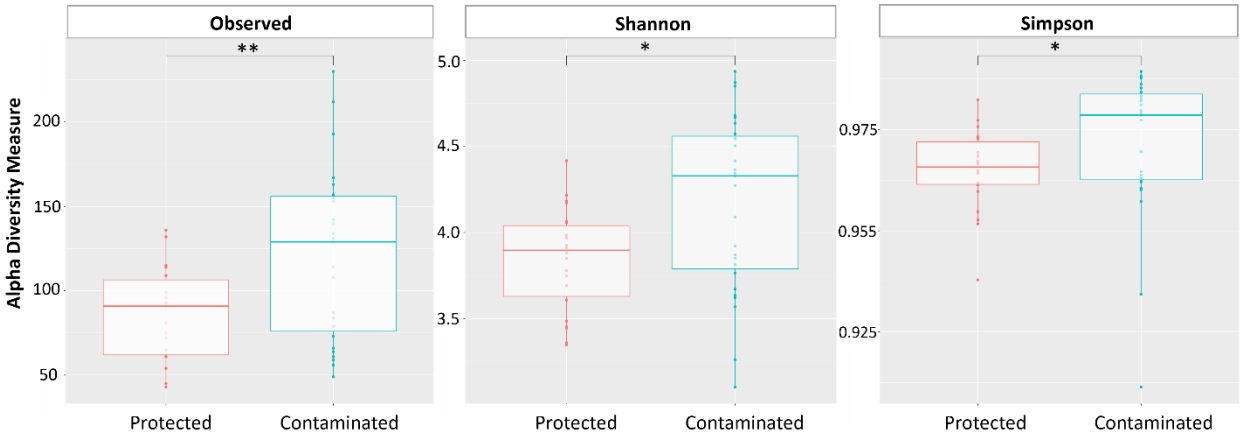

Fig 2. Box plots of distribution values for different indexes (Observed, Richness and Shannon), illustrating the alpha diversity of bacterial communities associated with the cuticle of *A. chartifex* ants from different environments. The Wilcoxon significance test represents p<0.01 (**) and p<0.05 (*).

The analysis of beta diversity shows that ants from the Protected and Contaminated areas differ in the cuticle-associated bacterial communities (Permanova; pseudo-F=1.77; p=0.001). Variation in the composition of the ants' bacterial communities is visualized using principal coordinate analysis (Fig 3a). The non-metric multidimensional scaling (NMDS) shows differences in the ants' bacterial communities between the environments, as well as variation in bacterial communities associated with ants from the main and satellite nests (Fig 3b). The Anosim test for the composition of bacterial taxa showed significant differences in ants' bacterial communities from the Protected and Contaminated areas (Bray-Curtis index, R=0.6087, p=1e-04), and between the nest types (Bray-Curtis index, R=0.2045, p=1e-04).

