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# Disentangling the assembly mechanisms of ant cuticular bacterial communities of two Amazonian ant species sharing a common arboreal nest

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## Abstract

Bacteria living on the cuticle of ants are generally studied for their protective role against pathogens, especially in the clade of fungus-growing ants. However, little is known regarding the diversity of cuticular bacteria in other ant host species, as well as the mechanisms leading to the composition of these communities. Here, we used 16S rRNA gene amplicon sequencing to study the influence of host species, species interactions and the pool of bacteria from the environment on the assembly of cuticular bacterial communities on two phylogenetically distant Amazonian ant species that frequently nest together inside the roots system of epiphytic plants, *Camponotus femoratus* and *Crematogaster levior*. Our results show that (a) the vast majority of the bacterial community on the cuticle is shared with the nest, suggesting that most bacteria on the cuticle are acquired through environmental acquisition, (b) 5.2% and 2.0% of operational taxonomic units (OTUs) are respectively specific to *Ca. femoratus* and *Cr. levior*, probably representing their respective core cuticular bacterial community, and (c) 3.6% of OTUs are shared between the two ant species. Additionally, mass spectrometry metabolomics analysis of metabolites on the cuticle of ants, which excludes the detection of cuticular hydrocarbons produced by the host, were conducted to evaluate correlations among bacterial OTUs and *m/z* ion mass. Although some positive and negative correlations are found, the cuticular chemical composition was weakly species-specific, suggesting that cuticular bacterial communities are prominently environmentally acquired. Overall, our results suggest the environment is the dominant source of bacteria found on the cuticle of ants.

**KEYWORDS**

ant gardens, bacterial communities, cuticular microbiome, insect cuticle, metabarcoding

## 1 | INTRODUCTION

The composition of skin microbiota has been studied on a diverse array of animals. The regulatory role of host secretion in skin microbiota composition has been reported across a large range of animals such as humans (Bouslimani et al., 2015), birds (Jacob et al., 2018), bats (Lemieux-Labonté, Simard, Willis, & Lapointe, 2017) and frogs (Medina et al., 2017). For amphibians, skin-associated

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microbes are structured first by the amphibian species, followed by environmental factors (Kueneman et al., 2014; Weitzman, Gibb, & Christian, 2018). It is important to highlight that a large fraction of the variance is unexplained (Kueneman et al., 2014), which suggests that bacteria might be acquired through random immigration mechanisms from the surrounding pool of bacteria. In other words, the unexplained variance of the skin microbiota of animals supports neutral acquisition mechanisms. For example, in bats geographical location is the major contributor to the structure and composition of skin and fur bacterial communities (Winter et al., 2017).

In-depth studies of the human skin microbiota have revealed a strong influence of the individual's behaviour and environment (Schommer & Gallo, 2013), and in particular how they interact with other humans and their pets (Cuscó et al., 2017). This also supports neutral acquisition mechanisms because the microbial composition of human skin results, at least partly, from passive immigration from the surrounding pool of bacteria (Kim et al., 2018; Sieber et al., 2019). In addition, cohabitation has been shown to be a strong determinant of skin bacterial composition both within host species (Ross, Doxey, & Neufeld, 2017; Song et al., 2013; Torres et al., 2017) and across host species (Lemieux-Labonté et al., 2017; Song et al., 2013) in mammals, but not amphibians (Kueneman et al., 2014; McKenzie, Bowers, Fierer, Knight, & Lauber, 2012).

Chemicals present on the surface of the host, whether they are produced by skin bacteria or excreted by the host directly, can also determine the colonization success of environmental bacteria on the host body surface. For example, the cuticular Actinobacteria of fungus-growing ants produce antimicrobials that inhibit the growth of entomopathogenic fungi on the cuticle, thus preventing the transport of undesirable pathogens inside the nest (Boya et al., 2017). Likewise, secretions of the metapleural gland, a complex glandular structure present in most species of ants, are known to have role in sanitation with broad antimicrobial effects (Bot, Ortius-Lechner, Finster, Maile, & Boomsma, 2002; Yek & Mueller, 2011).

Associations between insects and antibiotic-producing Actinobacteria present on their cuticle are widespread across Neotropical Hymenoptera species, and are usually thought to supplement insect immune defence against infections caused by entomopathogens (Matarrita-Carranza et al., 2017). Unlike intracellular symbiotic microorganisms, which are most often involved in obligate and maternally transmitted symbiotic relationships, extracellular symbiotic microorganisms may have multiple transmission pathways in insects which could explain the high fidelity of the partnership between insects and antibiotic-producing Actinobacteria (Salem, Florez, Gerardo, & Kaltenpoth, 2015). Besides the vertical transmission of symbionts across generations, from parents to offspring, cuticular bacteria can also be transmitted horizontally through individual host interactions or recruited from the environmental bacterial species pool (Ebert, 2013). These environmentally acquired bacteria may then be inhibited by antimicrobial compounds from cuticular bacteria or secreted by the host. In fungus-growing ants and beewolf wasps, it is now accepted that the cuticle contains a higher proportion of dominant beneficial Actinobacteria strains than expected by chance (Scheuring & Yu, 2012), but the diversity, assembly and maintenance of the cuticular bacterial community in social insects remain unclear across large phylogenetic scales. Across most social insects it is unclear if the cuticular bacterial community is structured by the evolutionary history of the host, social behaviour (i.e., contact between individuals), or determined by the set of bacterial taxa living in the surrounding environment (e.g., in the material the nest is made of such as branches, plants and soil), or a combination of these mechanisms as suggested by Scheuring and Yu (2012).

Here, we studied two ant species, *Camponotus femoratus* (*Ca. femoratus*) and *Crematogaster levior* (*Cr. levior*), cohabiting the same nest across multiple locations, from the local to the regional scale. As originally described by Ule (1901) in Brazil, ant gardens are epiphytic plants housing an ant colony in their root system. First, ants integrate epiphyte seeds into the walls of a paper nest built on trees. The seeds then germinate, and the plant eventually forms the nest structure (Orivel & Leroy, 2011). In the Neotropics, the most abundant ant species living in ant gardens are *Ca. femoratus* and and *Cr. levior* and the most common epiphytic plant species in ant gardens are *Aechmea mertensii*, *Anthurium gracile*, and *Codonanthe crassifolia* (Corbara, Dejean, & Orivel, 1999). Ant gardens represent a rare case of parabiosis, where colonies of both species tolerate each other and share the same nest and nesting resources while keeping their broods separate (Forel, 1898; Wheeler, 1921). Both species are beneficial to the host plant, by enriching the root environment with minerals and organic matter (Belin-Depoux, 1991), dispersing seeds, and providing protection from herbivores. The ant colony benefits from having an arboreal nesting site close to nutritive resources, such as fruit, extra-floral and foliar nectars of the host plants themselves, and from surrounding plants. Such an ant garden parabiosis is hence an ideal system to investigate the factors structuring the bacterial community associated with the cuticle of ants, as it allows controlling for the bacterial composition variability between nests in different habitats or geographical locations, as well as across phylogenetically distant ant species sharing the same nests.

