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Dietary specialization in mutualistic acacia-ants affects relative abundance but not identity of host-associated bacteria

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Abstract

Acacia-ant mutualists in the genus *Pseudomyrmex* nest obligately in acacia plants and, as we show through stable isotope analysis, feed at a remarkably low trophic level. Insects with diets such as these sometimes depend on bacterial symbionts for nutritional enrichment. We, therefore, examine the bacterial communities associated with acacia-ants in order to determine whether they host bacterial partners likely to contribute to their nutrition. Despite large differences in trophic position, acacia-ants and related species with generalized diets do not host distinct bacterial taxa. However, we find that a small number of previously undescribed bacterial taxa do differ in relative abundance between acacia-ants and generalists, including several Acetobacteraceae and Nocardiaceae lineages related to common insect associates. Comparisons with an herbivorous generalist, a parasite that feeds on acacias and a mutualistic species with a generalized diet show that trophic level is likely responsible for these small differences in bacterial community structure. While we did not experimentally test for a nutritional benefit to hosts of these bacterial lineages, metagenomic analysis reveals a *Bartonella* relative with an intact nitrogen-recycling pathway widespread across *Pseudomyrmex* mutualists and generalists. This taxon may be contributing to nitrogen enrichment of its ant hosts through urease activity and, concordant with an obligately host-associated lifestyle, appears to be experiencing genomewide relaxed selection. The lack of distinctiveness in bacterial communities across trophic level in this group of ants shows a remarkable ability to adjust to varied diets, possibly with assistance from these diverse ant-specific bacterial lineages.

KEYWORDS
acacia-ants, *Ca. Tokpelaia*, Formicidae, metagenomics, microbiome, *Pseudomyrmex*, *Vachellia*

1 | INTRODUCTION

Animals often depend on endosymbiotic bacteria to digest, process and enrich their diets (Flint, Bayer, Rincon, Lamed, & White, 2008; Hansen & Moran, 2011; Moran & Baumann, 2000), and insects provide some of the best understood cases of these relationships. Termites, for example, depend on endosymbiotic bacteria to fix nitrogen (Benemann, 1973; Breznak, Winston, Mertins, & Coppel, 1973), and pea aphids (*Acyrthosiphon pisum*) depend on the obligate intracellular symbiont *Buchnera aphidicola* to produce amino acids without the need for dietary input beyond nitrogen-poor plant phloem sap (Hansen & Moran, 2011; International Aphid Genomics Consortium, 2010; Sandström & Moran, 2001; Sandström & Pettersson, 1994). Tight cooperative relationships such as these take a diversity of

forms and are quite common in insects (Douglas, 2011). However, work in ants and butterflies makes clear that such relationships are far from universal (Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017; Hu et al., 2017).

The widely varied diets of ants, from strictly predatory to based almost entirely on protein-poor plant exudates (Davidson, Cook, Snelling, & Chua, 2003), make them a useful model for exploring the interaction between diet and bacteria. For example, the *Blochmannia* symbionts of carpenter ants in the genus *Camponotus* contribute to the nutrition of their hosts through nitrogen recycling and amino acid upgrading (Feldhaar et al., 2007) and *Westeberhardia* bacteria appear to provide tyrosine precursors to their host *Cardiocondyla* ants (Klein et al., 2016). More recently, species in the genus *Cephalotes* were shown to benefit from the nitrogen recycling capacity of their specialized microbial communities (Hu, Łukasik, Moreau, & Russell, 2014; Hu et al., 2018; Russell et al., 2009; Sanders et al., 2014). Bacteria in the *Cephalotes* gut degrade waste urea into ammonia and incorporate this recycled ammonia into glutamate that is then used in the synthesis of amino acids essential to the ant hosts (Hu et al., 2018). This nitrogen recycling capability via a complete urease pathway is also present in *Candidatus Tokpelaia* symbionts of ants in the predatory genus *Harpegnathos* (Neuvonen et al., 2016) and the herbivorous genus *Dolichoderus* (Bisch et al., 2018). Although previously hypothesized to serve in reducing the acidity of the ant gut, increasing its hospitality for *Ca. Tokpelaia* (Neuvonen et al., 2016) this pathway could also contribute to nitrogen recycling (Bisch et al., 2018). Symbionts with such nitrogen-recycling capability may be common across the ant family. While the potential for direct nitrogen fixation by bacteria exists in *Acromyrmex* (Sapountzis et al., 2015), this process has yet to be demonstrated by internally housed symbionts in any ant (Hu et al., 2018).

Acacia-ants in the genus *Pseudomyrmex* have been previously proposed to benefit from symbiotic interactions with bacteria (Eilmus & Heil, 2009). The relationship between acacia-ants and their acacia host plants (genus *Vachellia*) is one of the best known ant-plant mutualisms (Janzen, 1966, 1967). In this symbiosis, ants nest in hollow swollen thorns and feed on food bodies and extrafloral nectar provided by the plant. In exchange for these resources, resident ants aggressively protect their hosts by attacking herbivores, trimming encroaching plants and removing pathogenic fungi (Janzen 1967). While most ants are opportunistic predators and animal-protein scavengers, *Pseudomyrmex* acacia-ants appear to have an unusually strict diet derived entirely from their host plants (Clement, Köppen, Brand, & Heil, 2008; Heil, Krüger, Baumann, & Linsenmair, 2004). Congeneric nonmutualists (generalists) will, in contrast, readily consume animal protein. This dietary difference among closely related species provides a compelling system for studying the bacterial symbionts associated with the variable diets of ants.

In order to examine the possibility that bacteria may be important nutritional partners of acacia-ants, we first assess the dietary specificity of these ants using stable nitrogen isotope ratios. We then employ amplicon sequencing of the bacterial 16S rRNA gene to characterize the microbial communities within acacia-ants and their congeneric relatives in the genus *Pseudomyrmex*, determining the specificity of symbiont relationships by including sympatric ants from 11 other genera. Finally, we use metagenomic sequencing to assemble the genomes of specific taxa of interest, revealing their capacities to contribute to the nutrition of their host ants.

2 | MATERIALS AND METHODS

2.1 | Sampling

All ant samples were collected from the Area de Conservación Guanacaste (ACG) in northwestern Costa Rica at the Santa Rosa Biological Station (10.8°N, 85.6°W) during June of 2012 and from the Florida Keys, USA (25.1°N, 80.5°W) between 2011 and 2014. Within the ACG, four collecting sites were used: Tanqueta trail, Barrachas trail, River trail and Playa Naranjo. Three species of acacia-ants were collected from the ACG in Costa Rica: *Pseudomyrmex flavicornis*, *P. nigrocinctus* and *P. spinicola*. These species nest obligately in acacia (genus *Vachellia*) plants and behave as mutualistic partners. *Pseudomyrmex gracilis*, a widely distributed generalist, co-occurs in this area and was also a focal taxon for collection to allow for comparisons between acacia-ants and this closely related generalist. Although rare, we also collected *P. nigropilosus* whenever possible. This species is more closely related to the generalist *P. gracilis* than to acacia-ants (Figure 1) but nests obligately in acacias, acting as a parasite on the mutualism by taking advantage of both the nesting space and food provided but contributing no protection to the host plant (Janzen, 1975). Plant-ant mutualism has convergently evolved three times within *Pseudomyrmex* (Chomicki, Ward, & Renner, 2015; Rubin & Moreau, 2016; Ward & Downie, 2005), with three types of plants: acacias, *Triplaris*, and *Tachigali*. A single *Triplaris*-nesting

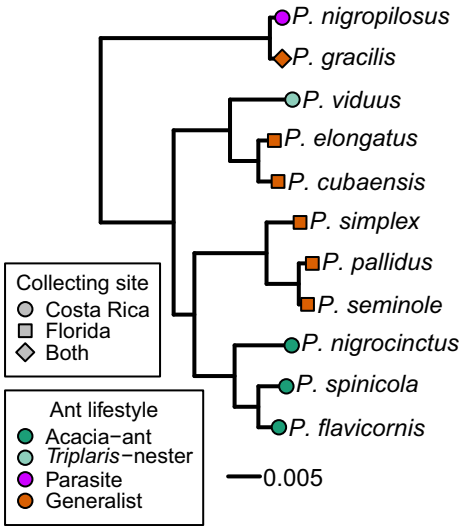

FIGURE 1 Phylogenetic relationships of *Pseudomyrmex* species included in this study (Chomicki et al., 2015) and the geographic sites from which they were collected. Branch lengths represent amount of change in nucleotide sequences from 10 nuclear markers. This crown group diverged ~35 Mya [Colour figure can be viewed at wileyonlinelibrary.com]

species, *P. viduus*, is also present in the ACG and was collected when possible. *Triplaris* plants do not provide direct food rewards to resident ants, and *Triplaris* nesters are known to have far more generalized diets, feeding on scale insect provided honeydew as well as scale insects themselves (Wheeler & Bailey, 1920). Comparisons between this species and acacia-ants can, therefore, reveal whether mutualism is influential in determining bacterial communities or whether the dietary specialization of acacia-ants plays a larger role. Finally, we also included a single unidentified *Pseudomyrmex* generalist found incidentally.

