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Environmental Contamination Affects Associated Bacterial Communities in a Neotropical Arboreal Ant

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ABSTRACT

Environmental stressors such as contamination from mining tailings can alter microbial communities associated with insects, including social ants. Ants, as widespread and ecologically influential insects with stable microbial associations, offer a relevant model to examine these effects. We investigate whether exposure to iron-rich tailings from a mining disaster is linked to changes in the diversity and composition of cuticle-associated bacterial communities in the arboreal ant *Azteca chartifex*. Using 16S rRNA gene amplicon sequencing, we compared ants from contaminated and protected areas in the Atlantic Forest. We tested whether bacterial diversity and community composition differ between environments and whether contamination is associated with changes in the ants' population-level core microbiota and the occurrence of indicator taxa. Ants from the contaminated area exhibited higher alpha diversity and a more variable microbiota across populations, while those from the protected area showed more similar microbiota. Community composition differed significantly between protected and contaminated environments, and distinct bioindicator bacteria were associated with each site. While our design was constrained by temporal and spatial separation between sites, the consistent core bacterial community across protected populations suggests that contamination, rather than distance, primarily explains the observed patterns. These results indicate that environmental contamination may influence ants' bacterial communities, potentially reflecting a response to ecological stress and altering microbe–host–environment interactions. This study provides a first assessment of how exposure to mining tailings may shape the microbiota associated with a dominant arboreal ant species and contributes to our understanding of insect–microbe dynamics in disturbed tropical ecosystems.

RESUMO

Estressores ambientais, como a contaminação por rejeitos de mineração, podem alterar as comunidades microbianas associadas à insetos, incluindo formigas sociais. As formigas, por serem insetos amplamente distribuídos e ecologicamente importantes, com associações microbianas estáveis, constituem um modelo relevante para investigar esses efeitos. Neste estudo, investigamos se a exposição a rejeitos ricos em ferro provenientes de um desastre de mineração está associada a mudanças na diversidade e na composição das

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comunidades bacterianas associadas à cutícula da formiga arborícola *Azteca chartifex*. Utilizando o sequenciamento de amplicons do gene 16S rRNA, comparamos formigas provenientes de áreas contaminadas e áreas protegidas na Mata Atlântica. Testamos se a diversidade bacteriana e a composição da comunidade diferem entre os ambientes e se a contaminação está associada a alterações na microbiota central (core microbiota) em nível populacional e na ocorrência de táxons indicadores. As formigas da área contaminada apresentaram maior diversidade alfa e uma microbiota mais variável entre populações, enquanto aquelas da área protegida exibiram microbiotas mais semelhantes entre si. A composição da comunidade diferiu significativamente entre os ambientes protegido e contaminado, e bactérias bioindicadoras distintas foram associadas a cada local. Embora nosso delineamento tenha sido limitado pela separação temporal e espacial entre as áreas, a consistência da comunidade bacteriana central entre as populações da área protegida sugere que a contaminação, e não a distância, explica predominantemente os padrões observados. Esses resultados indicam que a contaminação ambiental pode influenciar as comunidades bacterianas associadas às formigas, potencialmente refletindo uma resposta ao estresse ecológico e alterando as interações microrganismo–hospedeiro–ambiente. Este estudo fornece uma primeira avaliação de como a exposição a rejeitos de mineração pode moldar a microbiota associada a uma espécie dominante de formiga arborícola e contribui para a compreensão da dinâmica inseto–microrganismo em ecossistemas tropicais perturbados.

1 | Introduction

Ants are abundant and present across the planet, playing a fundamental role in the functioning of terrestrial ecosystems (Hölldobler and Wilson 1990; Underwood and Fisher 2006). Because most ants live in stationary nests and are easy to collect, they frequently serve as bioindicators of environmental contamination (Andersen 1997; Grześ 2010; Kaspari and Majer 2000; Skaldina et al. 2018). Bioindicators are living organisms whose abundance, physiology, or behavior change in predictable ways in response to pollutants or other stressors, making them useful for assessing the health and quality of ecosystems (Zaghloul et al. 2020). However, most studies using ants as bioindicators have focused on ant diversity, abundance, or contaminant accumulation, while largely overlooking how their associated bacterial communities respond to environmental conditions and human disturbance.

Ants have diverse and, in some instances, functional host-associated microbiota playing important roles for their hosts, with both host-specific and environmentally acquired components (De La Fuente and Marquis 1999; Van Borm et al. 2002; Heil 2008; González-Teuber et al. 2014; Russell et al. 2017; Birer et al. 2020; Moreau 2020). In many species, part of this microbiota forms a stable "core" community that is consistently associated with the host across individuals and environments (Moreau 2020; Russell et al. 2017). These microbiota 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 2009; Wernegreen et al. 2009; Kellner et al. 2015; Li et al. 2018; Díez-Méndez et al. 2019; Ashigar and Ab Majid 2021).