Using this unique system, we sampled ant gardens substrates and their inhabitants in three geographically distant sites in French Guiana ($N$ = 6 ant gardens, two per site) to characterize their bacterial community through 16S rRNA gene sequencing. We aimed to identify the portion of the cuticular microbiota that is specific to each ant species, shared by ant species and randomly acquired from the environmental pool of bacteria (i.e., shared between hosts and the nest material) across geographical locations. To better understand the cuticular bacterial community assembly process, we further studied the cuticular metabolomes of the two ant species using mass spectrometry to test the hypothesis that the cuticular chemical compounds, produced either by the host or by resident bacterial strains, could be involved in the recruitment or exclusion

of environmental bacteria or, more generally, involved in shaping the cuticular bacterial community.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling of hosts and nest material

Garden ant workers of *Camponotus femoratus* and *Crematogaster levior* (*Ca. femoratus*: ~5 mm, *Cr. levior*: ~2 mm) were collected in their ant garden nest at Montagne de Kaw (nest K1: Latitude: 04°33′300″N, Longitude: 052°10′303″W; nest K2: Latitude: 04°33′320″N, Longitude: 052°10′290″W), at Petit Saut (nest P1: Latitude: 05°02′890″N, Longitude: 052°57′938″W; nest P2: Latitude: 05°02′949″N, Longitude: 052°57′667″W) and at Nouragues (nest N1: Latitude: 04°02′350″N, Longitude: 052°40′070″W; nest N2: Latitude: 04°02′532″N, Longitude: 052°40′605″W, Figure S1). These three sites are located in the Amazonian forest in French Guiana and were sampled between February and March 2016. All gardens were at a maximum height of 2.5 m above ground level. For each ant species, 20 individuals were collected within the same nest for studies of both the cuticular bacterial community and the cuticular metabolome. Individuals of each species separately were transferred into sterile plastic tubes (i.e., a maximum of 10 individuals of the same species per tube) with sterilized tweezers that were flamed and rinsed in ethanol between individual collections, and the ants were killed the same day at −20°C. Dead ants were used to avoid a stress response caused by the washing step, which may contaminate the cuticular bacterial community with faecal evacuations. Furthermore, four samples of nest material were collected within each of these six ant garden nests for analysis of nest bacterial community. Nest material samples consisted of ~1 g of a mix of soil and roots where ant workers of both species were collected.

### 2.2 | Bacterial community study

#### 2.2.1 | Ant washing and DNA extractions

All the experiments were carried out in a sterile environment (i.e., biosafety cabinet with laminar flow). Bacterial DNA extraction of the cuticle of individual ants was performed using an optimized protocol and the Qiagen QIAamp DNA mini kit (QIAamp; Qiagen) as described previously Birer, Tysklind, Zinger, and Duplais (2017). Ten individuals of each *Ca. femoratus* and *Cr. levior* colony were washed separately, as well as four samples of nest material. To ascertain potential sources of DNA contamination, two negative extraction controls were included in each DNA extraction batch (i.e., one per nest). We thus extracted the DNA of a total of 156 samples: 60 individual cuticular washes for each ant species *Ca. femoratus* and *Cr. levior*, 24 samples of nest materials, and 12 negative extraction controls.

#### 2.2.2 | PCR amplifications and sequencing

We used the V5–V6 regions of the 16S rRNA gene to assess bacterial community diversity and composition. The universal primer pair used was BACTB-F (GGATTAGATACCCTGGTAGT) and BACTB-R (CACGACACGAGCTGACG), which amplifies a fragment of about 295 bp in length (Fliegerova et al., 2014). Both forward and reverse primers were labelled with a combination of two different 8-nt tags (Taberlet, Bonin, Zinger, & Coissac, 2018) before amplicon multiplexing and sequencing in order to reassign amplicons to their respective samples during bioinformatics analyses (see below). PCR (polymerase chain reaction) amplifications were duplicated for each sample. The PCR mixtures contained of 1 µl of extracted DNA, 0.5 µM of each primer (Eurogentec), 31.25 µM of each dNTP, 2.5 mM of $MgCl_2$, 0.05 U/µl AmpliTaq Gold Polymerase (Applied Biosystems), and 1× AmpliTaq Gold Buffer, and UHQ DNA-free water to reach a final volume of 20 µl. The PCR thermocycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 7 min. In addition to negative extraction controls, six PCR controls were introduced to evaluate DNA contamination incorporated at the PCR step. All PCRs, including controls, were pooled and purified, and the library was prepared with the Illumina TruSeq Nano kit for Illumina sequencing without a library enrichment step to reduce tag-switching events (reviewed by Taberlet et al., 2018). The library hence comprised a total of 324 PCRs. The library was then sequenced on one lane of an Illumina MiSeq platform using paired-end 2× 250-bp technology at the Genotoul platform.

#### 2.2.3 | Sequence analysis

The 2,552,604 obtained sequencing reads were subjected to a standard data curation process mainly based on the OBITOOLS package (Boyer et al., 2016). First, paired-end reads were assembled into extended single sequences, taking into account read overlap quality, with the FLASH algorithm (Magoč & Salzberg, 2011). Second, sequences were assigned to their respective sample by allowing 0 errors in tags and a maximum of two errors in primers. Sequences that did not fulfill these criteria were discarded, as well as those that were likely to be sequencing errors (i.e., containing ambiguous nucleotides or shorter than 100 bp). Finally, the remaining sequences were dereplicated and clustered into OTUs (Operational Taxonomic Units) with SUMACLUST (Mercier, 2013) with a sequence similarity threshold at 97%. The most abundant sequence of each OTU was considered as the OTU representative and was taxonomically assigned with the SILVA database (release 128; http://www.arb-silva.de), using the default parameters of the NGS analysis pipeline of the SILVA rRNA gene database project (SILVANGS 1.3; Quast et al., 2013).

For the whole data set, (a) we removed artefactual OTUs (e.g., degraded or chimeric fragments) by excluding OTUs that were not assigned to bacteria. Amongst the remaining OTUs, we further identified and removed (b) contaminant OTUs (i.e., present either

in chemical reagents or in technical material), which were defined as having a higher read abundance in at least one negative control as compared to biological samples. This criterion relies on the idea that any contaminant OTU from reagents or aerosols should be amplified more efficiently in negative controls, where there is no competition with other fragments for PCR amplification. The 12 negative extraction controls and six PCR controls were subsequently removed from the data set. Then, (c) false positive OTUs that can be generated during the library preparation of the sequencing or during sequencing itself (Esling, Lejzerowicz, & Pawlowski, 2015; Schnell, Bohmann, & Gilbert, 2015) were set to 0 in downstream analyses. Sample PCR duplicates were summed. More details are available in Birer et al. (2017). Finally, to exclude potential failed or low-success PCRs for which amplification was potentially inhibited, we excluded any PCR that yielded fewer than 500 reads.

After these data curation steps, we retained 59 *Ca. femoratus*, 58 *Cr. levior*, and 21 nest material samples for downstream analysis. We also discarded OTUs that accounted for less than 0.01% of the entire OTUs relative abundance, because uncertainties of low-abundance/occurrence species can alter estimates of species richness (Haegeman et al., 2013) and indicator species Cáceres and Legendre (2009). The additional filtering produced an OTU table with 926 OTUs. Samples harbouring fewer than 100 OTUs were also considered as potential failed or low-success PCRs and excluded from the analysis. After curation, 121 biological samples comprising 51 *Ca. femoratus*, 50 *Cr. levior*, and 20 nest material samples were used for further analysis (instead of 60, 60, and 24) with 922 OTUs and 949, 631 reads (see Table S1 for details).