No mutualistic species of *Pseudomyrmex* range into Florida, but several *Pseudomyrmex* generalists do occur there, including *P. gracilis*. This species was again a focal point for collection so as to allow within species comparisons across a large geographic distance. In addition, *P. cubaensis*, *P. elongatus*, *P. pallidus*, *P. seminole* and *P. simplex*, all generalists, were also collected from this site.

In both areas, we also performed general collections of ants, focusing in particular on three taxa that are known to host bacterial symbionts (*Camponotus*, *Cephalotes* and Dorylinae, the army ants) but included all taxa. We found that many acacias were also parasitized by a species of *Crematogaster* and we collected this species wherever possible. In general, we made collections from at least three colonies of each target taxon from each of the four local collecting sites in the ACG. Phylogenetic relationships of all *Pseudomyrmex* species sampled here and their location of origin are shown in Figure 1. The most recent common ancestor of the *Pseudomyrmex* species sampled existed ~35 Mya (Chomicki et al., 2015), and *Pseudomyrmex* diverged from all other ant species sampled ~100 Mya (Moreau & Bell, 2013).

Lastly, we also collected known herbivores (e.g., caterpillars, termites and millipedes), carnivores (e.g., spiders and centipedes) and plants, including a large number of acacias, at each sampling site so as to calibrate the stable isotope analyses. All collections were made into 95% alcohol in the field and kept at −20°C until processing. All samples used in this study are shown in Supporting Information Table S1, and vouchers have been deposited in the scientific collections of the Field Museum of Natural History, Chicago, IL, USA.

2.2 | Trophic levels

We collected a total of 359 samples for stable isotope analysis. These included leaves from 59 acacia plants occupied by *Pseudomyrmex* acacia-ants, ants from 54 acacia-ant colonies, 12 colonies of the generalist *P. gracilis* from the same sites as the acacia-ants, 40 colonies of *Cephalotes*, as well as 30 known herbivores and 15 predators. These samples were collected from all four ACG sites. Additional generalist *Pseudomyrmex* and codistributed insects from Florida were also included.

We pooled between three and 20 heads and thoraces (mesosomas) of adult ants from individual colonies for each isotope measurement. Samples were dried before analysing. For one colony of *P. nigropilosus*, the acacia parasite, we did not have sufficient adults and instead used pupae. Plants were collected in the same fashion and ~1 cm^2 of leaf material was used to estimate isotope ratios. All samples were analysed at the University of Utah Stable Isotope Ratio Facility for Environmental Research (Salt Lake City, UT, USA). For other insects, segments containing guts were removed when possible. For caterpillars, millipedes and centipedes, the front half of the insect was used.

For each site in the ACG, we had at least 11 samples of acacia leaves. In order to reduce the impact of variation in the isotopes present in the environment at individual sites, we, therefore, standardized all ACG samples to the mean $\delta^{15}N$ ratio of the acacias at the same site. We then used Wilcoxon rank-sum tests to compare nitrogen ratios between taxa. As many tests were performed, results were corrected for multiple testing by the Benjamini–Hochberg FDR procedure using the p.adjust function in R (R Core Team, 2018). To make the samples from Florida comparable to those from Costa Rica, we subtracted the difference in median values measured for all plants sampled in Costa Rica from the median value for plants from Florida. We used the median because the spread of plant values was quite large. We, again, used Wilcoxon rank-sum tests to compare between taxa.

2.3 | DNA extraction

All DNA extractions were performed using the Mo Bio PowerSoil Kit with minor modifications as described previously (Rubin et al., 2014) and in the Supporting Information. Abdomens (gasters) were removed and surface-sterilized in 5% bleach for one minute before DNA extraction. Gasters were used in an attempt to enrich for gut-associated microbes. For the adults of larger species, including all *Pseudomyrmex*, a single gaster was included in each extraction, although multiple gasters were pooled for the smaller species. Several larvae from single colonies were pooled whenever possible. Although this pooling may have introduced additional variation, individuals within colonies have similar bacterial communities and pooling tends to increase sequencing success rates for ants with low bacterial titres such as *Pseudomyrmex* (Rubin et al., 2014). Four "blank" samples with no insect material were extracted side by side and included in the same sequencing lanes to aid in the identification of contaminant sequences. These extractions were performed exactly as all others in the absence of insect material (i.e., empty tubes were used in the initial digestion step).

2.4 | Bacterial quantification

Quantification of the bacterial 16S rRNA gene was done as previously (Rubin et al., 2014). The EMP primers 515f (5′-GTGCC AGCMGCCGCGGTAA) and 806r (5′-GGACTACHVGGGTWTCTAAT) were used to amplify the bacterial 16S rRNA gene (http://www.earthmicrobiome.org/protocols-and-standards/16s/). QPCRs were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) using SsoAdvanced 2X SYBR green supermix (Bio-Rad) and 2 μl of DNA extract. We used serial dilutions of linearized plasmid-

containing inserts of the *E. coli* 16S rRNA gene to generate standard curves. We required that all qPCRs had efficiencies and R^2 between 90% and 110%. Each sample was run at least three times and, when one of these did not have reaction efficiency within the required range, run a fourth time. The mean of all values was used for further analysis. qPCR results were standardized to the overall quantity of DNA measured using a Qubit fluorometer (Rubin et al., 2014). Differences in quantity of bacteria between taxa were assessed using Wilcoxon rank-sum tests with FDR correction for multiple testing.

2.5 | Bacterial 16S rRNA gene sequencing

Sequencing was done following the protocol of the Earth Microbiome Project (Caporaso et al., 2012). The bacterial 16S rRNA gene was amplified using the universal primers 515f (5′-GTGCCAGC MGCCGCGGTAA) and 806r (5′-GGACTACHVGGGTWTCTAAT) and sequenced using two lanes of Illumina MiSeq 150-bp paired-end sequencing.

Despite the PCR amplification inherent in amplicon sequencing of the bacterial 16S rRNA gene, successfully obtaining informative data from this procedure is challenging, particularly when the concentration of bacterial DNA is low, as is known from many ants (Hu et al., 2017; Rubin et al., 2014; Sanders et al., 2017). In order to guarantee that we would obtain data from a sufficient number of colonies, we, therefore, performed three DNA extractions from each colony for both adults and larvae whenever possible. Overall, we sequenced adults and larvae from 176 ant colonies from 11 ant genera, resulting in 589 sequencing samples.

2.6 | 16S rRNA gene sequence processing

The two lanes of sequencing results were pooled into a single data set for all analyses. 16S rRNA gene sequences were demultiplexed using QIIME (Caporaso, Kuczynski, et al., 2010; Navas-Molina et al., 2013) and then forward and reverse reads were merged using UPARSE (Edgar, 2013), truncating at the first base with quality of three or less. The resulting contigs were then filtered for quality by discarding reads with more than 0.5 expected errors using the fastq_filter function of UPARSE.

Contamination can be a major issue for microbiome studies based on bacterial 16S rRNA sequencing (Salter et al., 2014). We decontaminated our data set while avoiding overfiltering using the procedure of Hu et al. (2017). First, we identified unique sequences from the four blank samples. Sequences that made up <0.2% of all sequences from blank samples were removed. When the ratio of the maximum relative abundance in the four blank samples to the maximum relative abundance in all other samples was >0.1, those sequences were discarded from the entire data set. For calculating maximum abundances, only those ant samples with at least 1,000 raw sequences were included. Samples for which more than 50% of sequences were classified as contaminants at this stage were completely discarded.