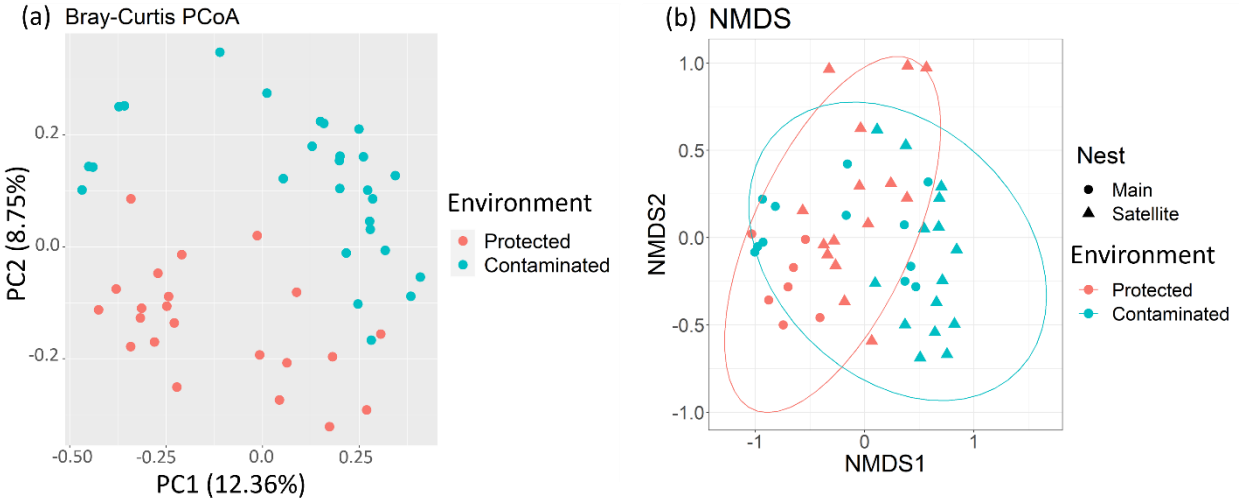

Fig 3. Ordination plots based on the Bray-Curtis similarity index, depicting the dissimilarity of bacterial community compositions associated with the cuticle of *A. chartifex* across two environments/treatments. (a) Principal Coordinates Analysis (PCoA); (b) Non-metric multidimensional scaling (NMDS), also shows the difference between nest types (Main and Satellite).

Composition of the ants' bacterial communities

In general, we found that ant-associated bacterial communities were dominated by Gram-negative Proteobacteria (30.5%). The gram-positive Actinobacteria was also in high relative abundance (16.23%), followed by the Gram-negative Bacteroidota (15.88%) and the Gram-positive Firmicutes (10.61%) (Fig. 4).

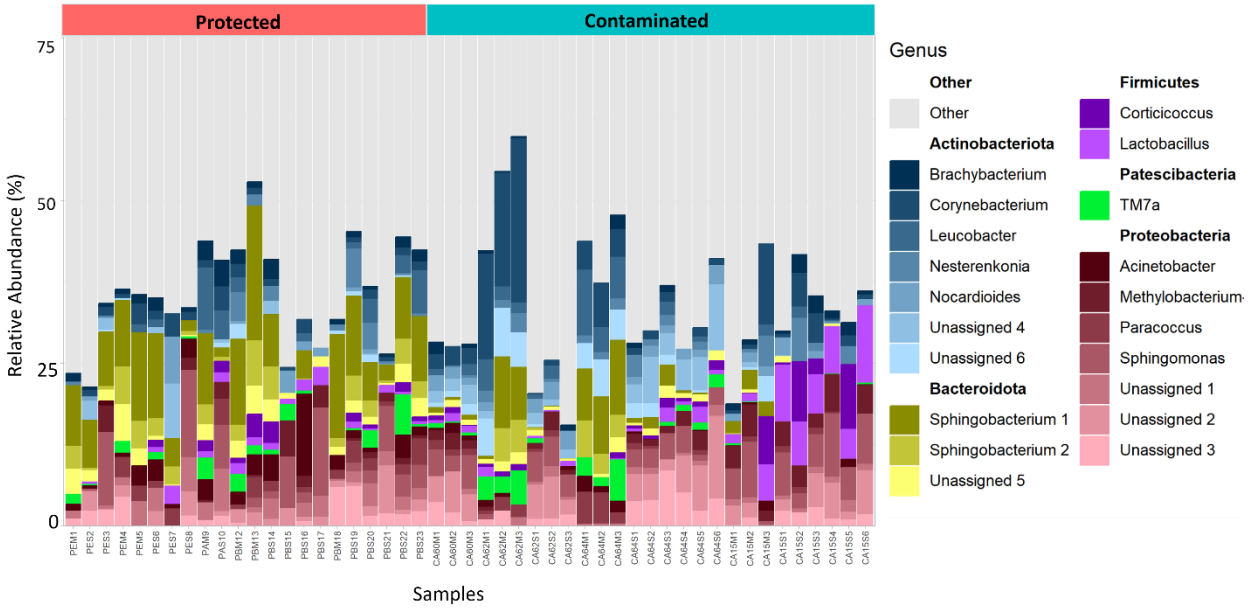

Fig 4. Phylum and genera (when possible) barplot of bacterial communities associated with *A. chartifex* from Contaminated and Protected areas. The graph shows the 20 most abundant bacteria genera from these samples.

Among the 20 most representative phyla, the ant cuticle bacterial communities from the Protected area had 73.39% Gram-negative bacteria, 20.61% Gram-positive bacteria, and 6% bacteria classified as unknown. Ants from the Contaminated area had 52.96% Gram-negative bacteria, 38.01% Gram-positive bacteria, and 9.03% unknown bacteria.