## 2.3 | Cuticular metabolites study

### 2.3.1 | Ant washing and metabolites extraction

Individual ants cannot be used at the same time for the microbial molecular ecology study and the metabolomic analysis of the cuticle because each protocol is specific and noncompatible for the two analyses. Because we aim to find shared biological or chemical *m/z* features across individuals from the same species or nest, we assume that the variance in the signal of association between biological and chemical features is smaller at the individual within-species/nest level than the variance among species/nest level. Dead individual ants were washed in an organic solvent mixture (ethyl acetate/methanol/water; 2:1:1) selected for its ability to solubilize metabolites with different polarities, from hydrophilic to lipophilic. Ten individuals of *Ca. femoratus* and *Cr. levior* were washed separately and shaken for 1 min on a vortex in order to facilitate the solubilization of cuticular metabolites. The solvent was then removed and introduced into glass vials and kept at −80°C until injection into the mass spectrometer. The complete experimental design is composed of 120 extractions: 60 individual cuticular metabolite-washed solutions for each ant species (i.e., *Ca. femoratus* and *Cr. levior*).

### 2.3.2 | Metabolomics analysis through mass spectrometry

The metabolomic analysis was run on a liquid chromatograph coupled to a SYNAPT G2 HDMS mass spectrometer (Waters) in August 2016 at the pharmacy faculty Paris-Descartes. The 120 biological samples were injected randomly with one quality control (QC) injection every five biological sample injections. QC is a representative pool of all biological samples, which makes it possible to follow the stability of the system throughout the analysis. In positive ionization mode, 5 µl was injected on a column (Waters Acquity UPLC, 2.1 × 100 mm, porosity 1.7 µm) at 40°C. The soft electrospray ionization source (ESI) of this instrument precludes the ionization (i.e., detection) of cuticular hydrocarbons.

The mobile phase was delivered with an elution gradient at 0.3 ml/min with water (solvent A) and acetonitrile (solvent B), both containing 0.1% of formic acid. The solvent gradient was: 5% until 100% of B (0–7 min). Mass range acquisitions were between 100 and 1,500 *m/z*, and data were collected in centroid mode.

### 2.3.3 | Chemometric data analysis

After LC-MS analysis, raw data were converted to mzXML with PROTEOWIZARD software (Chambers et al., 2012). All chemometric analysis were carried out in R (R Core Team, 2017). LC-MS spectra processing was carried out using the XCMS package (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006). Parameters used for peak picking were: Chemical feature detection: *centWave*, signal to noise ratio cutoff (snthresh = 3), chromatographic peak width (peakwidth = [5,12]), Retention time correction: retcor method *obiwarp*. The resulting data matrix was processed under a workflow following Di Guida et al. (2016). Filtering of data included the following steps: (a) removing features with relative standard deviation greater than 30% in QC, (b) removing features with more than 95% of missing values, (c) data normalization with Probabilistic Quotient Normalization (PQN), (d) imputing missing values with Random Forest, and (e) transforming data with a generalized logarithm (glog).

## 2.4 | Statistical analyses

### 2.4.1 | Microbial diversity analyses

To evaluate whether the cuticular bacterial communities were appropriately sampled for each individual, we built rarefaction curves on curated data for each individual of *Ca. femoratus* and *Cr. levior* and for nest material samples (Figure S2). As none of the rarefaction curves reached a plateau, we chose to interpret alpha diversity with the exponential of the Shannon diversity index, which is robust to differences in biodiversity sampling effort (Chao, Chiu, & Jost, 2014; Haegeman et al., 2013).

To generate normalized sequence counts, we performed Cumulative Sum Scaling (CSS) normalization on the raw sequence counts (Paulson, Stine, Bravo, & Pop, 2013) using the package METAGENOMESEQ (Paulson, Talukder, Pop, & Bravo, 2017). CSS was specifically developed for sparse microbiota metabarcoding data, such as the data obtained here. CSS is an extension of the quantile normalization approach suited to marker gene survey data. It consists of scaling raw counts, which are relatively invariant across samples, up to a given percentile (determined empirically from the data) in order to mitigate the influence of larger abundance values.

The diversity of the cuticle microbial community (alpha diversity) of each ant individual was computed using an exponential Shannon index as explained above using the vegan R-package (Oksanen et al., 2016). The exponential function was applied to the Shannon diversity index to calculate the effective number of species (Chao et al., 2014; Haegeman et al., 2013). We then performed a linear regression using exponential Shannon diversity index as the explanatory variable. To test the effect of sample type and nest identity, as well as their interactions, we used a two-way ANOVA. Post-hoc pairwise comparisons were then performed with Tukey post-hoc tests with FDR (false discovery rate) *p*-value corrections for multiple testing.

We then assessed the effect of the locality (site and nest identity) and host ant species on bacterial beta-diversity (i.e., community composition variation across samples). Beta-diversity was defined as pairwise Bray–Curtis dissimilarities (Legendre & Legendre, 2012) on CSS-normalized data. Community differences were visualized using a Principal Coordinates Analysis (PCoA), and the effects of the tested factors and their interaction were assessed with a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001). Similarly, the effect of the host species (i.e., sample type excluding the nest material [*Ca. femoratus* and *Cr. levior*]) was assessed by PERMANOVA to further characterize how host species differed within each nest.

We then identified the bacterial OTUs that were significantly associated with each host species, that is statistically more abundant and most commonly found in each of the following bacterial communities: (a) *Ca. femoratus* core bacteria, (b) *Cr. levior* core bacteria, (c) nest material bacteria and (d) ant shared bacteria, (e) *Ca. femoratus*-nest shared bacteria, and (f) *Cr. levior*-nest shared bacteria (Figure S3). To identify core bacteria of each group, we used the Indicator Value test (*IndVal*), specially designed to assess the predictive value of a taxon, here an OTU, as an indicator of one or a combination of sample types (*Ca. femoratus*, *Cr. levior*, or nest material). *IndVal* tests are based on the comparison of occurrences and abundances of OTUs across sample types (Cáceres & Legendre, 2009). The *multipatt* function of the package *indicspecies* (Cáceres & Legendre, 2009) was used to compute indicator values, and significance was assessed with 9,999 permutations. Resulting *p*-values were corrected for multiple comparisons. OTUs with *IndVal* > 0.4 and corrected *p*-value <.05 were retained as indicators of their respective groups.