We then used the default UNOISE3 (Edgar, 2016; Edgar & Flyvbjerg, 2015) pipeline to cluster the remaining sequences into zero-radius OTUs (ZOTUs), discarding those represented by fewer than eight sequences. These ZOTUs should represent unique biological sequences. Raw sequences were clustered against these ZOTU sequences using VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) at a 97% similarity threshold to allow for some sequencing errors. This clustering algorithm matches each sequence to the most similar ZOTU. Representative sequences were aligned using PyNAST (Caporaso, Bittinger, et al., 2010) against the Greengenes core set (DeSantis et al., 2006), and a phylogeny was inferred using Fasttree as implemented in QIIME.

We removed all samples with fewer than 3,400 sequences after quality controls and split all ant samples into two data sets of adults and larvae. In many cases, we sequenced multiple individuals from each colony. We combined these nonindependent samples into single representatives of colonies by summing reads in all individuals from each colony.

We tested for differences in bacterial communities using several approaches. First, we used supervised learning as implemented in QIIME to determine whether communities are identifiable as originating from particular types of ants. We ran these analyses on 100 replicate OTU tables rarefied to 3,400 reads and used the average error ratio to assess their performance. We used an error ratio (the ratio of the baseline error rate if the classifier worked randomly to the observed errors) ≥2 to determine when significant differences in communities exist (Navas-Molina et al., 2013; Van Treuren et al., 2015). We also compared beta diversities (measured as weighted and unweighted Jaccard and UniFrac distances) within and between groups to determine how consistent communities were, testing for differences using nonparametric *t* tests with Monte Carlo permutation. Beta diversity measures of distance between communities were also examined for correlations with differences in trophic level (i.e., $\delta^{15}N$) using Mantel tests. Differences in communities were visualized using principal coordinates analyses (PCoA). We tested for differences in the abundance of individual taxa using Wilcoxon rank-sum tests and the frequency of the presence of individual taxa using chi-square tests. Both types of tests were corrected for multiple testing using the Benjamini–Hochberg procedure using the R function p.adjust. Finally, Pearson correlation coefficients were used to identify correlations of bacterial community characteristics and trophic level. All statistical tests were performed using R (R Core Team, 2018).

2.7 | Metagenome sequencing

To assess the functional capacity of the bacterial communities associated with *Pseudomyrmex*, we performed shotgun metagenomic sequencing of two libraries constructed with Illumina's Nextera XT kit. The first sequencing library, which included DNA extracted from 25 larval meconia (the pouch storing faeces until pupation) from *P. nigropilosus* colony BER0554, was sequenced using 150-bp paired-end reads on the MiSeq platform. The second library, with DNA

from 30 adult worker guts (combined crop, midgut and hindgut), from *P. flavicornis* colony BER0517, was sequenced using two lanes of HiSeq paired-end 100-bp sequencing. We chose these colonies based on available material and the relative abundances of bacteria of interest estimated from the amplicon data set.

The resulting sequences were filtered extensively. We first removed duplicate reads using FastUniq (Xu et al., 2012). The remaining sequences were filtered using Trimmomatic (Bolger, Lohse, & Usadel, 2014) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:10 MINLEN:36. In addition, we filtered Illumina adapter sequences with ILLUMINACLIP:2:25:7. This filtering introduced a small number of additional duplicate sequences so we reran FastUniq on the filtered sequences. We then merged paired reads into single reads using fastq-join from the ea-utils package. We removed ant-derived sequences by mapping the resulting sequences to the *P. gracilis* genome (Rubin & Moreau, 2016) using STAMPY v. 1.0.23 (Lunter & Goodson, 2011) allowing 3% divergence between reads and reference. Reads that failed to map were used in all subsequent analyses. If one read in a pair was successfully mapped to *P. gracilis*, both reads in that pair were discarded.

2.8 | Metagenome assembly

We followed the general procedure implemented by multi-metagenome (Albertsen et al., 2013) to assemble the genomes of individual bacterial taxa. Extracting ORFs from initial assemblies and comparing them with BLASTP against the GenBank database revealed the presence of both *Enterococcus faecalis* and *Enterobacter cloacae* genomes, two common human commensals, in the assembled data. We excluded reads that mapped to either of these genomes (NCBI genomes NC_004668.1 and NC_014121.1) using BWA (Li & Durbin, 2009) with very close matches (-O 100 -B 100). The remaining reads were assembled using the uneven depth version of IDBA (Peng, Leung, Yiu, & Chin, 2012) with a maximum kmer size of 115. Scaffolds <500 bases long in the resulting assembly were discarded. Initial scaffold taxonomy was determined by finding closest hits of ORFs to the set of ~100 essential genes provided in the multi-metagenome package using HMMER v3.1 (Eddy, 2011; hmmer.org) and MEGAN to compile scaffold taxonomies (Huson, Auch, Qi, & Schuster, 2007). For separation of scaffolds into taxonomic bins, we used DIAMOND (Buchfink, Xie, & Huson, 2014) to find closest BLASTP hits of ORFs to NCBI's nonredundant database of bacterial proteins downloaded on October 30, 2016. We required that at least 60% of proteins on a scaffold have consistent taxonomic classification. Scaffolds were separated into genomes based on this taxonomy, GC-content and mapped sequence coverage determined by BWA. Genome completeness was determined again using HMMER and the essential gene set provided by multi-metagenome.

2.9 | Metagenome analysis

Unassembled sequences were submitted to the MG-RAST webserver (Meyer et al., 2008) to characterize taxonomic composition and functional potential. These results were of particular interest for those bacteria that we failed to assemble directly.

Assembled genomes were annotated for genes, functions and pathways by uploading to the RAST server (Aziz et al., 2008; Overbeek et al., 2014). For comparative analysis of the assembled *Ca. Tokpelaia* genome, we downloaded the protein coding sequences from 11 other closely related Rhizobiales genomes (Neuvonen et al., 2016) representing diversity in both evolutionary history and life history strategy. These included five *Bartonella* pathogens of mammals: *B. australis* (NC_020300), *B. bacilliformis* (NC_008783), *B. birtlesii* (NZ_CM001557), *B. henselae* (NC_005956) and *B. quintata* (NC_005955); three free-living relatives: *Agrobacterium tumefaciens* (GCF_000016265), *Ochrobactrum anthropi* (GCF_000017405) and *Brucella melitensis* (GCF_000007125); the honeybee symbiont *Bartonella apis* (NZ_CP015625); and the ant symbiont *Ca. Tokpelaia hoelldoblerii* (CP017315) isolated from *Harpegnathos saltator*. We also included *Bartonella tamiae* (GCF_000278275), a pathogen of mammals that appears to have an intermediate stage in ticks (Billeter, Miller, Breitschwerdt, & Levy, 2008; Kabeya et al., 2010; Leulmi et al., 2016). For comparisons of the assembled Orbales genome, we downloaded *Actinobacillus succinogenes* (NC_009655), *Escherichia coli* (NC_000913), *Haemophilus parainfluenzae* (NC_015964), *Vibrio mimicus* (NZ_CP014042), and the bee symbionts *Frischella perrara* (NZ_CP009056), *Gilliamella apicola* (NZ_CP007445), *G. intestini* (GCF_900094935), *G. bombicola* (GCF_900094945), *G. bombi* (GCF_900103255) and *G. mensalis* (GCF_900103085).

Orthologous gene groups were identified across taxa using PROTEINORTHO v5.16 (Lechner et al., 2011) with minimum connectivity of 0.5. Orthologous groups with a single sequence representative from every species were aligned using FSA (Bradley et al., 2009), and poorly aligned regions were removed with Gblocks (Castresana, 2000). Phylogenies were estimated in RAXML v7.3 (Stamatakis, 2006) with a concatenated matrix of all protein sequences and the PROTGAMMAWAG substitution model with 100 bootstrap replicates. To assess the degree of relaxed selection in putative symbionts, we estimated dN/dS ratios using the free-ratios model of PAML v4.9 (Yang, 1997, 2007). Strict quality controls were imposed: dN/dS values were only included when they were <4.0 and estimated dS values were at least 0.001. Differences between distributions of dN/dS ratios in different genomes were assessed using paired Wilcoxon rank-sum tests.