ASVs that most contribute to differences between environments

The 16S rRNA gene sequencing of the bacteria associated with the cuticle of *Azteca chartifex* ants resulted in 1,048 ASVs from a total of 8,417,738 sequences in 49 samples. The total number of ASVs was calculated to investigate the bacterial diversity in the ants' cuticle from both environments, and the ASVs shared between them. The shared bacteria identified in our dataset is determined by the presence or absence of ASVs, and their identities can be accessed in (Supplementary Material). Ants from Protected and Contaminated areas shared 165 ASVs (Fig. 5). On the other hand, analysis of differential abundance showed that each area had different bacteria guilds and taxa specifically associated to them. From the most abundant bacteria genera, the Gram-negative *Methylobacterium* and the Gram-positive *Lactobacillus*, were found only on the ants' cuticle from the Contaminated area, which had 3.8% of its bacteria species as habitat indicatives

(i.e., specifically associated to it). The Gram-negative: *Sphingobacterium*, *Acinetobacter*, *Sphingomonas*, as well as the Gram-positive *Brachybacterium*, and *Leucobacter* were exclusively associated with the Protected area, which had 17.9% of indicative bacteria species (Fig. 5).

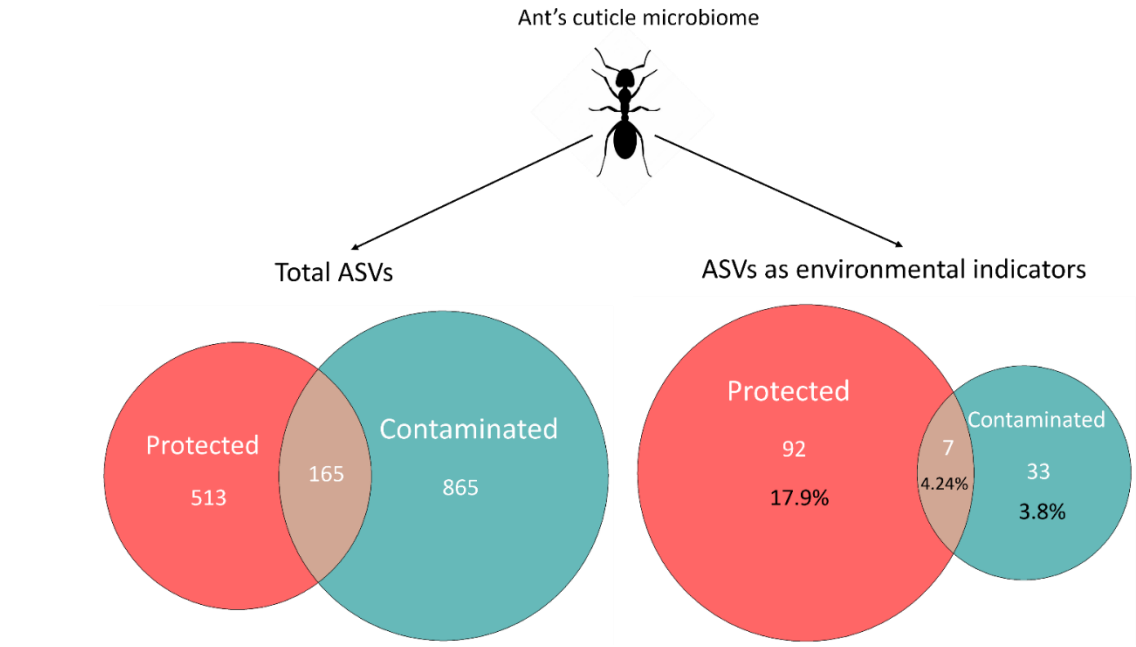

Fig 5. Venn-Euler Diagram illustrates specific and shared bacterial groups within each treatment category. Based on Indicator Species Analysis, it identifies distinct bacterial taxa as indicators for each group.

Discussion

In the present study we provide a first assessment of mining tailings impacts from a dam disaster on the bacterial communities of a dominant arboreal ant species in the Atlantic forest ecosystem. Our data reveal a greater number of unique bacterial taxa on ants' cuticles from the Contaminated area compared to Protected area. The Shannon index, known for its sensitivity to rare taxa within a community (Kim *et al*., 2017; Finn, 2024), highlights significant differences. Specifically, the bacterial communities associated with ants from the Contaminated area exhibited higher alpha diversity across all analyzed indexes. Gram-negative bacteria dominated the ants' cuticles from both environments. Furthermore, ants from each environment presented species-specific bacteria on their cuticles, often referred to as environmental bioindicator bacteria.