Ant colonies are particularly vulnerable to environmental disturbances because high density, genetic similarity, and eusociality increase their susceptibility to diseases and parasites (Van Meyel et al. 2018). To counteract this vulnerability, social insects have evolved collective immunity behaviors that reduce the risk of infection at the colony level (Currie et al. 1999; Cremer et al. 2007; Van Meyel et al. 2018). Among environmental stressors, heavy metal contamination poses a major threat to insects, including ants (Galloway and Depledge 2001; Sorvari et al. 2006; Feldhaar and Otti 2020). Exposure to heavy metals can reduce colony size and survival and impair individual immune function (Eeva et al. 2004; Sorvari et al. 2007; Grześ 2010), although some ant species have some tolerance to these contaminants (Rabitsch 1995; Eeva et al. 2004; Grześ 2009). For example, Sorvari et al. (2006) demonstrated that *Formica aquilonia* exhibits enhanced immune encapsulation of contaminants in polluted areas, and Klimek et al. (2021) showed that *Lasius niger* activity in contaminated soils can reduce heavy metal concentrations at a microscale and increase soil microbial biomass. Although ants are widely used to assess environmental contamination, the role of their associated microbiota in mediating responses to environmental stress remains poorly understood.

In ants, the cuticle is not only a physical barrier but also a chemically complex interface that mediates nestmate recognition, caste differentiation, and colony organization through cuticular hydrocarbons (Sprenger and Menzel 2020). These multiple functions make the cuticle a key interface between ants and their environment and provide a strong rationale for examining the microbiota associated with it, even though the links between chemical signaling and microbial community composition remain largely unexplored. Cuticular microbes also contribute to immune defense and protection against environmental threats (Currie et al. 2006; Mattoso et al. 2012; Li et al. 2024), and changes in these communities may therefore influence how ants cope with heavy metal pollution. In 2015, the rupture of an iron mine tailing dam from a big mining industry (Samarco) resulted in the largest global environmental disaster associated to mining activity (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 (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

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et al. 2022). So far, few independent studies have examined the impact of this disaster in fine-tuned ecological interactions in riparian Atlantic forests (Omachi et al. 2018; Cruz et al. 2020; Ribeiro et al. 2023). Consequently, investigations into ant species in this region affected by the mud remain limited, with only one study analyzing ant biodiversity and ecosystem functions (Fietto et al. 2024).

The Neotropical ant *Azteca chartifex* Forel, 1896 is an arboreal dominant species that exhibits aggressive territorial behavior and builds arboreal "carton" nests, with cellulose and processed fibers (Longino 2007). The main nest, where the queen lives, can reach more than 2 m in length 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). Bitar et al. (2021) showed the microbiota associated with the cuticle surface of these ants, especially on the legs, had a key role in the interaction of this species and the leaf surfaces on the trees by them occupied.

We investigated whether environmental exposure to heavy metal-rich mining tailings influences the diversity and composition of the cuticle-associated bacterial community in the arboreal ant *Azteca chartifex*, in an Atlantic rainforest of southeastern Brazil. 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 cuticle? (2) Do ants from the contaminated area exhibit changes in their core bacterial community and have indicator bacteria associated with their cuticle?

2 | Methods

2.1 | Study Area

Field work was carried out in 2020 and 2022, in the protected Atlantic forest reserve of the Doce River State Park (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.

2.2 | Sampling Design

We compared the cuticle bacterial communities of *Azteca chartifex* ants from forests exposed or not to the tailings. Protected areas were taken from Doce River State Park (PERD) and contaminated areas along the Doce river and a secondary river where the tailings invaded the margins (Figure 1). As it is impossible to get before-after data of microbiota, as the subject studied is a disaster, we kept both protected and contaminated sample sites within a very similar forest physiognomy, exclusively in the water-forest ecotone habitats, and ants taken from only one tree species. Also, the largest distance between sites was 26 km away. This location is situated in the mid river basin, 100 km (62.59 mi) from the dam breach. For this reason, the tailings wave had already lost momentum when it reached the Park, accumulated in the margins, covering the forest soil at various lengths, but did not damage the trees or the understory's tallest bushes. Due to technicalities, there were no possible restoration actions capable of removing the immense volume of materials accumulated from this disaster inside the forests and along the low and mid-river margins. Hence, the toxic mud remains in place, causing long-term contamination through the river (Gabriel et al. 2021).

We investigated three *A. chartifex* populations in the protected area (hereafter Pop 1, Pop 2, Pop 3; spatial distances shown in Figure 1b) and two in the contaminated area (hereafter Pop 4, Pop 5; see text above for distances). For each population, ants from polydomous colonies were sampled from both the main nest and the corresponding satellite nests. In the protected area, sampling was carried out in 2020, and ants were collected from five main nests and 13 satellite nests across three populations/locations (we used previous data from Bitar et al. 2024). These polydomous colonies were distributed across neighboring trees: Pop 1 comprised two main nests, Pop 2 one main nest, and Pop 3 two main nests, with four to five satellite nests selected from each location (see Figure 1b).