3 | RESULTS

3.1 | Acacia-ants have an extremely low trophic level

For all species outside of *Pseudomyrmex*, $\delta^{15}N$ ratios were pooled by genus and compared to *Pseudomyrmex* species individually. The three acacia-ant species each had significantly lower trophic levels when compared to every other ant genus sampled as well as the generalist *Pseudomyrmex* species *P. gracilis* and *P. elongatus* (FDR-corrected $p < 0.05$, Supporting Information Table S2, Figure 2). In addition,

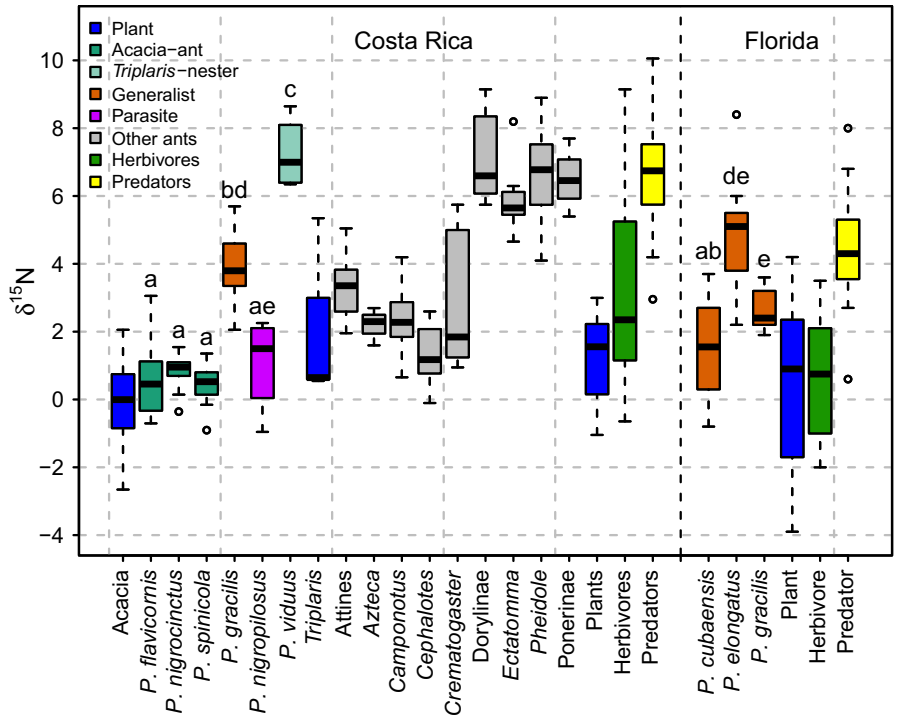

FIGURE 2 Distribution of δ^{15}N across samples collected at the ACG in Costa Rica and in Florida. Letters show significance between ant species of *Pseudomyrmex* based on Wilcoxon rank-sum tests with FDR correction. All three acacia-ant species (*P. flavicornis*, *P. nigrocinctus* and *P. spinicola*) also have significantly lower δ^{15}N than every other ant taxon included in this study. *Pseudomyrmex viduus* is a plant-ant mutualist that nests obligately in trees in the genus *Triplaris*, and *P. nigropilosus* is an obligate parasite of acacia plants [Colour figure can be viewed at wileyonlinelibrary.com]

acacia-ants had a significantly lower trophic level than the *Triplaris*-nesting *P. viduus* (FDR-corrected $p < 0.05$), confirming more generalized diets in *Triplaris*-nesting *Pseudomyrmex* than acacia-ants. The only taxa with δ^{15}N ratios not significantly higher than acacia-ants were a generalist collected in Florida, *P. cubaensis* (Figure 2), and the acacia parasite, *P. nigropilosus*, supporting previous observations that this latter species depends on the nutritional resources provided by acacias (Janzen, 1975).

3.2 | Bacterial quantity varies by genus

The quantity of bacteria varied widely in adults by ant genus (Figure 3). Notably, those taxa with clearly established symbiotic relationships with bacteria (*Camponotus* and *Cephalotes*) had the highest number of bacteria and significantly more than all species of *Pseudomyrmex*, regardless of collection site (FDR-corrected $p < 0.0006$, Supporting Information Table S3).

There was far less variation within larvae, but the same general patterns were consistent (Figure 3). *Camponotus* had significantly greater numbers of bacteria than all *Pseudomyrmex* taxa except *P. pallidus*, although significant differences did not exist for *Cephalotes*. The significantly lower numbers of bacteria in *Pseudomyrmex* from Florida compared with those from Costa Rica, while not as consistent as for adults, occurred for the majority of comparisons, including for the comparison of *P. gracilis* collected at the two sites (FDR-corrected $p < 0.05$, Supporting Information Table S3).

3.3 | Adults and juveniles host different bacterial communities

Adult and juvenile *Pseudomyrmex* bacterial communities were dominated by Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Supporting Information Figure S1). Using beta diversity, machine learning, and tests of relative abundance, we found that ant adults host distinct bacterial communities when compared to juveniles (Supporting Information Appendix S1). We, therefore, treated these life stages separately in all analyses. This finding differs from previous conclusions in *Pseudomyrmex* by Eilmus and Heil (2009), although this discrepancy is likely the result of the higher resolution provided by 16S rRNA sequencing as opposed to the restriction fragment length polymorphisms used to assess diversity previously.

3.4 | *Pseudomyrmex* hosts variable communities compared to other ants

Strong similarity in community composition within adults from the genus *Camponotus*, those in the genus *Cephalotes* and those in the Dorylinae (army ants) led to clear clustering of microbiomes from these hosts in principal coordinates analyses (Figure 4a). Concordant with these results, we also identified a number of bacterial taxa that were significantly more abundant in each of these genera than all other ant taxa (Supplementary Information). No other ant genera, including *Pseudomyrmex*, formed clear clusters of similar