The environment can play a significant role in shaping the diversity and composition of ants' bacterial communities (Ramalho *et al*., 2019; 2020). We observed differences in both diversity and composition of bacterial communities associated with ants from the Protected and Contaminated areas. Variation in bacterial guild assemblages and composition may occur even between similar habitats within the same ecological community (Lee *et al*., 2008; Martins & Moreau, 2020). However, the differences found in the microbiota of *Azteca chartifex* in two riparian locations (Protected, Contaminated) within the same river basin and forest type, only 26.15 km apart, suggest differences could have been affected by the tailing impact rather than by chance. In addition, environmental heterogeneity can be the primary cause of differences in microorganisms' communities at a local scale (<1,000 km) (Wu *et al*., 2013; Chen *et al*., 2018).

Environmental stressors and soil microbiomes can have a significant influence on insects' bacterial communities (Hannula *et al*., 2019; Wu *et al*., 2020). Bacterial community structure can be characterized by the proportions of Gram-negative and Gram-positive bacteria (Kramer & Gleixner, 2008; Zhang *et al*., 2008; Fanin *et al*., 2019). Gram-negative bacteria were more abundant in the bacterial communities of ants from both locations. Although, ants from the Protected area exhibited a higher abundance of Gram-negative Proteobacteria. Degli Esposti & Martinez Romero (2017) traced the metabolic profiles of Proteobacteria in arthropods and concluded that the high abundance of this bacterial phylum is correlated with a stable microbiome in terrestrial animals.

Despite significant differences in overall bacterial community composition, *A. chartifex* from Protected and Contaminated environments shared a total of 165 ASVs. Considering the abundance of bacterial taxa, we identified environmental bioindicators associated with ants from each area and for the shared bacteria between them. The identities and abundance of bacteria can be influenced by environmental factors and pollution in the area (Sumampouw & Risjani, 2014). Ants from Contaminated area harbored fewer bioindicator bacteria on their cuticles compared to ants from Protected area. Environmental contamination may select for fewer abundant taxa over a higher number of rare taxa (Jiao *et al*., 2017; Yuan *et al*., 2022).

Several bacterial genera were found to be abundant in ants from Contaminated area, suggesting that these bacteria may play a significant role in shaping the bacterial community structure of ants in the presence of heavy metal pollution. For instance, *Methylobacterium* sp. has been isolated from soil contaminated by Zn, showing high resistance to this metal (Kunito *et al*.,

1997). This genus is commonly associated with the plant phyllosphere (Vorholt, 2012), raising the possibility that ants acquire these bacteria while foraging (Bitar *et al*. 2014). Additionally, a *Lactobacillus* species, prevalent in ants from the Contaminated areas and within the *A. chartifex* shared bacteria, has demonstrated potential as an indicator for heavy metal contamination and a candidate for bioremediation of zinc and copper in aqueous environments (Hasr Moradi Kargar & Hadizadeh Shirazi, 2020). Experimental assays involving these strains and heavy metals are essential to test their resistance.

This study represents a first assessment of the impacts of mining tailings resulting from a dam disaster on the bacterial communities associated with a dominant arboreal ant species in the Atlantic forest ecosystem. Ants from the Contaminated area exhibited higher alpha diversity of their cuticle bacterial community, along with a lower abundance of bacterial taxa classified as bioindicators of Contaminated areas. The composition of the ants' bacterial communities differed between the Protected and Contaminated areas, with a general dominance of Gram-negative Proteobacteria across both sites. Despite variations observed between the bacterial communities of ants from distinct environments, *A. chartifex* exhibited some shared bacteria associated with their cuticles. In conclusion, we propose that certain bioindicator bacteria associated with ants from contaminated areas may play a significant role in shaping the structure of ants' bacterial communities. Further investigation into the functional aspects of these bacteria is necessary to fully understand the interactions between ants, bacteria, and the environment.