In the contaminated area, the sampling took place in 2022. In Pop 4 (located 60 m from the river), we collected ants from three main nests and three satellite nests. In Pop 5 (15 m from the river and 900 m from Pop 4), we collected ants from one main nest and two satellite nests. In this area, each colony had one or two satellite nests, typically located on the same tree as the main nest (see Figure 1c).

2.3 | DNA Extraction and 16S Sequencing

The DNA extraction of bacteria associated with the ant cuticle was performed under sterile conditions, following the protocols of the Quick-DNA Miniprep Kit (Zymo Research No. D3024) for samples from the protected area and QIAamp DNA Micro Kit (Qiagen Ltd.) for samples from the contaminated area. For contaminated sites, ants from each nest were pooled into two replicate samples prior to DNA extraction and sequencing. These replicates represent repeated measurements of the same nest and were not treated as independent biological units in downstream analyses. Both protocols had the following modifications: 30 workers of *A. chartifex* from each nest (main and satellite) were pooled in 2 mL tubes and washed with the extraction kit buffer. The samples were gently shaken (no vortex) 10 times at 5 min intervals over 30 min, such that all the DNA of cuticle-associated bacteria was extracted. A total of 36 samples of ants had DNA extracted and visualized in agarose gels.

Bacterial identification and relative read abundance were made using high-throughput sequencing of the 16S rRNA gene. Library preparation followed two protocols (Appendix S1). In the first protocol (ants from the protected area), the oligonucleotides 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used. In the

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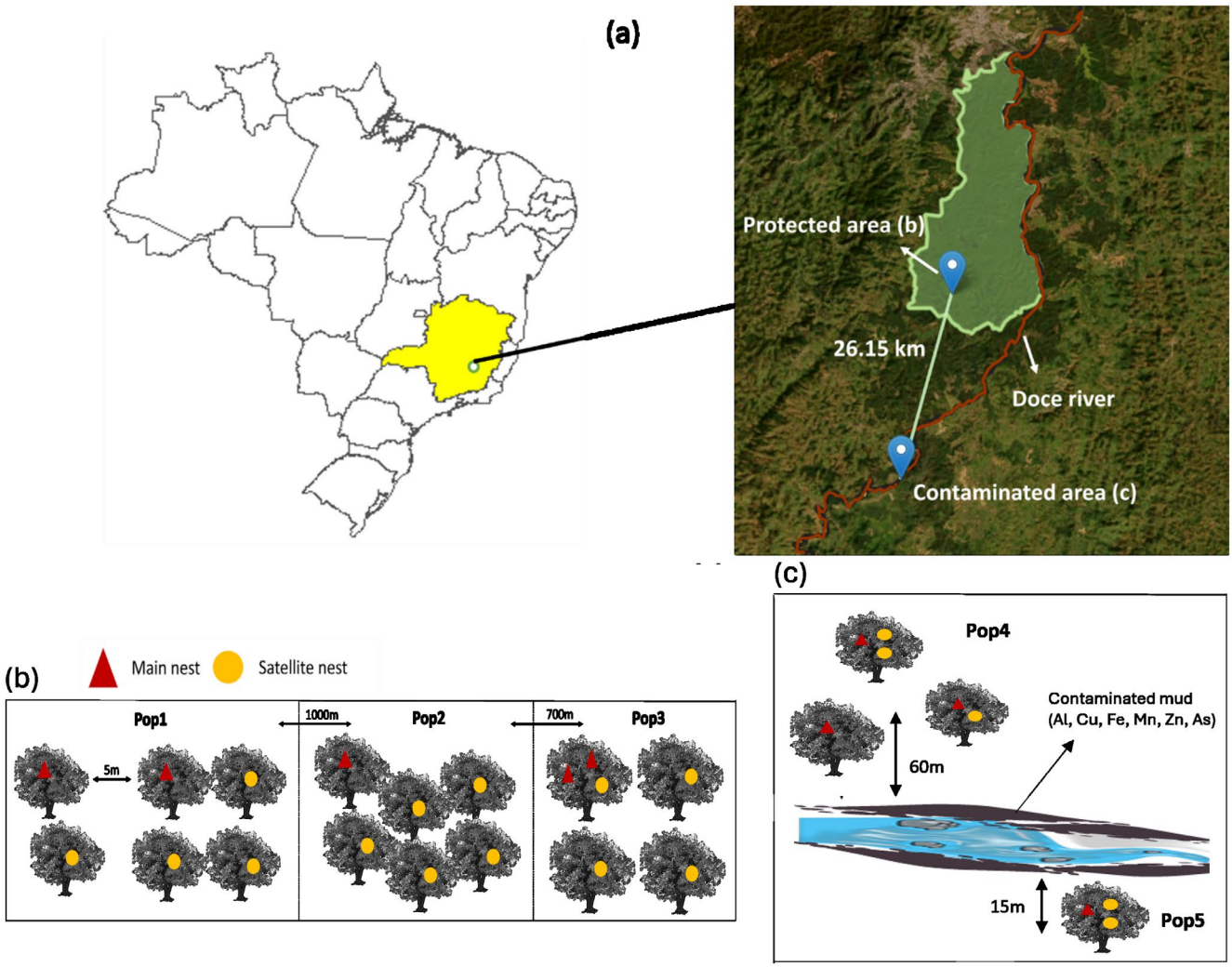