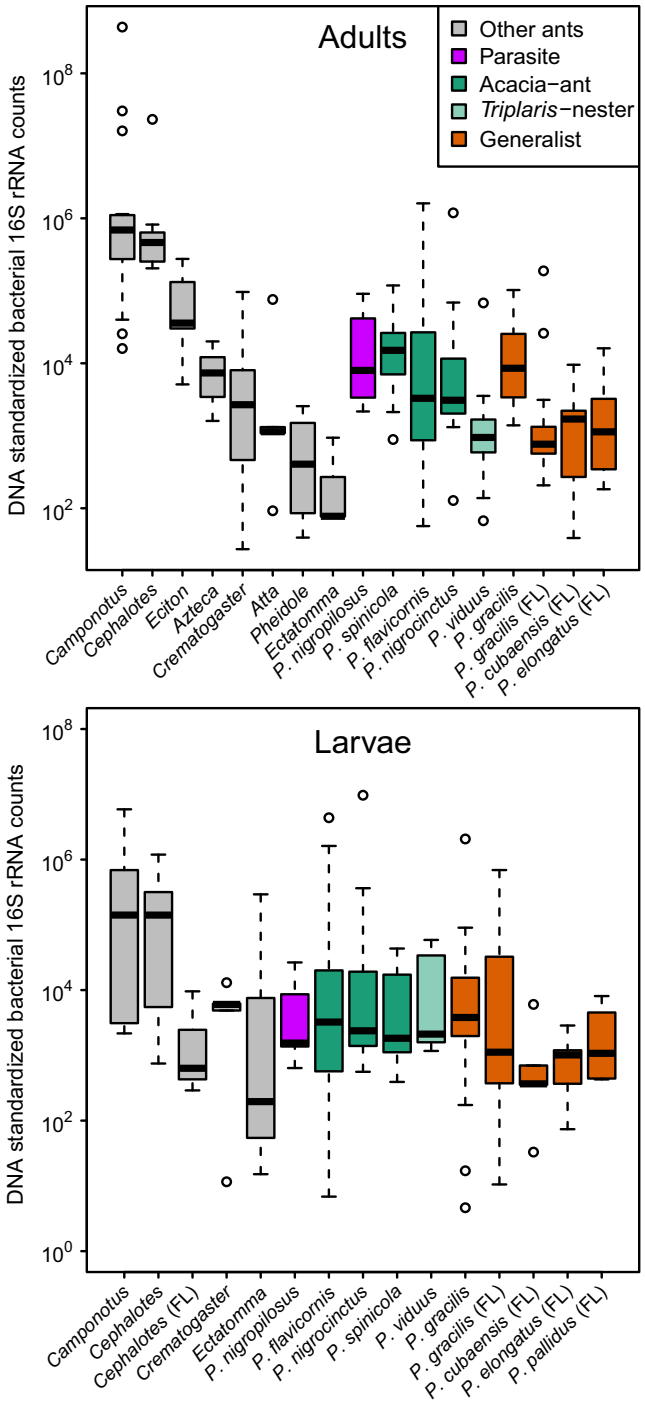

FIGURE 3 Quantities of the bacterial 16S rRNA gene in the ant taxa examined using qPCR in adults and larvae. All *Pseudomyrmex* adults, including obligate mutualists, have significantly lower numbers of bacteria than both *Camponotus* and *Cephalotes*, the two genera with known beneficial bacterial symbionts (Feldhaar et al., 2007; Hu et al., 2018). Significance was determined by Wilcoxon rank-sum tests with FDR correction ($p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

communities. Consistent with this apparent clustering, beta diversity among all *Pseudomyrmex* samples was significantly higher than beta diversity among all samples in the genus *Cephalotes* and, separately, than beta diversity among samples in the Dorylinae ($p < 0.01$; Supporting Information Table S4). For comparisons involving *Camponotus*, only weighted UniFrac beta diversity was significantly greater in *Pseudomyrmex*, likely because each *Camponotus* species is dominated by a distinct *Blochmannia* taxon, thus increasing the value of the nonphylogenetic Jaccard metric of beta diversity. The same patterns held generally true for between genus comparisons of larvae but to a lesser extent (Supporting Information Table S4; Figure 4b).

3.5 | Influence of geography at multiple scales

Differences in local collecting site within a geographic area could potentially influence bacterial communities. We, therefore, compared communities of samples of the same species collected from each of the four sites used in the ACG in Costa Rica to determine whether any consistent differences exist. We found little evidence for an influence of collecting site on bacterial communities at this scale (Supporting Information Appendix S1) and, therefore, combined samples from across all Costa Rican (ACG) sites in all further analyses.

However, differences were apparent across large geographic distances by comparing *P. gracilis* generalists collected from both Costa Rica and Florida. We compared all metrics of beta diversity within and between sites using nonparametric t tests with Monte Carlo permutation (Supporting Information Table S5). For adults, there was significantly lower beta diversity among samples from the same country than between all pairs of samples from Florida and the ACG (FDR-corrected $p < 0.05$) for both weighted UniFrac and Jaccard metrics. Unweighted metrics were not significantly different for adults. For larvae, within country beta diversity was significantly lower than between-country beta diversity for unweighted UniFrac, and both weighted and unweighted Jaccard (FDR-corrected $p < 0.05$). Despite making this comparison within a single species, it is likely that the individuals collected in the two sites were more closely related to other individuals from the same site, potentially increasing similarity within site. Regardless, these subtle differences meant that direct comparison of bacterial communities from samples collected at the two distant sites could potentially bias results. We, therefore, limited initial comparisons between groups of *Pseudomyrmex* to just those samples collected in Costa Rica.

3.6 | Acacia-ant vs. generalist communities

To determine whether there were bacterial groups unique to the acacia-ant microbiome, we compared these species to the generalist sympatric species *P. gracilis* using only samples from the ACG in Costa Rica. Although communities appeared to be largely similar based on overall taxonomic composition (Supporting Information Figure S1), principal coordinates analyses revealed some degree of difference in community structure (Figures 4 and 5a). Indeed, comparisons of weighted beta diversity metrics (Jaccard and UniFrac) within all acacia-ants and between acacia-ants and the generalist *P. gracilis* were significantly different for adults (FDR-corrected $p < 0.001$), although unweighted metrics were not significantly

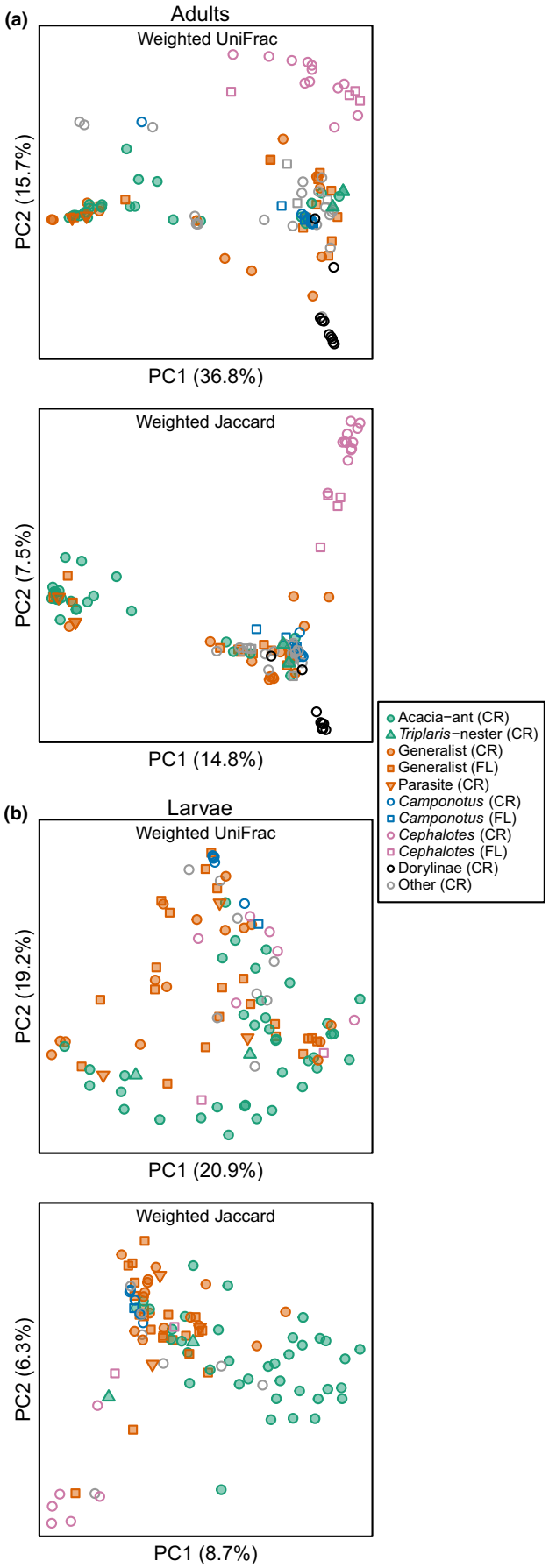

FIGURE 4 PCoAs of weighted UniFrac and Jaccard beta diversities for bacterial communities from adults (a) and larvae (b). Particularly for adults, *Cephalotes*, *Camponotus* and Dorylinae (the army ants) clearly host bacterial communities that have different composition than other ants. Collecting locality is indicated in parentheses (FL = Florida; CR = Costa Rica) [Colour figure can be viewed at wileyonlinelibrary.com]

different (FDR-corrected $p > 0.5$). Within larvae, comparisons of all metrics were significantly different (FDR-corrected $p < 0.001$).

Despite the apparent differences in degree of similarity within and between groups, supervised learning was unable to distinguish the generalist *P. gracilis* from acacia-ants with an error ratio of 1.03 for adults and 1.02 for larvae. Some consistency was apparent in these analyses, however. In the 100 iterations of the supervised learning, ZOTU0 was identified as the most influential taxon for distinguishing acacia-ants and *P. gracilis* 11 times and ZOTU2 was the most important taxon in the other 89 cases. Among larvae, ZOTU0 was always the most influential.