Finally, we conducted a phylogenetic analysis of all OTUs, including OTUs identified in one of our groups, together with their three most similar sequences in the GENBANK *nr* database based on their alignment coverage and e-values (GENBANK assessed in November 2017). We did so to determine if these specific OTUs were phylogenetically more related to each other, but also to further predict their putative ecological traits (based on GENBANK descriptions, e.g., isolation source), assuming bacterial traits may be conserved phylogenetically (Martiny, Jones, Lennon, & Martiny, 2015). The aim here is to infer the potential ecology of the core bacterial communities by comparing their phylogenetic relatedness with 16S rRNA gene sequences from GENBANK with known habitat preferences (i.e., the habitat/organism from which they were isolated). For each major bacterial phylum, we performed a multiple sequence alignment of each corresponding OTU and their three closest GENBANK sequences with *MUSCLE* as implemented in GENEIOUS version 10.2.3 (http://www.genious.com; Kearse et al., 2012) and reconstructed a maximum likelihood (model GTR [Generalized Time Reversible]) phylogenetic tree with *phangorn* (Schliep, 2011). Phylogenetic trees were visualized with *ggtree* (Yu et al., 2016). All statistical analyses were carried out in R (R Core Team, 2017).

### 2.4.2 | Metabolites analysis

Just as an OTU does not characterize unambiguously a bacterial taxon, the term "*m/z* chemical feature" used for our metabolomics approach (instead of metabolite) represents the ionized small molecules (i.e., molecular weight <1,000 Da) which are variables with a unique *m/z* and retention time.

Because some samples were discarded during filtering steps in both the metabolomics and metabarcoding analysis workflow, we obtained an unbalanced number of individuals between the two data sets, which precludes conducting cross-comparisons. To circumvent this limitation, we included a total of 46 *Ca. femoratus* individuals and 45 *Cr. levior* individuals, leading to the same number of individuals for each ant species for each nest in both data sets (metabolomic analysis and metabarcoding; Table S2).

We then assessed the effect of ant species and nest identity on the cuticular diversity features (i.e., variation in chemical composition across samples) by computing pairwise Bray–Curtis dissimilarities on normalized chemical data and conducting a PCoA and PERMANOVA (Anderson, 2001) using the same scheme as explained above for microbial data.

### 2.4.3 | Metabarcoding and metabolomic analysis data integration

As we did not collect bacterial communities and metabolites on the same individuals (but individuals from the same nest), we conducted each of the following analyses 10 times, by shuffling chemical samples within the same species and nest while keeping bacterial community samples fixed. (a) We first assessed if chemical features profiles co-varied with the cuticular bacterial

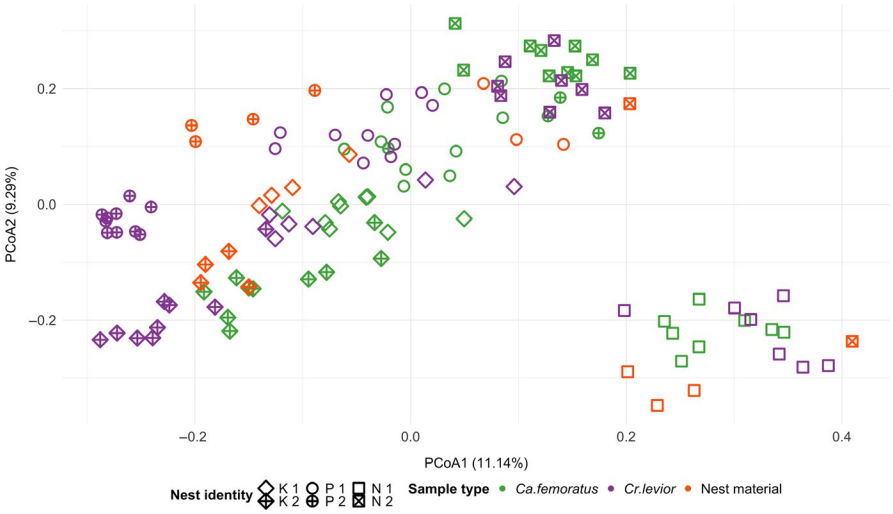


**FIGURE 1** Principal Coordinate Analysis showing the differences in bacterial community composition (i.e., beta diversity) across sample type and nests. Bacterial community composition similarities between *Camponotus femoratus* (green), *Crematogaster levior* (purple) and of nest material (orange). Two nests per site were sampled and are denoted by shape and fill [Colour figure can be viewed at wileyonlinelibrary.com]

community composition, and whether the co-variation depends on ant species-specific metabolites (i.e., derived from the host or its bacterial community). To this end, we summarized the chemical features composition using the first four PCoA axes of the chemical PCoA as described above, and used those as explanatory factors, together with the ant species as a cofactor in a PERMANOVA analysis on bacteria community dissimilarities. We averaged the resulting 10 PERMANOVA analyses. (b) Next, we looked for specific correlations between OTUs and chemical features by performing a Regularized Generalized Canonical Correlation Analysis (rGCCA). This generalized approach for the integration of multiple data sets was conducted using the *wrapper.rgcca* and *network* functions from the *mixOmics* R package (Rohart, Gautier, Singh, & Cao, 2017; Tenenhaus & Tenenhaus, 2011). Using the resulting 10 rGCCAs, we constructed a correlation consensus network by averaging correlations values for each chemical feature–OTU. Only correlations detected as significant in at least eight rGCCAs and of which absolute correlation values were >0.6 were considered as valid. The correlation consensus network was visualized with CYTOSCAPE 3.5.1 (Shannon et al., 2003). Each *m/z* of chemical features implicated in a correlation was compared against the METLIN database (https://metlin.scripps.edu) to propose a putative chemical identification of the metabolite.

## 3 | RESULTS

### 3.1 | Taxonomic composition and alpha diversity

The bacterial community of both *Camponotus femoratus* and *Crematogaster levior* was mainly composed of four phyla, Actinobacteria, Acidobacteria, Alphaproteobacteria, and Gammaproteobacteria, which each represented more than 10% of the total relative abundance for both species. The relative abundances of the different bacterial phyla were also similar across ant species and the nest materials (Figures S4 and S5). The proportion of the total richness per nest (i.e., gamma diversity) to the total OTUs table varied from 0.76 to 0.87 (Table S3). Within nests, OTUs of each ant's species overlapped from 32.80% to 78.90% (Table S3).

Alpha diversity, as defined with the exponential Shannon index, did not significantly differ among sample types (i.e., ant species samples *Ca. femoratus* and *Cr. Levior*, and nest material samples) (ANOVA $F$ = 0.0180, $df$ = 2, $p$ = .982), and among nest identities (ANOVA, $F$ = 1.7438, $df$ = 5, $p$ = .131) but did differ significantly between ant species inside each nest (i.e., the interaction of ant species and nest identity; ANOVA, $F$ = 2.0933, $df$ = 10, $p$ = .031; Figure S4). Significant differences in diversity were found only between ant species within nest K1 from Kaw (Figure S5; Table S4, Tukey post-hoc test, $p$ < .05).