FIGURE 1 | (a) Map of Brazil and aerial view of the Atlantic forest in Eastern Minas Gerais state (yellow); the area of the Parque Estadual do Rio Doce (PERD) is highlighted. (b) Samplings of main and satellite nests were made in the protected area inside the PERD, and in (c) the contaminated area outside the park along the Doce river (note distance scale bars). Inside the park, 5 main and 13 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.

second protocol (ants from the contaminated area), the oligonucleotides 341F (CCTACGGGDGGCWGCAG), and 806R (GACTACNVGGGTMTCTAATCC) were used. The different 806R sequences represent degenerate variants of the same reverse primer used in distinct library preparation kits and target the same conserved site in the V3–V4 region. Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA). Paired-end runs of 500 and 600 cycles were performed using V2 × 500 or V3 × 600 sequencing kits (Illumina, USA) on average > 100,000 read coverage per sample. Although, two library-preparation protocols were used, all samples were processed under controlled laboratory conditions and analyzed using the same bioinformatic pipeline to ensure methodological consistency and minimize technical bias.

2.4 | Bioinformatic and Statistical Analyses

All the data were imported into Qiime2-2022.2 (Bolyen et al. 2019) using the Casava 1.8 paired-end demultiplexed fastq protocol. The sequence reads were trimmed (forward reads trim 280, reverse reads trim 5 and trunc 220) 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 (4940 reads) as a normalization step in data analysis. We used the phyloseq package to create the phyloseq object (McMurdie and Holmes 2013), which was then used to generate the phylum relative read abundance graph. We calculated the ASV's richness to use in alpha diversity measures with the package vegan (Oksanen et al. 2005) and used the ggpubr package (Kassambara 2020) to add Wilcoxon comparisons on the alpha diversity graphs. Alpha diversity was assessed using observed ASVs for richness and the Shannon and Simpson indices for richness and evenness.

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For beta diversity analysis, we used the *vegan* package to calculate Bray-Curtis distances. We applied the ANOSIM test to assess broad differences in bacterial community composition between the two environments and between ants from main and satellite nests. PERMANOVA was then used to assess finer-scale differences among populations within environments and to perform pairwise population-level comparisons. We used NMDs graphs to visualize the similarity of host-associated bacterial communities between the environments. All the graphs were made using the ggplot2 package (Wickham 2009).

To identify the cuticle core bacterial community of *A. chartifex*, ASV presence–absence data from the protected area were analyzed under four grouping schemes: (1) across all populations (population-level core), (2) across all populations within main nests, (3) across all populations within satellite nests, and (4) across all population × nest-type combinations (strict core). Core ASVs were defined as those detected in 100% of samples within each grouping scheme. Nest type (main vs. satellite) was therefore incorporated as an explicit ecological factor rather than being treated as a component of colony identity. For each core set, relative read abundance and contribution to total read counts were calculated. Core ASVs identified in the protected area were subsequently screened in contaminated populations to assess losses associated with mining impact.

We calculated the total number of ASVs, as presence and absence, to understand the bacteria diversity found in the ant samples from each environment and which are shared between them. We used the package indispecies to perform a differential abundance analysis by testing the association between bacterial ASVs and environmental categories (protected vs. contaminated) (De Cáceres 2013). This approach evaluates how strongly each ASV is associated with one or both environments based on its occurrence and relative read abundance across sites. Association strength was quantified using the IndVal index and its group-equalized variant (r.g), which corrects for unequal sampling effort by assigning equal weight to each site group (De Cáceres et al. 2010). Finally, we used the package eulerr (Chen and Boutros 2011) to create a Venn Euler diagram with the values of specific and shared bacteria genera.

3 | Results

3.1 | Alpha and Beta Diversity

The sequencing of the 16S rRNA region of bacterial communities generated a total of 6,518,770 raw reads in 36 samples. After rarefaction to 4940 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: $p = 0.0001$; Shannon: $p = 0.0001$; Simpson: $p = 0.0001$). In general, ants from both environments presented highly diverse bacterial communities. Nevertheless, ants from the contaminated area showed higher alpha diversity in all estimates (Figure 2).