These two taxa, ZOTU0 and ZOTU2, were both members of the Acetobacteraceae and did show association with lifestyle in adult samples (Figure 5b). The closest BLAST hit to ZOTU0 was a sequence recovered from the ant *Linepithema humile* (KX984918) with a match of 98%. The best hit for ZOTU2 was to sequence recovered from the ant *Lasius flavus* (MG831360) with 97% similarity. These taxa made up the clear majority of Acetobacteraceae in *Pseudomyrmex*, and those samples that lacked substantial numbers of this family of bacteria failed to cluster by behaviour (Figure 5b). Among acacia-ants and *P. gracilis*, there were 12 colonies for which the abundance of Acetobacteraceae in adults was <20%. Seven of these were acacia-ants, and the other five were from *P. gracilis*. There was little consistency across these samples; only three taxa were present in at least 1% relative abundance across four or more colonies. These were ZOTU28 (*Acinetobacter*), ZOTU50 (*Cytophaga*) and ZOTU55 (Xanthomonadaceae). None of these made up more than 10% of more than one colony.

3.7 | Relative abundance in acacia-ants and a generalist

We tested those ZOTUs present in a total of at least 10 samples for differences in abundance between acacia-ants and samples of the generalist *P. gracilis* from the ACG. Of the 226 bacterial taxa tested, one was significantly more abundant in acacia-ants: ZOTU0 (Acetobacteraceae; FDR-corrected $p = 0.04$). This taxon was present in adults of all 29 acacia-ant colonies and all 14 *P. gracilis* colonies, but median relative quantity was drastically different. ZOTU0 made up a median of 78% of acacia-ant communities and only 0.035% of *P. gracilis* communities. This taxon was also significantly more abundant in acacia-ant larvae than *P. gracilis* larvae, although it was present at much lower relative quantities (median of 4.9% in acacia-ants and 0.021% in *P. gracilis*). ZOTU0 was present in all 39 acacia-ant and all 15 *P. gracilis* larval samples. Notably, this lineage was also present in

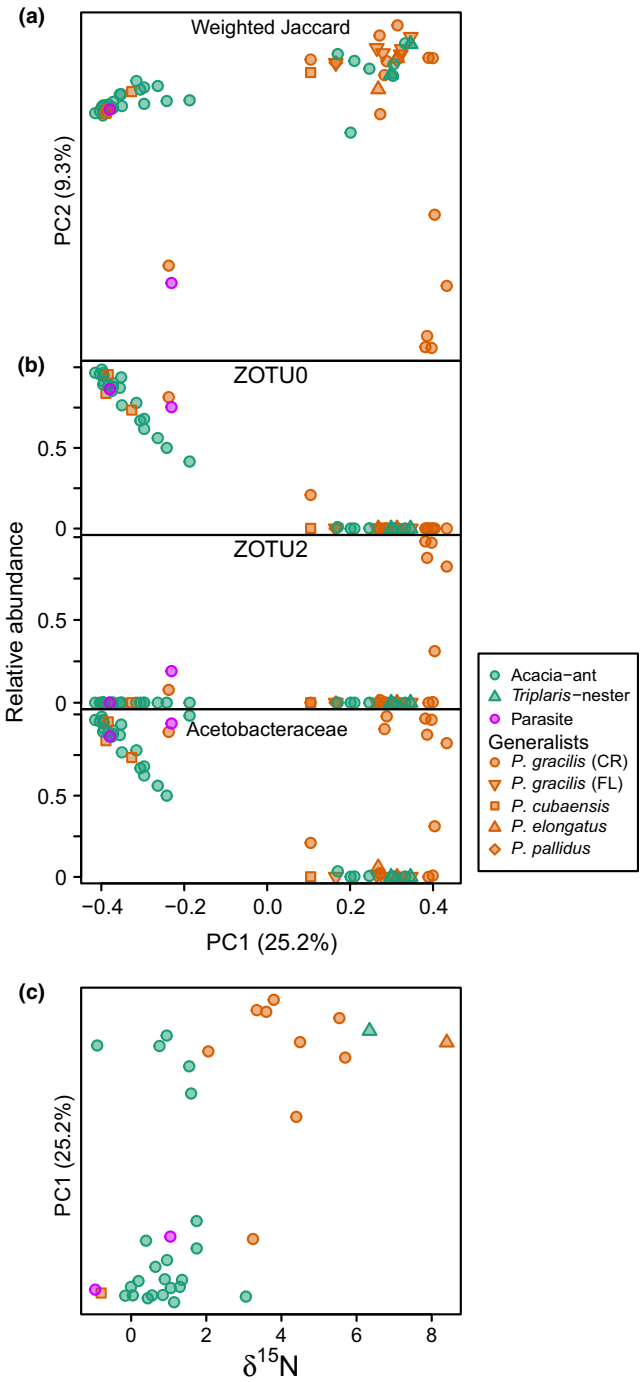

FIGURE 5 Weighted Jaccard PCoA of *Pseudomyrmex* samples (a). All acacia-ants, *Triplaris*-nesting *P. viduus* and *P. nigropilosus* parasites were collected at the ACG in Costa Rica, whereas all *P. cubaensis*, *P. elongatus* and *P. pallidus* were collected in Florida. *Pseudomyrmex gracilis* was collected from both sites. Relative abundances of ZOTU0, ZOTU2 and all Acetobacteraceae as a function of PC1 (b). PC1 as a function of nitrogen isotope ratios for those *Pseudomyrmex* colonies where both sequencing data and $\delta^{15}N$ data were collected (c). The composition of *Pseudomyrmex*-associated bacterial communities, often dominated by Acetobacteraceae, is correlated with trophic level [Colour figure can be viewed at wileyonlinelibrary.com]

adults of all 4/4 colonies of *Crematogaster* found nesting in acacias but at a relative abundance of only 0.033%. Finally, ZOTU0 was also present at similar levels in adults of *P. cubaensis* generalists from Florida with a median relative abundance of 78%, although the median for larvae was lower at 0.03%.

We also detected one taxon with significantly greater abundance in adults of the generalist *P. gracilis* (FDR-corrected $p = 0.04$). This was ZOTU4176 (Sphingomonadaceae) with median relative abundance of zero in acacia-ants and 0.0014% in *P. gracilis*. It was only present in adults from two of 29 acacia-ant and eight of 14 *P. gracilis* colonies.

There was a single additional taxon (of 195 tested) significantly more abundant in acacia-ant larvae (FDR-corrected $p = 0.02$). This taxon (ZOTU19) had a 16S rRNA sequence that was 96% similar to Nocardiaceae sequence recovered from the ant *Polyrhachis robsoni* (KC137132). ZOTU19 was present in 30 of 37 acacia-ant larvae and six of 14 *P. gracilis* larvae and had a median relative abundance of 0.02% in acacia-ants and zero in *P. gracilis*. This taxon was also present in all three larval samples from *P. nigropilosus* with a median abundance of 1.9% although it was not significantly greater in abundance in parasites than generalists after FDR correction, likely as a result of the small sample size. ZOTU19 was also present in larvae from three of five *Crematogaster* colonies at a median abundance of 0.0033%.

We also tested for differences in relative abundance of bacterial orders between *Pseudomyrmex* acacia-ants and generalists. None of the 38 orders tested in adults were significantly different after multiple test correction. In larvae, Entomoplasmatales was significantly more abundant in *P. gracilis* generalists and was present in all 14 colonies with a median abundance of 0.06% (FDR-corrected $p = 0.005$). Entomoplasmatales was absent from eight of 37 acacia-ant colonies. Bacillales, Pseudomonadales, Sphingomonadales and Rhodospirillales were all significantly more abundant in acacia-ant larvae (FDR-corrected $p < 0.03$). Although these orders were all present in all acacia-ant and *P. gracilis* colonies, the median relative abundances were quite different. Rhodospirillales, the order that includes ZOTU0 was particularly different, making up 6.1% of acacia-ant larval communities and only 0.18% of *P. gracilis* generalist communities. In Bacillales, the medians were 0.75% in acacia-ants and 0.12% in *P. gracilis*; in Pseudomonadales, relative abundances were 2.8% vs. 0.65%; and in Sphingomonadales, they were 0.85% vs. 0.29%.