Supplementary Information

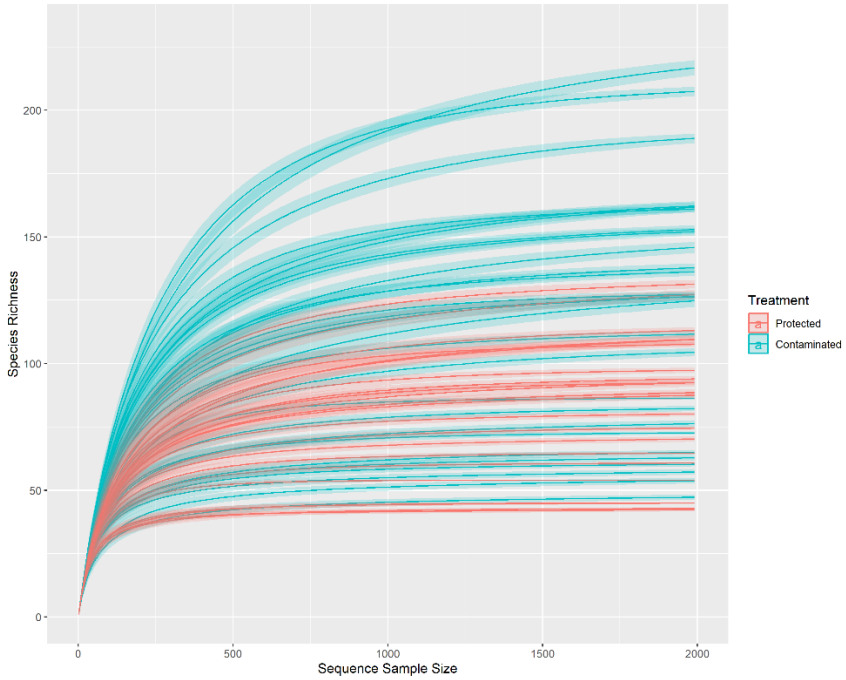

Fig S1 – Rarefaction curves of bacteria amplicon sequence variants, showing species richness in 49 ant samples (Protected area = 22; Contaminated area = 27).

Considerações Finais

Os três diferentes estudos desta tese de doutorado analisaram as comunidades bacterianas associadas às formigas e forneceram importantes discussões sobre a ecologia e impactos ambientais nestes microbiomas. Estes estudos, coletivamente, enfatizam a importância de compreender as interações entre formigas, bactérias e o ambiente ao redor, inclusive como potencial para bioindicação de impactos ambientais. Formigas compõe um grupo taxonômico classicamente utilizado como bioindicadores de impacto. A inclusão do microbioma dos corpos das formigas em análises de contaminação ambiental pode agregar um elemento sutil para detecções de impactos de difícil visualização ou detecção na escala da diversidade de animais.

O primeiro capítulo mostra que a proporção de bactérias Gram-negativas nas comunidades bacterianas associadas às formigas, varia com diferentes condições ambientais. Formigas que habitam ambientes imprevisíveis que produzem condições extremas para a sobrevivência dos insetos, como regiões temperadas e dosséis (formigas arborícolas), têm maior proporção de bactérias Gram-negativas nos seus microbiomas comparadas às formigas das regiões tropicais e que habitatm os chãos. Estes resultados sugerem que as bactérias Gram-negativas, por possuirem mais mecanismos de resistência em ambientes extremos, têm importante papel em adaptar e resistir a variabilidade ambiental em ambas micro e macro-escalas.

O segundo estudo revela uma significante variação entre ninhos nas comunidades bacterianas dos corpos de uma formiga polidômica, *Azteca chartifex*. As bactérias generalistas compartilhadas entre formigas de ninhos matriz e satélites, podem ter sido adquiridas diretamento do ambiente ao redor ou entre forrageiras dos diferentes ninhos. Alé disso, a presença das formigas nas árvores influenciam a composição das comunidades bacterianas das superfícies das folhas, diminuindo a diversidade de bactérias e compartilhando bactérias entre formigas e folhas. Estes resultados indicam que existe complexas vias de interação entre formiga, planta e bactérias.

O terceiro e último capítulo examina os efeitos dos rejeitos de metais nas comunidades bacterianas associadas à *Azteca chartifex.* Formigas dos ambientes contaminados exibiram maior alfa diversidade nas comunidades bacterianas associados ao seus corpos, e também apresentaram uma diferente composição bacteriana comparadas às formigas das áreas protegidas. Apesar destas diferenças, houve uma dominância bactérias Gram-negativas do filo Proteobactéria em ambos os

ambientes (contaminado e protegido). A presença de bactérias bioindicadoras específicas das áreas contaminadas sugerem o potencial destas bactérias em moldar as comunidades bacterianas associadas às formigas.