### 3.2 | Bacterial community composition variation across samples

Bacterial beta diversity varied strongly varied according to the locality, that is the site identity and the nest identity (Figure 1, site identity: $F_{2,100}$ = 20.69, $p$ = .001, $R^2$ = .18, nest identity: $F_{3,100}$ = 15.42, $p$ = .001, $R^2$ = .21). Although the effect of sample type (i.e., each species and nest material) was smaller, it was still highly significant (sample type: $F_{2,100}$ = 8.94, $p$ = .001, $R^2$ = .074), and the interaction between the nest identity and the sample type contributed also to explain the variance (nest identity × sample type: $F_{6,100}$ = 3.33, $p$ = .001, $R^2$ = .083), whereas the interaction between the site identity and the sample type explained less variance than the sample type itself (site identity × sample type: $F_{4,100}$ = 2.93, $p$ = .001, $R^2$ = .05). When repeating the analysis for each nest separately (i.e., beta diversity at the scale of the nest), the effect of host species was consistent (all $p$-values <.005, Table S5). This shows that bacterial communities between *Ca. femoratus* and *Cr. levior* within each nest are statistically different. Despite the small variance explained by the sample type in the PERMANOVA between cuticular washes and nest material, we still observed significant differences between ant species within each nest. Similar

results were obtained once nest material samples were removed of the explanatory variable sample type in the PERMANOVA analysis (Table S6). Mean pairwise Bray–Curtis distances between different host species within the same nest were less than the distance among nests, demonstrating the same results as those obtained in the Permanova (Table S7).

### 3.3 | OTUs indicators of a host-specific ant cuticle core community

Although the nest (i.e., the environment) is the dominant source of the cuticular microbes, we identified two sets of bacterial OTUs as putative core bacteria of each ant species when all colonies were pooled without any geographical distinction (all of groups a and b; Figure 2). These corresponded to 47 OTUs *for Ca. femoratus* (group a), of which 14 belonged to Gammaproteobacteria, eight to Actinobacteria (including three OTUs classified as *Streptomyces* OTUs), and seven to Alphaproteobacteria OTUs (including two *Wolbachia* OTUs; Table S8). For *Cr. levior* we identified 18 OTUs that are putative core bacteria that mainly corresponded to 10 Acidobacteria OTUs (Table S8, group b). The percentage of the total ant OTUs specific to each ant species was 5.2% for *Ca. femoratus* and 2.0% for *Cr. levior*. We also identified 33 OTUs shared by the two ant species across nests, but that are not representative of the nest material (Table S8, group d). The percentage of the total ant OTUs shared between the two species was 3.6%. Each of the two ant species also shared specific bacteria with the nest, but not with the other species of ant (i.e. present on either ant species and nest), corresponding to a total of 17 OTUs for *Ca. femoratus* and 35 OTUs for *Cr. levior* (Table S8e,f). Table S9 provides a full description of each group at the OTU level.

### 3.4 | Phylogenetic analysis

Phylogenetic analysis of the OTUs from our study with their three closest GenBank reference sequences suggested that most of the sequences from the present study are phylogenetically related to bacteria isolated from soils or rhizospheres.

For Firmicutes (Figure S7), the OTU seq_121012, which is one of the *Cr. levior* core bacteria and identified as a *Lactococcus*, is phylogenetically related to other *Lactococcus* sp. sequences isolated from insect guts (unpublished GenBank data for the termite *Globitermes sulphurous*, and cockroach *Salganea esakii* [Bauer et al., 2015]).

Within the phylogenetic analysis of the order Rhizobiales (Alphaproteobacteria) (Figure S8), four GenBank references were retrieved from ant samples of other studies. Two taxa correspond to uncultured Bartonellaceae found in whole ants extracts from *Megalomyrmex glaesarius* and *Megalomyrmex symmetochus* (Liberti et al., 2015), and the two others corresponded to uncultured Rhizobiales retrieved from the ants *Terataner* sp. and *Cardiocondyla emeryi* sp. (Russell et al., 2009). These four published sequences clustered with two OTUs from the present study, seq_0646 and seq_5181, both taxonomically assigned to *Mesorhizobium* sp., but they were not part of the core bacterial community of any of the groups (groups a–f).

Within the remaining sequences from the class Alphaproteobacteria phylogenetic tree (Figure S9), one clade corresponding to the genus *Wolbachia* contained eight GenBank references, all isolated from insects, and two OTUs of our study: seq_0153 belonging to none of the core bacterial community fractions of our study, and seq_134874 belonging to the *Ca. femoratus* core bacteria. The eight *Wolbachia* GenBank references were isolated from four ant species: *Tetraponera penzigi* (Russell et al.,

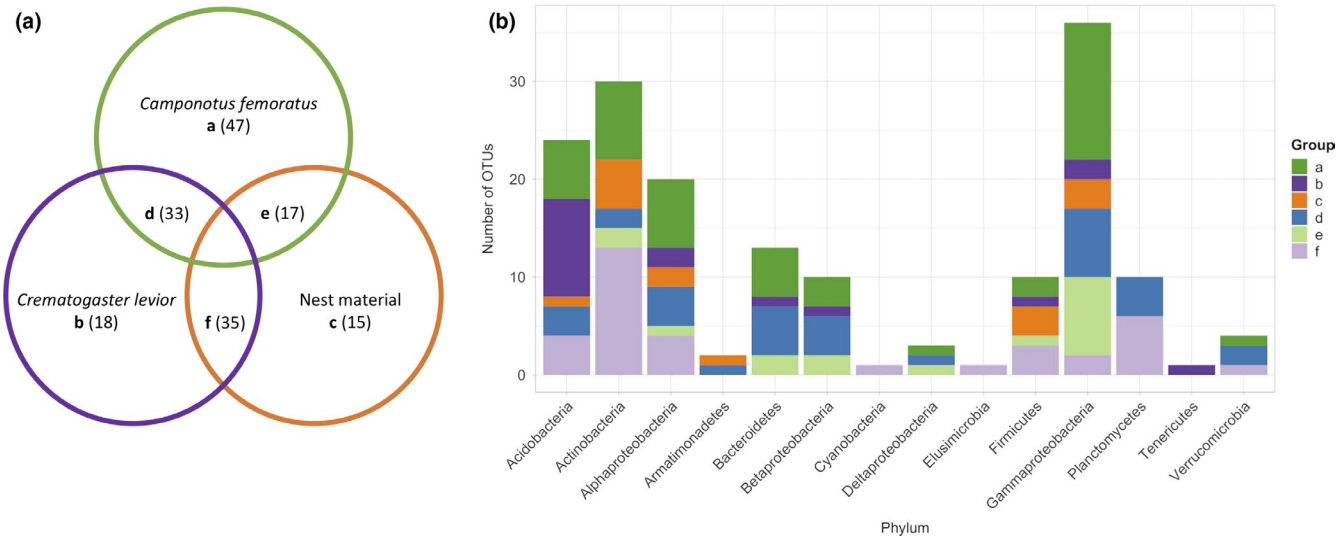


**FIGURE 2** Number (A) and taxonomy (B) of bacterial OTUs identified from [a] *Camponotus femoratus* core bacteria, [b] *Crematogaster levior* core bacteria, [c] nest material bacteria, [d] ant shared bacteria, [e] *Ca. femoratus*-nest shared bacteria, and [f] *Cr. levior*-nest shared bacteria. OTUs were identified as part of the potential core bacteria community from each sample by Indicator Value tests (*IndVal*), indVal >0.4 and corrected $p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

2012), *Heteroponera microps*, *Polyergus breviceps* and *Trachymyrmex urichii* (Russell et al., 2009); one psyllid: *Mycopsylla proxima* (unpublished data); one beetle: *Oreina cacaliae* (Montagna et al., 2014); one wasp: *Philanthus gibbosus* (Martinson et al., 2011); and one mite: *Torotrogla cardueli* (Glowska, Dragun-Damian, Dabert, & Gerth, 2015). Another Alphaproteobacteria clade (*Pseudomonas* sp.) contained three sequences from the *Ca. femoratus* core bacteria and GenBank references obtained from environmental samples.