The analysis of beta diversity shows that ants from the protected and contaminated areas differ in the cuticle-associated bacterial communities (Permanova; pseudo-F = 8.12; $p = 0.001$). 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, $R = 0.6462$, $p = 1e\text{-}04$), and between the nest types (Bray-Curtis index, $R = 0.179$, $p = 0.006$). When we compared bacterial communities associated with ants among populations within the protected area (Pop1, Pop2, Pop3), we found no significant differences. Furthermore, significant differences were observed between the microbiota of each ant population within the contaminated area (Table 1). 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 (Figure 3a), and among populations (Figure 3b).

3.2 | Composition of the Ants' Bacterial Communities

In general, we found that ant-associated bacterial communities were dominated by Gram-negative Proteobacteria (36.0%). The gram-positive Actinobacteriota was also in high relative read abundance (25.3%), followed by the Gram-negative Bacteroidota (23.4%) and the Gram-positive Firmicutes (4.71%). Also, the marked increase in Actinobacteriota and

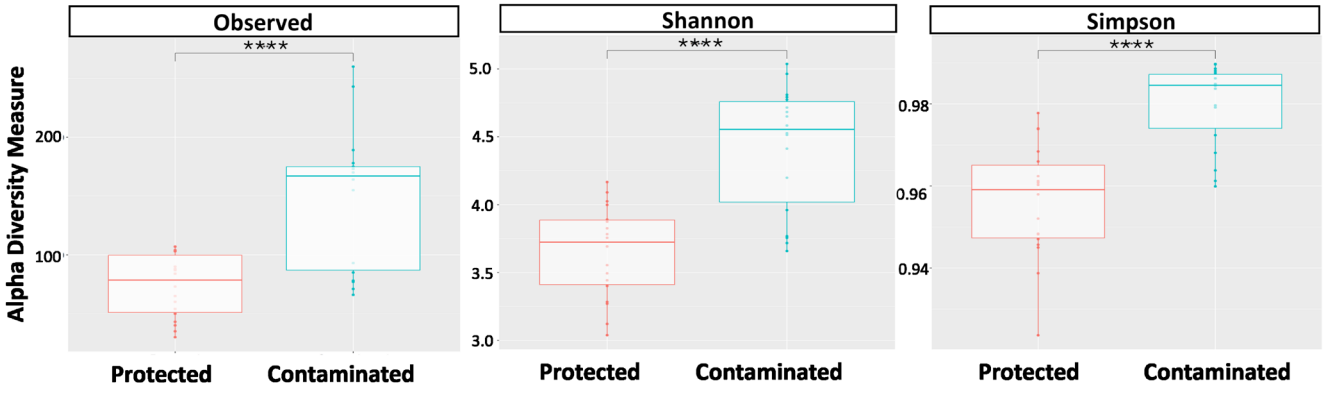

FIGURE 2 | Box plots of distribution values for different indexes (Observed, Richness and Shannon), illustrating the alpha diversity of bacterial communities associated with the cuticle of *A. chartifex* ants from different environments. The Wilcoxon significance test represents $p = 0.0001$ (****).

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TABLE 1 | Pairwise PERMANOVA results comparing bacterial community composition associated with *Azteca chartifex* across different ant populations.

Comparison	Pseudo-F (*F*)	*p* (Pr(>F))	R^2
Pop1 vs. Pop2	0.7735	0.681	0.07179
Pop1 vs. Pop3	1.2129	0.201	0.10817
Pop1 vs. Pop4	3.9913	**0.001**	0.19965
Pop1 vs. Pop5	4.1771	**0.003**	0.29464
Pop2 vs. Pop3	1.5806	0.099	0.13649
Pop2 vs. Pop4	3.9624	**0.001**	0.19849
Pop2 vs. Pop5	3.2702	**0.007**	0.24643
Pop3 vs. Pop4	6.5307	**0.001**	0.28986
Pop3 vs. Pop5	6.7856	**0.003**	0.40425
Pop4 vs. Pop5	3.3105	**0.004**	0.17143

Note: Populations Pop1, Pop2, and Pop3 are from the protected area, while Pop4 and Pop5 are from the contaminated area. The table presents pairwise comparisons, including pseudo-F values, *p*-values, and R^2 (proportion of variance explained) to assess differences in microbial communities between ant populations. Significant values ($p < 0.05$) are indicated in bold.

concomitant reduction of Bacteroidota in contaminated ants further suggests strong taxonomic filtering under metal stress (Figure 4).

3.3 | Core Bacterial Community and ASVs That Contribute Most to Differences Between Environments

A total of 415 ASVs were detected on the cuticle of ants from the protected area. When nest type was not considered, 104 ASVs (25.1% of total richness) were present in all populations and defined as the population-level core, accounting for 78.4% of total reads. Incorporating nest type substantially reduced the size of the core bacterial community. 33 ASVs were consistently detected across all populations in main nests, and 75 ASVs in satellite nests. The intersection between these groups comprised 19 ASVs, representing the strict core shared across all populations and both nest types (4.6% of richness and 36.6% of reads). When these protected-area core ASVs were evaluated in contaminated ants, four ASVs from the population-level core were completely absent, whereas only one ASV was lost from the main-nest core, one from the satellite-nest core, and none from the strict population × nest-type core (Table S1).