Os resultados desta tese sobre as comunidades bacterianas associadas às formigas, abrem janelas para futuras investigações. Algumas perspectivas chaves são importante para compreender melhor estas interações complexas, como:

- O papel funcional das bactérias Gram-negativas nos microbiomas das fomigas, especificamente nos ambientes variáveis. Investigar como estas bactérias contribuem para a resiliência e adaptação das formigas em ambientes extremos, pode melhorar nosso entendimento sobre a ecologia de microrganismos e sobre simbiose.

-Mecanismos de aquisição e transmissão de bactérias. Estudos devem explorar os mecanismos usados pelas formigas para adquirir e trasmitir bactérias entre ambientes. Isto inclui entender ambientes quais as fontes destas bactérias e o papel do comportamento das formigas sobre as assembléias das comunidades bacterianas. Abordagens experimentais e técnicas moleculares podem ajudar a entender estes processos.

-Interações tripartidas entre formigas-plantas-bactérias. Futuros estudos precisam entender melhor a dinâmica destas interações. Isto inclui examinar como o comportamento de forrageio das formigas influencia as comunidades bacterianas nas superfícies das plantas, e o potencial serviço ou deserviço que estas interações conferem para as plantas.

-Impacto de estressores ambientais. O impacto da contaminação das atividades humanas sobre as comunidades bacterianas das formigas é algo que nunca fora investigado. Estudos devem acessar como os poluentes alteram as comunidades bacterianas e a funcionalidade, e o potencial papel das bactérias bioindicadoras na manutenção da saúde ecossistêmica.

-Conservação do microbioma. Entender quais são as espécies chaves das comunidades bacterianas associadas às formigas de diferentes habitats são essenciais para elucidar aspectos de saúde das colônias. Consequentemente, este mapeamento ajudam a desenvolver estudos de funcionamento dos ecossistemas e na conservação de espécies.

-Estudos de longa-duração e experimentais. Conduzir estudos de longa duração e experimentos controlados podem promover a comprensão da estabilidade e dinâmica das comunidades

bacterianas associadas às formigas ao longo do tempo. Estes estudos podem ajudar a identificar quais os fatores são responsáveis pelas mudanças na composição dos microbiomas e quais as consequências ecológicas.

-Estudos interdisciplinares e colaborativos. Encorajar esforços colaborativos entre microbiólogos, entomólogos, ecólogos e cientistas ambientais pode gerar uma abordagem holística e mais completa no estudo das interações entre formiga e bactérias.

Com os resultados aqui apresentados e com as futuras perspectivas, os pequisadores podem avançar no entendimento dos papeis das bactérias associadas as formigas, que emergem para as interações das formigas com outros organismos.

Referências

Introdução

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ANEXOS

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DECLARAÇÃO

Em observância ao **§5° do Artigo 1° da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Canopy dominant ant species and associated microbiota : commensal adaptations to harsh environments*** ", desenvolvida no Programa de Pós-Graduação em Ecologia do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 12/10/2014

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Canopy dominant ant species and associated microbiota : commensal adaptations to harsh environments** , não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 12/10/2024

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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

Marília R. Bitar, Luiz-Marcelo R. Tomé, Fernanda V. Costa, Rodrigo B. Kato, Paulo S. Oliveira, Aristóteles Góes-Neto & Sérvio P. Ribeiro

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 associated with ants and the leaves they forage on. We found that ants from main and satellite nests have different 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, Kaltenpoth 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 (Currie & 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 (Kembel & 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 > 2 m) 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