3.8 | Trophic level influences bacterial community in *Pseudomyrmex*

Principal coordinates analysis of all *Pseudomyrmex* regardless of geographic origin showed that the species *P. cubaensis*, the only generalist for which trophic level was not significantly greater than that for acacia-ants, hosts more similar bacterial communities to acacia-ants than to other generalists (Figure 5a). For both weighted metrics (Jaccard and UniFrac), beta diversity was significantly greater between *P. cubaensis* and the generalist *P. gracilis* ($p < 0.005$) than between

P. cubaensis and acacia-ants ($p < 0.005$) for both adults and larvae. Unweighted metrics were nonsignificant ($p > 0.05$) for all comparisons except when using the unweighted Jaccard metric for larvae ($p = 0.001$).

We obtained both nitrogen isotope ratios and bacterial community composition data for adults from 36 colonies of *Pseudomyrmex* and for larvae from 50 colonies. In these samples, Mantel tests for correlations between weighted Jaccard distances and differences in standardized $\delta^{15}N$ were significant for adults and larvae ($p < 0.005$), suggesting that trophic level may play a larger role in determining bacterial community than host behaviour. Indeed, divergence along the first principal coordinates axis derived from weighted Jaccard distances of both adults ($\rho = 0.62$; $p = 4.2 \times 10^{-5}$) and larvae ($\rho = -0.52$; $p = 0.0001$) was significantly predicted by $\delta^{15}N$ (Figure 5c). Consistent with this pattern, ZOTU0 was also significantly negatively correlated with trophic level in both adults ($\rho = -0.51$; $p = 0.001$) and larvae ($\rho = -0.38$; $p = 0.005$).

The *Triplaris*-nesting species *P. viduus* and the obligate parasite of acacias, *P. nigropilosus*, further supported the possibility that trophic level was influential in determining community composition. We only obtained sufficient sequence counts to include adults from two colonies each of these species in analyses of bacterial communities but the grouping of these samples in PCoA met expectations based on stable isotope ratios (Figure 5a). Although it is also a mutualist with plants, *P. viduus* had a higher trophic level than acacia-ants (Figure 2) and hosted bacterial communities more similar to *Pseudomyrmex* generalists than acacia-ants (Figure 5a). *Pseudomyrmex nigropilosus*, despite its close phylogenetic relationship to the generalist *P. gracilis* (Figure 1), grouped more closely with acacia-ants (Figure 5a), consistent with its low trophic level (Figure 2). Similar patterns were not apparent among larvae where two and three colonies yielded sufficient sequence reads for inclusion from *P. viduus* and *P. nigropilosus*, respectively (Supporting Information Figure S5). Bacterial communities of larvae from both of these taxa did not appear more similar to either acacia-ants or *Pseudomyrmex* generalists.

3.9 | Genomes of novel taxa from Rhizobiales and Orbales

We found scaffolds classified as Alphaproteobacteria and Gammaproteobacteria in the *P. nigropilosus* larval metagenome assembly. We first separated the Alphaproteobacteria genome by identifying scaffolds with at least one protein with a best BLASTP hit to Alphaproteobacteria in NCBI's nonredundant database (Supporting Information Figure S2), requiring at least 60% of all proteins on individual scaffolds to also be classified as Alphaproteobacteria. Of the 100–106 essential genes expected for individual genomes (Albertsen et al., 2013), 95 unique sequences were present with only a single duplicate, indicating a nearly complete genome with little contamination from other taxa. This genome was composed of 2.0 Mb in 279 scaffolds with an N50 length of 13 kb and 2,311 protein-coding genes. GC-content was 53.8%. We submitted several translated ORFs to NCBI's BLASTP server to estimate taxonomy, and all were best hits to *Ca. Tokpelaia hoelldoblerii*, a symbiont of the ant *Harpegnathos saltator* in the order Rhizobiales (Neuvonen et al., 2016). We therefore abbreviated this taxon as *Tpnig* (*Tokpelaia Pseudomyrmex nigropilosus*).

For the Gammaproteobacteria, plotting GC-content against mapping coverage of the initial reads revealed a cluster of nine large scaffolds that appeared to represent a single taxon (Supporting Information Figure S2). These scaffolds had 104 unique essential genes and only a single duplicate, again indicating completeness and lack of contamination. This genome was a total of 2.8 Mb in length with an N50 of 247 kb and 2,556 protein-coding genes. GC-content was 37.4%. Best BLASTP hits were to *Frischella perrara* (order Orbales), a symbiont of honeybees, suggesting a preliminary classification to this genus, and we abbreviated this genome as *Fpnig*. Remaining scaffolds were fragmented, and no clear clustering was apparent (Supporting Information Figure S2).

Unfortunately, our attempts to assemble the metagenomic data of *P. flavicornis* adults were less successful. Our assembly totalled 17 Mb in 14,080 scaffolds with an N50 of only 2,335 bases and a largest scaffold of 211 kb. Of the 53 scaffolds for which we were able to confidently assign taxonomy, 52 were classified as Acetobacteraceae and there was no clear clustering by coverage or GC-content (Supporting Information Figure S2). It appears that several closely related species were present in this data set, reducing assembly quality and the ability to distinguish genomes. We, nevertheless, attempted to elucidate the possible nutritional contribution of these taxa by submitting the 17 Mb set of scaffolds to RAST. No genes involved in either the urease or nitrogenase pathway were identified. Complete biosynthesis pathways for five of the 10 amino acids essential to insects were present.

3.10 | Phylogeny, host distribution and evolutionary rates

We identified 546 genes with single-copy orthologs in all 12 *Tpnig*-related genomes used for comparative analyses, yielding a sequence matrix of 168,724 amino acids for phylogenetic inference. All nodes in this phylogeny had bootstrap values of 100%. The inferred phylogeny showed that *Tpnig* was most closely related to the ant symbiont, *Ca. T. hoelldoblerii* (Figure 6a). Evolutionary rate analyses showed that both of these bacterial taxa had dN/dS distributions that were significantly greater than the symbiont of honeybees, *Bartonella apis* and *B. tamiae* ($p < 1 \times 10^{-4}$). These values were more similar to the obligately pathogenic *Bartonella* species.

The 16S rRNA sequence for *Tpnig* was a perfect match to ZOTU9 (Supporting Information). This taxon was present in adults from 20/29 acacia-ant colonies and seven of 14 *P. gracilis* generalist colonies. It was also present in larvae from 26/37 acacia-ant colonies and 12/14 *P. gracilis* colonies. It did not differ significantly in relative abundance between acacia-ants and *P. gracilis* in either adults or larvae ($p > 0.05$). However, median relative abundance in adult acacia-ants was 0.003% and 0.0007% in *P. gracilis* generalists and was a maximum of 87% in acacia-ants and 0.005% in *P. gracilis*. There was a similar pattern in larvae with a median abundance of 0.003% and