Ants from protected and contaminated areas shared 274 ASVs (Figure 5). On the other hand, analysis of differential abundance showed that each area had different bacteria guilds and taxa specifically associated with them (Table S2). Among the ASVs identified as significant habitat indicators, ASV291 (Gram-negative *Aureimonas* sp.), and ASV269 (Gram-positive *Microbacterium* sp.) were found exclusively on ants from the contaminated area, which contained 7.7% of its ASVs significantly associated with that environment. In contrast, several Gram-negative indicator ASVs, ASV16 (*Sphingobacterium* sp.), ASV15 (*Flavobacterium* sp.), and ASV21 (*Paracoccus* sp.) were exclusively associated

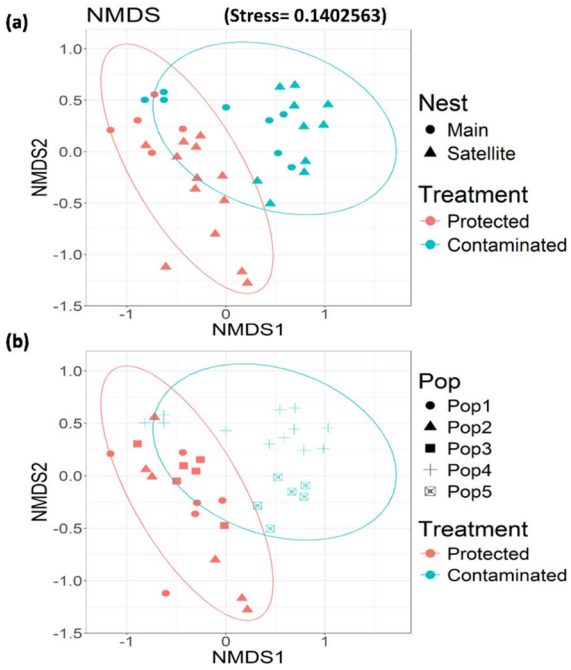

FIGURE 3 | Ordination plots based on the Bray–Curtis similarity index, depicting the dissimilarity of bacterial community compositions associated with the cuticle of *A. chartifex* across two environments: (a) Non-metric multidimensional scaling (NMDS), also shows the difference between nest types (Main and Satellite); (b) and the differences among populations.

with ants from the protected area, which contained 12.5% of ASVs significantly associated with that environment (Figure 5).

4 | Discussion

Our study presents a first assessment of how exposure to toxic mining tailings influences the cuticular bacterial communities of a dominant arboreal ant species in the Atlantic forest. Our data reveal a greater number of unique bacterial taxa on ants' cuticles from the contaminated area compared to the 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. The bacterial species composition differed between ants from the two areas, with a more similar microbiota among ant populations in the protected area and a more variable microbiota among ant populations in the contaminated area. Community differentiation also differed between the two contaminated populations: Pop4 showed consistently lower PERMANOVA effect sizes than Pop5 when compared with protected populations, and the Pop4–Pop5 comparison revealed moderate but significant divergence. This suggests that Pop4 may represent an intermediate or less impacted condition, whereas Pop5 shows stronger bacterial communities restructuring consistently with higher environmental stress. Additionally, contaminated populations exhibited a reduced population-level core bacterial community relative to protected populations. Finally, ants from each environment harbored specific ASVs on their cuticles, often