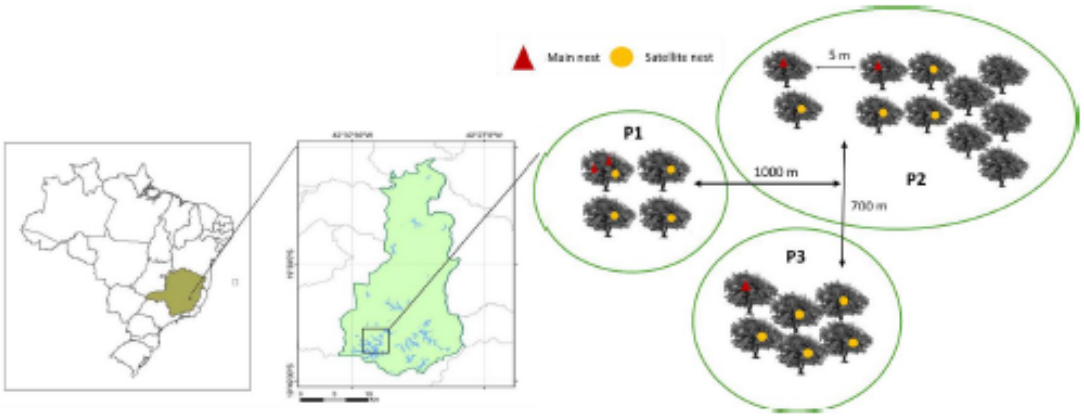

Fig. 1: Map of Brazil and Rio Doce State Park, showing the *Byrsonima sericea* tree and *Azteca chartifex* ant populations (P1, P2, and P3) across the study areas, located at two distinct forest-lake ecotones. Sampling design across the studied populations, showing trees with main and satellite nests 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

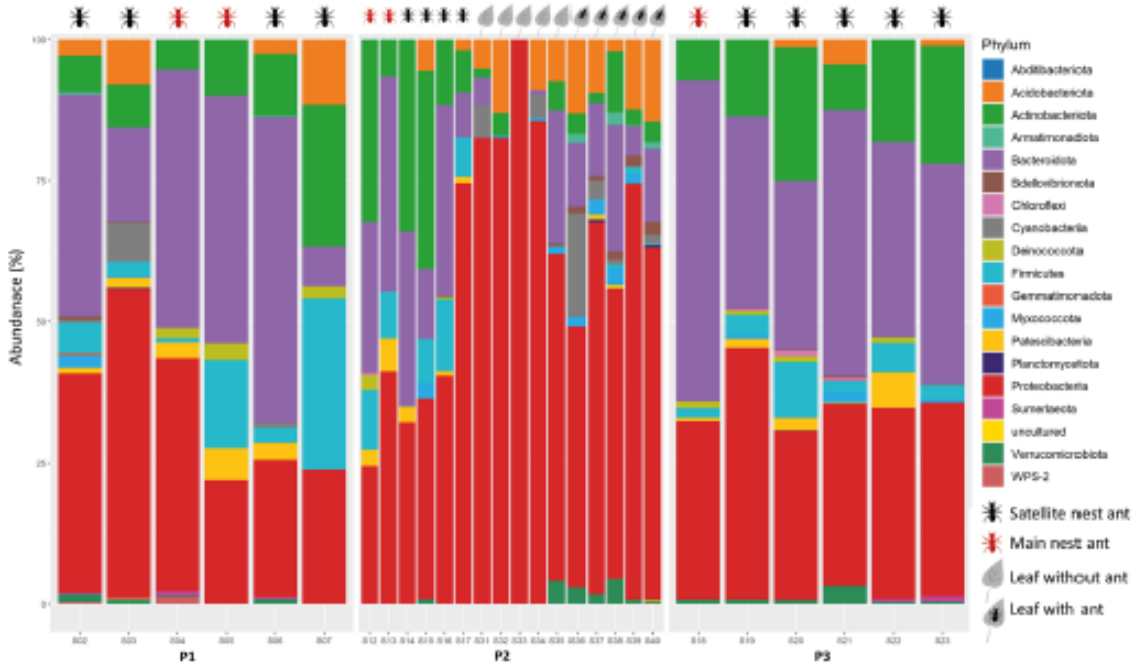

Fig. 2: Phylum variety analysis barplot of bacterial communities from ecotones samples: Samples of *Azteca chartifex* (main and satellite nests) from three locations (P1, P2 and P3) and samples of leaves of *Byrsonima sericea* trees with and without ant nests (location P2). Bars show the relative abundance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