Within the Gammaproteobacteria phylogeny (Figure S10), OTUs belonging to *Ca. femoratus* core bacteria were highly represented. Two branches clustered five and three OTUs with GenBank references obtained from various environmental samples. The rest of the tree included Gammaproteobacteria GenBank references obtained from the mosquito *Aedes aegypti*, the mite *Dermanyssus gallinaea*, and the tick *Ixodes tasmani*.

### 3.5 | Chemical features diversity across ant species and correlation with bacterial community composition

Chemical features (molecular entity with a unique *m/z* and retention time) of ant individuals only tended to differ across ant species and nest identities (Figure 3). These observations were confirmed statistically by the PERMANOVA analysis (species: $F_{1,90}$ = 12.27, $p$ = .001, $R^2$ = .10; nest identity: $F_{5,90}$ = 3.26, $p$ = .001, $R^2$ = .14; interaction nest identity × species: $F_{5,90}$ = 2.24, $p$ = .001, $R^2$ = .09).

The PERMANOVA analysis on bacterial communities with ant species and chemical profiles as explanatory factors showed that chemical feature diversity explained 9.3% of the variance in bacterial community composition (Table S10), while ant species explained 5.5% of the variance. The interactions of these two variables also contributed to significantly explain the variance of the bacterial communities (8.1%).

### 3.6 | Correlation between OTUs and chemical features

We identified 127 significantly correlated pairs of OTUs/chemical features (nine negatively and 118 positively) involving 12 OTUs and 19 chemical features (Figure 4). The 12 OTUs are reported in Table S11; three of them are assigned to Acidobacteria, seven to Proteobacteria, and two to Saccharibacteria. OTU seq_3030 is taxonomically assigned to the genus *Rickettsia* sp. OTU seq_25386 is taxonomically assigned to *Novosphingobium* sp., and a BLAST search against the GenBank database shows that it is close to bacterial sequences obtained from the refuse dump nest of the leaf cutter ant, *Atta colombica*. These 12 OTUs are not part of the either of the core bacterial communities identified here and were particularly more abundant in terms of the proportion of reads in samples (ant cuticle and nest material samples) collected in nest n°1 from the Nouragues site (Figure 4).

The METLIN database (https://metlin.scripps.edu) used to identify metabolites returned no hits to known chemical identities for most of our queries, except for five features that may correspond to small peptides composed of three to four amino acids (Table S12).

## 4 | DISCUSSION

Understanding which factors structure insect cuticle microbial communities can be challenging, but by leveraging a naturally occurring association of two ant species sharing ant garden nests, we were able to investigate the influence of host species, species interaction, and nest environment on the cuticular bacterial community. We found that while some OTUs were specific to each or both ant species, the majority of cuticular bacteria are probably acquired from the environment and depend on the locality, the nest and site. This suggests that the majority of these communities result from random colonization on the cuticle from the environmental pool of bacteria. This result is in line with the weak specificity of the cuticular chemical composition for each ant species and reinforces the hypothesis

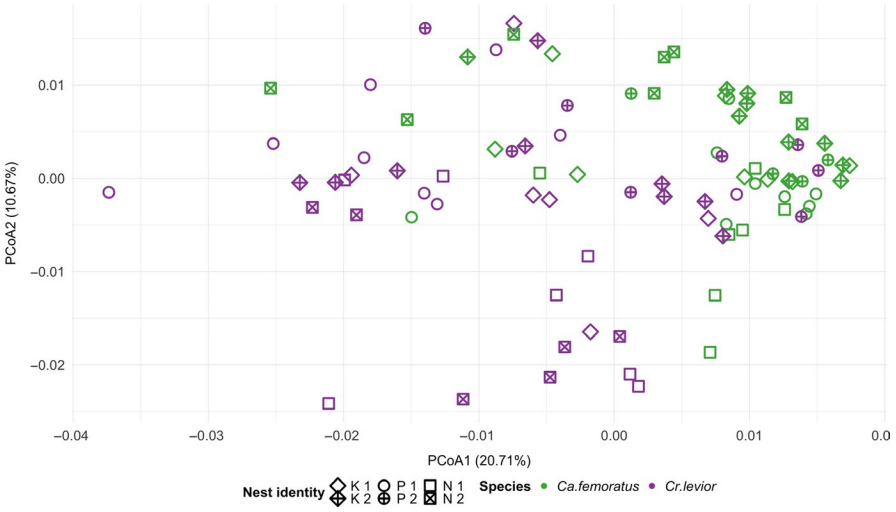


**FIGURE 3** Principal Coordinate Analysis of the cuticular chemical profiles according to ant species and nest identity. Cuticular chemical composition similarities between *Camponotus femoratus* (green) and *Crematogaster levior* (purple). Two nests per site were sampled and are denoted by shape and fill [Colour figure can be viewed at wileyonlinelibrary.com]

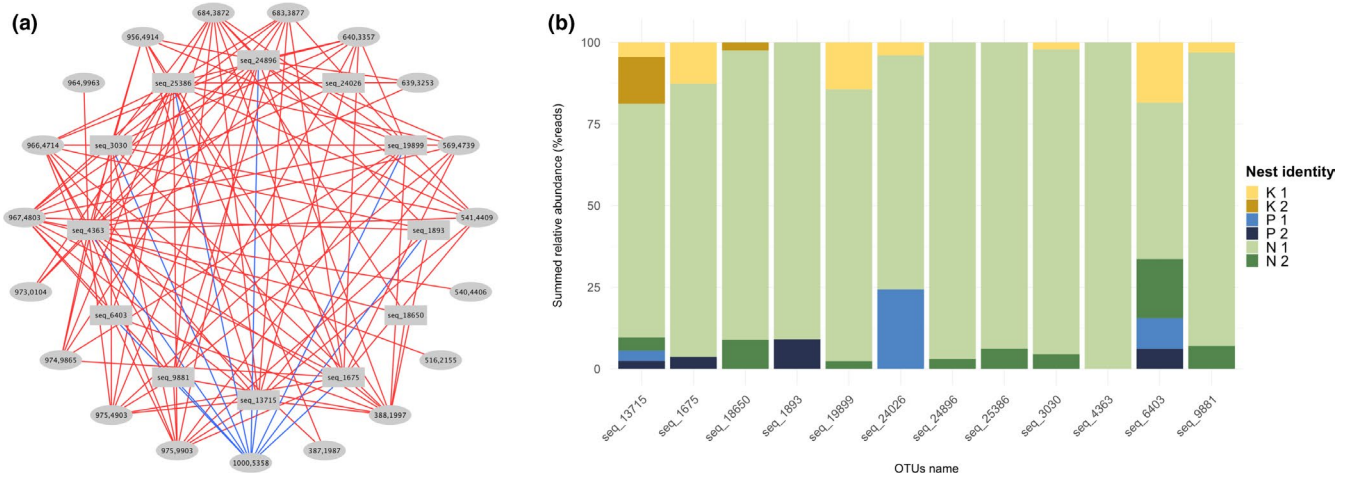


**FIGURE 4** (a) Consensus RGGCA correlation network between OTUs and chemical features. Positive correlations are represented by red edges, and negative correlations by blue edges. The taxonomic assignation of these 12 OTUs are in Table S11 and the chemical features putatively identified are in Table S12. (b) Summed relative abundance (% reads) of the 12 OTUs correlating with chemical features within the network shown in (a). Nest identity is represented by different colours [Colour figure can be viewed at wileyonlinelibrary.com]

that body surface bacterial communities are prominently neutrally assembled from the environmental pool of bacteria.