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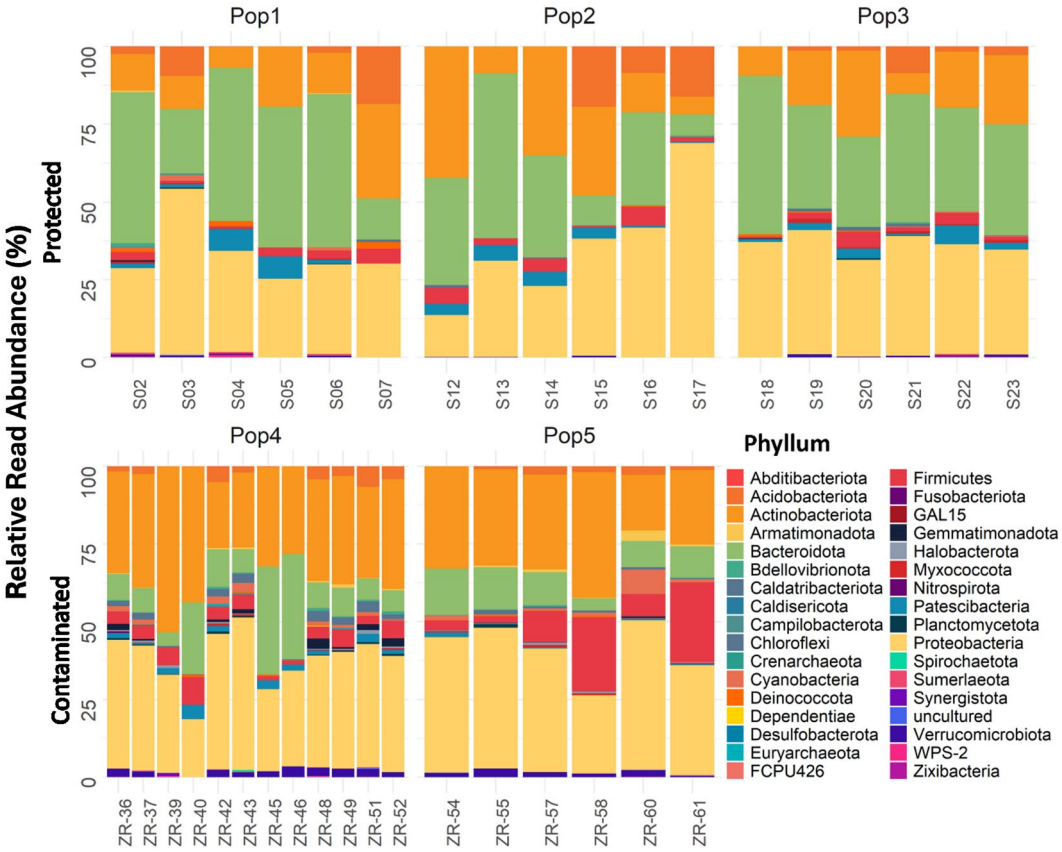

FIGURE 4 | Bar plot showing the relative read abundance of bacterial phyla associated with *Azteca chartifex* from contaminated and protected areas. Ant populations are grouped by the environment: Pop1, Pop2, and Pop3 from the protected area, and Pop4 and Pop5 from the contaminated area.

referred to as environmental bioindicator bacteria (Jiao et al. 2017; Shade and Handelsman 2012; Zaghloul et al. 2020).

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 and 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 and separated by 26.15 km suggest that factors beyond geographic distance may be contributing to community divergence, particularly environmental changes associated with mining tailings. Although microbial communities can exhibit distance–decay patterns (Hanson et al. 2012), the relative importance of geographic distance versus environmental heterogeneity is strongly scale dependent, and environmental factors often play a major role at local to regional extents (Wu et al. 2013; Chen et al. 2018). The environmental heterogeneity within the contaminated area may therefore generate distinct microbial source pools and promote greater variation in bacterial assemblages among ant populations.

Environmental stressors and soil microbiomes can significantly influence insect-associated bacterial communities (Hannula et al. 2019; Wu et al. 2020). A study conducted in an area impacted by the same mining tailings found that soil microbiota exhibited increased bacterial species richness (Vasconcelos 2021). Because *A. chartifex* ants also forage on the ground and on vegetation (Wheeler 1986; Bitar et al. 2021), changes in soil microbiota may indirectly affect canopy-dwelling ants through contact with contaminated substrates, plants, or associated arthropods. Such transmission provides a pathway by which soil disturbance reaches the canopy. Increased environmental heterogeneity and altered soil microbial source pools may promote stochastic colonization of ant cuticles, leading to greater variability in bacterial assemblages among populations (Zhou et al. 2014). At the same time, heavy metal stress may relax host filtering or select for tolerant taxa, such as Actinobacteriota, leading to bacteria phylum shifts detected in contaminated ants (Kannabiran 2017).

Despite the high overall richness observed in contaminated ants, we detected a measurable loss of bacterial taxa belonging to the population-level core bacterial community, defined as ASVs consistently present across all protected populations. Core microbial taxa are often interpreted as functionally important symbionts maintained by host filtering or long-term ecological associations (Neu et al. 2021; Shade and Handelsman 2012). Their disappearance therefore suggests that mining-derived environmental stress may disrupt stable bacteria even when total diversity increases. Notably, when nest type was incorporated into the core definition, the number of universally conserved taxa declined markedly, indicating that much of the apparent core community is structured by fine-scale spatial heterogeneity within colonies