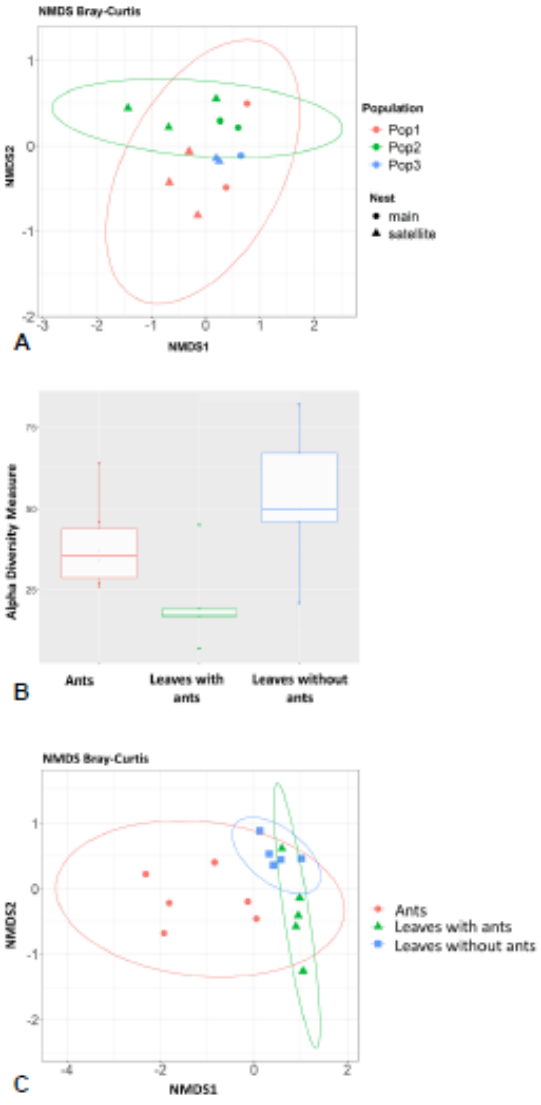

Fig. 3: (A) Non-metric multidimensional scaling (NMDS), with Bray-Curtis dissimilarity index, shows bacterial community composition of ants from different populations and nest types (main and satellite). (B) Observed alpha diversity of bacterial communities associated with ants, leaves with ants, and 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).

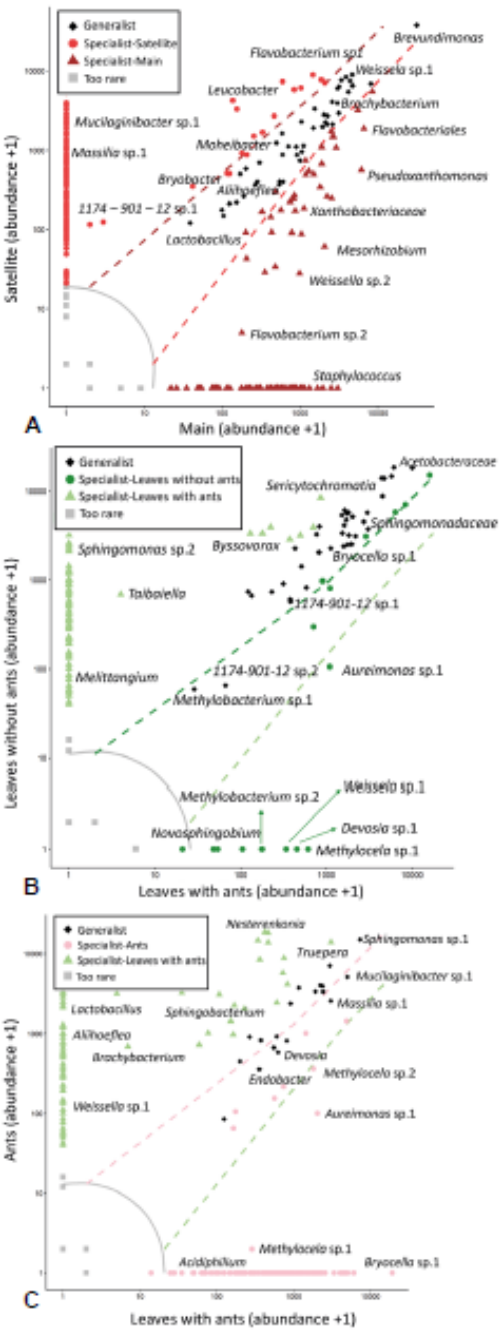

Fig. 4: CLAM test: (A) showing the specialist bacteria in ants' cuticles from satellite nests, specialist bacteria in ants' cuticles from main nests, and the generalist bacteria shared between the two sample types; (B) showing the specialist bacteria in leaves without ants, leaves with ants, and the generalist bacteria shared between the two sample types; (C) showing the specialist bacteria in ants, in leaves with ants, and the generalist 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, R^2 = 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; R^2 = 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, and 55.32% were classified as specialists on leaves 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 *Byrsonima 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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