The cuticle of ants is indeed an exposed surface prone to colonization by both host-specific bacteria directly transmitted by ant adults to offspring, probably through physical contact, that are often suggested to play a beneficial role for the host, but probably more commonly by transient environmental microorganisms that should not play a role for the host or could be pathogenic. Several mechanisms can structure the presence of environmental bacteria on the host cuticle. First, the geographical location and/or the nesting type (e.g., arboreal, soil nesting) define the immediate environmental pool of bacteria (Hanson, Fuhrman, Horner-Devine, & Martiny, 2012) in which the ant host dwells. Second, several traits of the host can recruit or exclude particular microbes from the environmental bacterial pool. This includes traits that are specific to host species (e.g., secretions/cuticle chemical composition), or that are related to the age of the colony and/or of the individuals (e.g., individual task within the colony or social behaviours such as mutual cleaning). These latter characteristics are also likely to influence the physicochemical characteristics of the cuticle (pH, moisture level, presence and chemical profile of glandular secretions) as well as the transmission of bacteria amongst individuals (Onchuru, Javier Martinez, Ingham, & Kaltenpoth, 2018).

## 4.1 | The environmental pool of bacteria as a major determinant

The bacterial communities living on animal surfaces may be strongly regulated by the host or may just represent a collection of bacteria acquired passively through random immigration and recruitment, which are typical neutral processes (Sieber et al., 2019). Our analysis supports this view, as the nest from which individuals were collected is the most important factor structuring the bacterial composition on the cuticle of both ant species, even across a small spatial scale (i.e., different nests from the same geographical location). This suggests that the vast majority of the cuticular bacterial community is mainly acquired from their immediate environmental bacterial pool, which depends of the geographical location and other abiotic/biotic factors. Here, we controlled for several sources of variance in the environmental bacterial pool, such as geographical location at small and larger scales, the presence of the two ant species in the nests, seasonality, and nest height. Another set of factors that could shape the composition of the environmental pool of bacteria is the floristic composition, the age, or the health status of each nest, which we unfortunately did not recorded in this study. Yet, the fact that the bacterial community is mainly acquired from the immediate environment, is consistent with other reports on body surface bacterial microbiota in other animals such as birds (van Veelen, Falcao Salles, & Tieleman, 2017), bats (Winter et al., 2017), and humans (Schommer & Gallo, 2013). Our results hence indicate that, in the case of ant cuticles in ant gardens, the environmental pool of bacteria is formed by larger-scale processes depending on the spatial and environmental conditions from the host's immediate environment, as proposed by van Veelen et al. (2017).

## 4.2 | Impact of the ant host

The unique nature of the cuticle, composed by chitin covered with cuticular hydrocarbons and small metabolites, serves (among other functions) as a mechanism to attract or exclude environmental microbiota, including pathogens, obtained from the surrounding nest or outside environment (Little, Murakami, Mueller, & Currie, 2006; Ortiz-Urquiza & Keyhani, 2013). The analysis of alpha diversity showed significant differences in the number of OTUs found on the cuticle between the two ant species for only one nest among the six nests studied. We expected the cuticle could act as

a selection filter leading to lower alpha diversity on ant cuticles of both species compared to nest material. However, our results do not support these predictions. Interpretation of the analysis on alpha diversity is, however, limited by the caveat that the microbial diversity of nest material may have been undersampled in our study, as suggested by our rarefaction analyses (Figure S2). The analysis of bacterial community composition between ant species and nest material does, however, suggest that few bacterial OTUs are present in the nest but absent on the cuticles of both species (Figure 2, group c = 15 OTUs). These are probably actively filtered out by the cuticle. We also identified some other OTUs shared by one ant species with the nest material, but not with the other ant species (Figure 2, groups e and f), probably suggesting species-specific active filtering mechanisms.

### 4.3 | Putative species-specific vertically and horizontally transmitted bacteria

Amphibian skin microbiota has been shown to be strongly determined by host species identity, and have little overlap with the microbiota of the surrounding environment or other cohabiting amphibian species (McKenzie et al., 2012; Walke et al., 2014; Weitzman et al., 2018). Conversely, the bacterial communities living on the skin of cohabiting mammals, such as humans and dogs (Song et al., 2013; Torres et al., 2017), different species of bats (Lemieux-Labonté et al., 2017) and lizards (Weitzman et al., 2018), have been shown to be extensively shared. In the case of cohabiting ants, the majority of the cuticular bacterial community was shared with the nest material. While the majority of the cuticular bacterial community seems neutrally acquired from the surrounding environmental pool of bacteria, we also identified several OTUs specific to the host species (Figure 2: group a = *Ca. femoratus* = 47 OTUs; group b = *Cr. levior* = 18 OTUs). This suggests that some OTUs are specific to ant species and may be acquired vertically or may be the result of horizontal transmission between workers or ant behaviours that modify the bacterial community to keep specific taxa. In particular, we identified several *Streptomyces* OTUs in the core bacterial community of *Ca. femoratus*, known for producing bioactive secondary metabolites used in many symbiotic interactions. We also identified *Wolbachia* OTUs, a notorious vertically transmitted endosymbiont of arthropods with major impacts on the host's evolution (Werren, Baldo, & Clark, 2008). The presence of these intracellular bacteria on the cuticle of *Ca. femoratus* is surprising and, hence, might result from contamination from decaying tissues or the presence of germ cells. For *Cr. levior* we found gut bacteria on the cuticle, as suggested by the presence of *Lactococcus*, which is phylogenetically related to other insect microbial gut bacteria and might originate from faeces or social interactions involving contact with secretions. This demonstrates that some of the symbionts probably associated with the digestive system can be found on the surface of the cuticle, probably through host faecal contamination.

### 4.4 | Bacteria common to both species of the parabiosis

Our study of ants living in the same nests also allowed us to identify OTUs specific of the parabiosis, that is found on individuals from both species across three collection sites and nearly/totally absent from the nest material (Figure 2: group d, 33 OTUs). These OTUs are unlikely to be contaminants because we have carefully removed potential contamination from the data set. In addition, they belong to bacterial clades that are not known to be common contaminants. We cannot determine whether these parabiosis-specific bacteria (group d) are transmitted vertically in both species, only in one species and acquired horizontally through contact with the other one, or if they are acquired by each of the two ant species from the environment. The fact that these OTUs are consistently shared between the two species does not necessarily mean that these bacteria are essential to the ant garden parabiosis, but any nonhost-specific bacteria pivotal to the parabiosis are likely to be in group d. For example, Actinobacteria genera present in the ant core bacterial community are known for producing bioactive secondary metabolites, and therefore, they could allow the ant hosts to defend themselves against entomopathogenic infections within the garden (Little et al., 2006).