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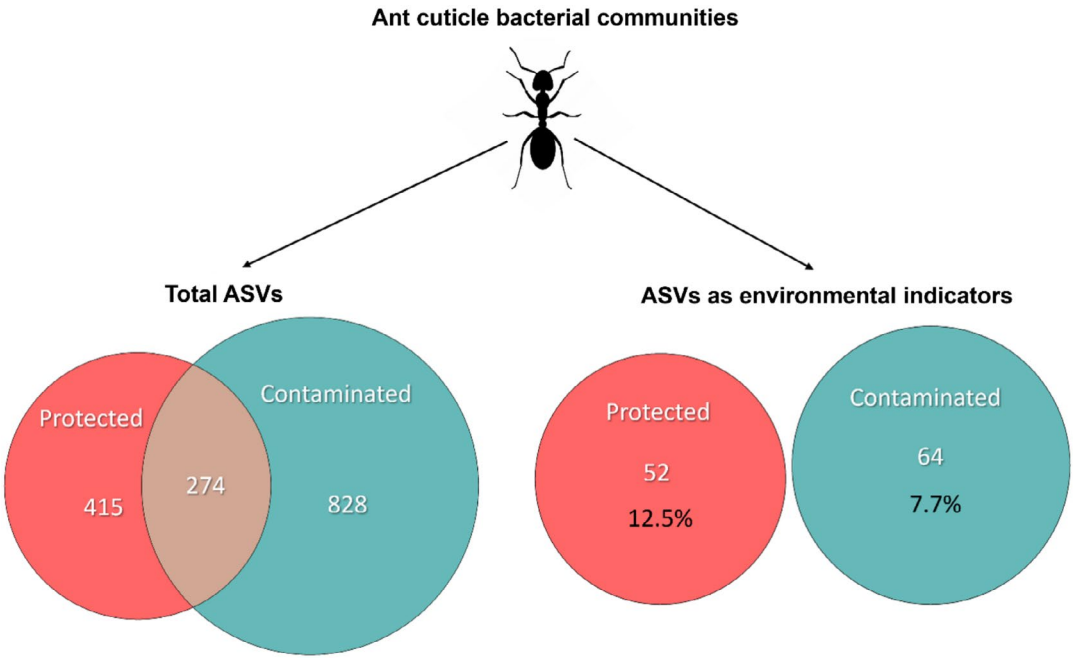

FIGURE 5 | Venn-Euler Diagram illustrates specific and shared bacterial groups within each treatment category. Based on Indicator Species Analysis, it identifies distinct bacterial taxa as indicators for each group.

rather than strict host dependence (Anderson et al. 2012). The persistence of a small subset of bacteria across populations and nest types further suggests the existence of a disturbance-tolerant microbial backbone, whereas the loss of population-level core taxa reflects reduced bacterial community stability under contamination.

Although the two environments differed markedly in bacterial community composition, *A. chartifex* from protected and contaminated environments shared a total of 274 ASVs. Considering the abundance of bacterial taxa, we identified environmental bioindicators associated with ants from each area. The identities and abundance of bacteria can be influenced by environmental factors and pollution in the area (Sumampouw and Risjani 2014). Ants from the contaminated area harbored fewer bioindicator bacteria on their cuticles compared to ants from the 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. In general, insect-associated microbiota can contribute to stress tolerance and detoxification (Engel and Moran 2013; Van Arnam et al. 2018). For instance, the bacterial genera *Aureimonas* and *Microbacterium* have been isolated from contaminated environments (Zhang et al. 2019; Rathour et al. 2020) and have shown potential roles in metal tolerance (Lin et al. 2016; Złoch et al. 2016). However, the association of these bacterial genera with ants remains poorly understood, as do their roles in structuring bacterial communities and their mechanisms of heavy metal tolerance.

Our findings suggest that environmental contamination from mining tailings may influence the structure and variability of bacterial communities associated with arboreal ants. These shifts in microbial diversity and composition could reflect a response to ecological stress, altering microbe–host–environment interactions. Given that cuticular bacteria are often linked to ant health, defense, and environmental sensing, changes in these communities may have broader implications for ant colony resilience and ecosystem functioning. While our design was constrained by temporal and spatial separation between sites, the consistent core bacterial community across protected populations suggests that contamination, rather than distance or sampling year alone, primarily explains the observed patterns. Future studies should explore the functional roles of these bacterial taxa, the long-term stability of contamination-driven microbial changes, and whether similar patterns emerge in other insect hosts across contaminated landscapes.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

The data that support the findings of this study are available in SRA at https://www.ncbi.nlm.nih.gov/, reference number PRJNA1117050. These data were derived from the following resources available in the public domain: Bioproject, https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1117050.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** Library Preparation and DNA Sequencing: **Table S1:** Core bacterial community composition associated with the cuticle of ants from the protected area, defined at different levels of stringency. The population-level core (Core_Pop) includes ASVs detected in all sampled populations regardless of nest type, whereas nest-type cores comprise ASVs consistently present across populations within main or satellite nests. The strict core (Core_Shared) corresponds to ASVs shared across all populations and both nest types. For each core definition, the table indicates ASV occurrence and their persistence in ants from contaminated environments, highlighting ASVs absent under contamination. **Table S2:** Indicator ASVs associated with protected and contaminated environments based on differential abundance analysis. Indicator value (IV) reflects the strength of association between each ASV and a given environment, with corresponding significance levels (*p*-values). Taxonomic assignments are provided at the lowest reliable rank. Only ASVs showing significant associations with either environment are included.

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