Furthermore, these OTUs could be involved in the behaviour of the two species. For example, gut bacteria have been found to synthesize components that modulate aggregation behaviour in cockroaches and locusts (Dillon, Vennard, & Charnley, 2002; Wada-Katsumata et al., 2015), as well as the cognitive functions and behaviour in humans (Dinan, Stilling, Stanton, & Cryan, 2015). In the ant garden system, the mechanisms by which the colonies of these two ant species tolerate each other in the same habitat are not known. Recent work on the cuticular hydrocarbons (CHCs) of these same ant garden species in French Guiana show little CHC overlap between the two species, suggesting that chemical mimicry does not explain the tolerance between these two ant species (Emery & Tsutsui, 2013, 2016). Another study on parabiotic ants showed that the production of soothing molecules, crematoenones, by *Crematogaster modiglianii* significantly reduces the aggressive behaviour of *Camponotus rufifemur* (Menzel et al., 2013). Apart from the crematoenones, it is possible that the microbial taxa shared by *Crematogaster* and *Camponotus* are involved in their cohabitation through production of volatile compounds involved in recognition mechanisms. Microbial-derived pheromones and volatile compounds are indeed well known in insects (Engl & Kaltenpoth, 2018). For example, Dosmann, Bahet, and Gordon (2016) experimentally tested whether microorganisms contribute to recognition among individuals of the same nest in the ant *Pogonomyrmex barbatus*. The external bacterial community of the ants was modified by topical application of either antibiotics or microbial cultures. The results show that ants with an increased external microbiota were rejected (Dosmann et al., 2016). In our study, whether shared bacteria between the two ant species play a defensive or recognition role could be tested

through experimental manipulation of the cuticle bacterial community (e.g., application of topical antibiotics), followed by GC-MS analyses of cuticle microbial volatiles and two-species interaction observations. Comparison of the bacterial community of each of the ant species living alone with that of cohabiting ants, would also pinpoint potential candidate OTUs facilitating the tolerance of the cohabiting ant species.

### 4.5 | Impacts of the cuticle chemistry

Ant cuticles are enriched with a diverse array of metabolites, which may originate from the ants, bacteria, nest, or the outside environment. Ants can produce antimicrobial acidic compounds in glands and these metabolites could limit the presence of certain microbial taxa (Ortiz-Urquiza & Keyhani, 2013). The metapleural gland, thought to be the main gland responsible for ant-derived antimicrobial products, is lost in most species of *Camponotus*, whereas it is present in *Crematogaster*, which may explain the difference between the number of core bacteria recovered from these two ant species. Other metabolites produced by ants, such as CHCs, could regulate the presence of bacterial taxa, through peptides or phospholipids, which form vesicles to transport CHCs from glands to the exocuticle (Canavoso, Jouni, Karnas, Pennington, & Wells, 2001). Conversely, metabolites biosynthesized by the bacterial community can also regulate the presence of certain taxa within the skin cuticular microbial community (Woodhams et al., 2018). First, we did not find a clear specific cuticular metabolome for each ant species (Figure 3). It should be mentioned that the soft electrospray ionization used in the MS analysis prevents the detection of apolar CHCs which would have strongly contributed in discriminating the two cuticular metabolomes, in order to favour the detection of bacterial metabolites. Second, our results show that the ant cuticle metabolome partially explains the composition of the cuticular bacterial community (Table S9). The correlations found between chemical features and bacterial OTUs were rare (Figure 4), and mainly occurred in nest N1 from the Nouragues station, which had a peculiar bacterial community composition (Figure 1). Almost all these correlations were positive, and only one chemical feature was negatively correlated to 10 OTUs. Unfortunately, the instrument used to identify the molecular structure of chemical features was not sufficient to provide full structural characterization. Only seven out 19 chemical features significantly associated with OTUs have been putatively identified as peptides in the chemical database (METLIN), thus limiting our understanding of the relationship between these chemical features and bacterial OTUs on the cuticle.

The biological functions of these chemical features can be inferred from their associations with bacterial OTUs. For example, *Rickettsia* sp. is a well-known nonobligate endosymbiont. In aphids, they have been suggested to be involved in host defence against parasitoid wasps and fungal entomopathogens (Oliver, Degnan, Burke, & Moran, 2010; Scarborough, Ferrari, & Godfray, 2005). A chemical feature associated with *Rickettsia* may hence potentially correspond to bioactive molecules. Likewise, some Sphingomonodaceae strains are able to degrade aromatic compounds (Kertesz & Kawasaki, 2010). In this case, the chemical features associated with Sphingomonodaceae could be a metabolic product of Sphingomonodaceae. Finally, the *Novosphingobium* sp. associated with all samples from nest N1 (OTU seq_25386) is closely related to GenBank bacterial sequences obtained from the refuse dump of nests of the leaf cutter ant, *Atta colombica*. Therefore, this particular OTU may be associated with chemical features resulting from microbial degradation of ant waste. Why nest N1 is so distinct remains unknown, but its particular bacterial and chemical signature, combined with the potential functions of the identified bacteria, suggests that it is undergoing a very different process from all other nests. Whether such divergence is due to different plant composition, nest age or the impact of sources of stress (e.g., disease or parasites) remains to be determined.

The Amazonian ant garden parabiosis, composed of two ant species sharing the same nest, is a unique system and allows us to disentangle the proportion of the cuticular bacterial community that is particular to each ant species, common to both species and generally found throughout the ant garden. We show here that the cuticular microbial diversity of ants is the result of several, nonexclusive processes. The majority of the cuticular bacterial biodiversity is shared between both species of ants and their nest and was dictated by variance among nests. This indicates that the immediate bacterial pool present in the vicinity of the host (e.g., its nest or its environment) is the main source of the cuticular bacterial community of the two cohabiting ants. However, we also identified several putative host-specific ant cuticle-dependent taxa, and some specific to the parabiosis. Despite significant positive and negative interactions, the comparison of cuticular bacterial communities and metabolomes was inconclusive, as all signals were dominated by a single nest, which seems to be an outlier. Further work, particularly experimentally manipulating the bacterial community, will certainly elucidate the interaction between the cuticular bacterial communities and metabolome. Indeed, field observations are greatly complemented by experiments in an artificial environment, in order to reduce complexity. In the ant garden system, such experiments would need surface-sterilized ants of both species brought together into a predefined microbiome terrarium. Also, using the tools and methods utilized here and including more ant species from a broader phylogenetic scale and nesting in contrasting environments may help to gain further resolution into the diversity and function of the cuticular bacterial community of ants, and the processes implicated in their assembly.

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## AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the study. C.B., C.D. and N.T. performed the sampling; C.B. carried out the molecular work; L.Z. performed the bioinformatics analyses; and C.B. and L.Z. conducted the statistical analyses; and C.B. and C.M. contributed to phylogenetics analysis. All authors have contributed to the writing of the manuscript.

## DATA AVAILABILITY STATEMENT

Sequence data and associated metadata are available in the Dryad Digital Repository (https://datadryad.org/) under https://doi.org/10.5061/dryad.wh70rxwjn.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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