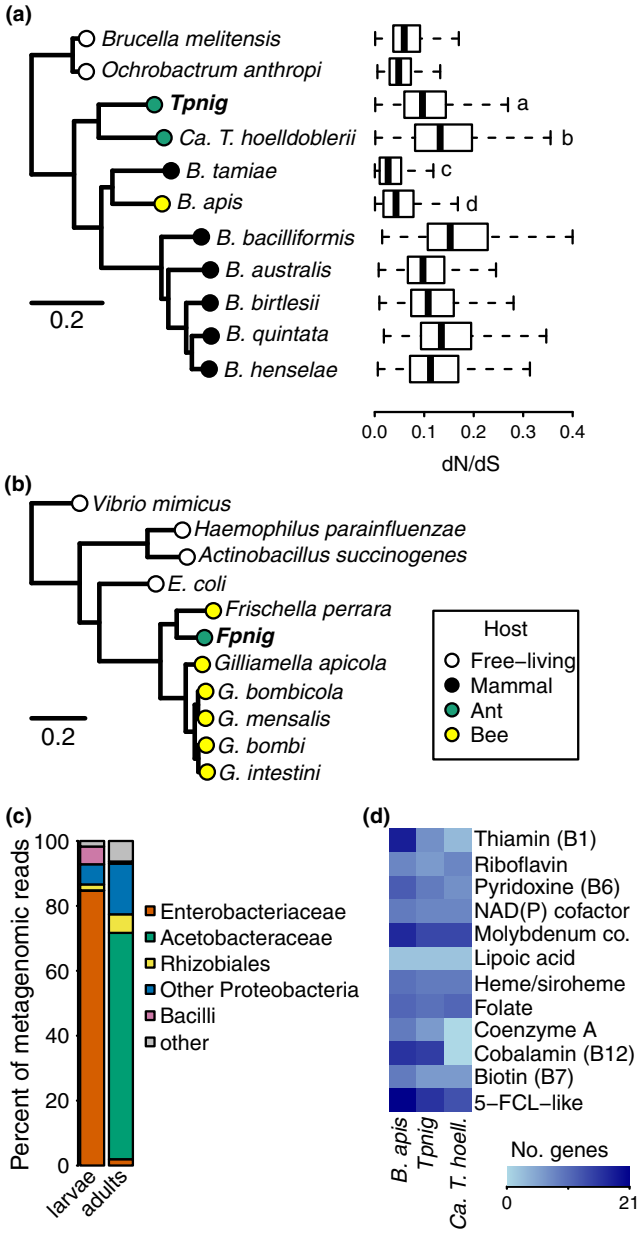

FIGURE 6 Phylogenies of symbionts *Tpnig* (a) and *Fpnig* (b) inferred from concatenated matrices of genomewide protein sequences. Boxplots show the distribution of dN/dS values for all coding sequences. Letters indicate significant differences in these distributions as determined by Wilcoxon rank-sum tests. Tests were only conducted between all combinations of the four taxa shown with letters. Taxonomic composition of the metagenomic sequence reads from *Pseudomyrmex nigropilosus* larvae and *P. flavicornis* adults (c). Numbers of genes annotated to vitamin and cofactor pathways in *Tpnig*, *Ca. T. hoelldoblerii*, the symbiont of the ant *H. saltator*, and the related symbiont of honeybees, *Bartonella apis* (d). *Tpnig* and *Ca. T. hoelldoblerii* appear to be subject to genomewide relaxed selection [Colour figure can be viewed at wileyonlinelibrary.com]

0.0003% in acacia-ants and *P. gracilis*, respectively, and maximum abundances of 98% and 13%, respectively. Exact sequence matches to *Tpnig* were also present in the 16S rRNA data of more distantly related taxa, including adults from five of 11 army ant colonies, 10/17 *Cephalotes* colonies and 7/16 *Camponotus* colonies as well as larvae from eight of nine *Cephalotes* and seven of eight *Camponotus* colonies, suggesting lack of species-specificity. Alternatively, such wide host occupation may instead be indicative of the slow evolution of the bacterial 16S rRNA sequence. The 253-bp sequence representing ZOTU9 was 98% similar to *Ca. Tokpelaia hoelldoblerii*, the symbiont of a very distantly related ant species and only 91% similar to Rhizobiales putatively domesticated by a related group of ants, *Tetraponera* (Borm, Buschinger, Boomsma, & Billen, 2002).

For *Fpnig*, 810 genes and 257,235 amino acids were used to infer phylogenetic relationships, again yielding bootstrap values of 100%. As expected, this taxon was most closely related to the honeybee symbiont *Frischella perrara* (Figure 6b). ZOTU120 was identical to the 16S rRNA sequence recovered from this assembly (Supporting Information). This lineage only occurred in 10 adult samples across our data set and adults from only seven *Pseudomyrmex*. In larvae, it was present in 21 colonies across our full data set, 16 of which were *Pseudomyrmex*. Again, *Fpnig* did not differ in relative abundance between acacia-ants and the generalist *P. gracilis* either among adults or larvae ($p > 0.05$) and only reached a maximum relative abundance of 0.03% in acacia-ants and 0.002% in *P. gracilis*. Median abundance was zero.

3.11 | Biosynthetic capabilities of *Pseudomyrmex* symbionts

We attempted to determine the impact of *Tpnig* on its hosts by examining the functional capacity of its genome. Complete pathways for seven of the 10 amino acids essential to insects (histidine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) were present in this taxon. No genes involved in the synthesis of branched chain amino acids (leucine and isoleucine) were detected, and the arginine synthesis pathway was missing a single gene: *argB*. The three subunits and four accessory proteins required for the urease pathway (which breaks urea down into ammonia) were all present, as was a glutamine synthetase type 1 gene required to catalyse the production of glutamine from the ammonium produced by urease. We found that many genes involved in vitamin synthesis pathways were absent from *Tpnig* relative to the related symbiont of honeybees *Bartonella apis* (Figure 6d). *Fpnig* had complete pathways for all 10 amino acids essential to insects. It did not include any urease subunits but did possess the ability to convert ammonia to glutamine.

4 | DISCUSSION

4.1 | Relative bacterial abundance affected by trophic level

We hypothesized that acacia-ants would host a unique set of gut microbes that allow them to better utilize their acacia-based diets, assessing this possibility by comparing their bacterial communities to a diversity of other ants. These included distantly related taxa from across the ant phylogeny, congeneric species with more generalized

diets, and even a parasitic congener that feeds on acacias but does not provide the protection given by acacia-ants. Comparing all of these ant species, we fail to find bacterial taxa associated specifically with acacia-ants; similar bacteria are present across all *Pseudomyrmex* species investigated in this study. A small number of bacterial taxa do differ in relative abundance among *Pseudomyrmex* ants including a strain of acetic acid bacteria in adults and a strain of Nocardiaceae in larvae but neither are exclusively associated with particular ant taxa. Rather, variation in bacterial communities appears to be better explained by differences in trophic level than by the dietary specialization of acacia-ants.

Although our sample sizes for these taxa were small, the *Triplaris*-nesting *P. viduus* and the acacia parasite *P. nigropilosus* provide insight into the source of differences in relative abundance of bacterial taxa between ant species. First, *Triplaris* plants do not provide the extrafloral nectar or food bodies of acacias and, as expected, *P. viduus* feeds at a much higher trophic level than acacia-ants (Figure 2). Likely as a result, the bacterial communities associated with these ants are more similar to generalists than to acacia-nesting mutualists, particularly with regard to the dominant strain of Acetobacteraceae (Figure 5). Second, although far more closely related to *P. gracilis* generalists (Figure 1), the parasite *P. nigropilosus* obligately nests in acacias, taking advantage of the nesting space and nutritional resources provided (Janzen, 1975), and feeding on a diet similar to that of acacia-ants (Figure 2). Concordant with a predominantly dietary mechanism for determining bacterial community composition, *P. nigropilosus* tends to host more similar communities to acacia-ants than generalists (Figure 5). However, an acacia-based diet is clearly not necessary for the bacterial community composition present in acacia-ants. Bacterial communities associated with *Pseudomyrmex cubaensis*, the only generalist for which trophic level was not significantly greater than that of acacia-ants, are much more similar to acacia-ants than other generalists (Figure 5). Together, these findings suggest that trophic level, regardless of diet specificity, is most influential in determining bacterial community composition.

Despite this variation in relative abundance of a small number of bacterial taxa between acacia-ants and *Pseudomyrmex* that feed higher on the trophic scale, the communities associated with these ants are not clearly distinct but instead share taxa, sometimes even in the same relative abundance. This may be due to the lack of a stable community of specific bacterial taxa associated with these ants. *Pseudomyrmex* species have low bacterial densities relative to those ants known to host specialized symbionts (i.e., *Camponotus*, *Cephalotes* and army ants; Figure 3) possibly making the gut environment more open for colonization by incidentally encountered taxa. Consistent with this possibility are both the relatively high beta diversity among *Pseudomyrmex* colonies (Figure 4) and high alpha diversity within these species (Supporting Information Figure S4) showing that *Pseudomyrmex* hosts more variable bacterial communities than ants with known relationships with bacterial symbionts. Thus, consistent with the results from the stable isotope analysis, the patterns found are likely the result of different bacterial taxa opportunistically colonizing ant guts depending on the type of nutrients present.

These patterns are in contrast to what has been found in other groups of ants where diet specialization is correlated with a clear change in bacterial community composition (Anderson et al., 2012; Funaro et al., 2011; Hu et al., 2018; Łukasik et al., 2017; Russell et al., 2009; Sanders et al., 2017). There are several possible explanations for why acacia-ants may not require similar shifts in microbial communities despite their large trophic shift. First, acacia plants may provide comparable nutrition to what generalists obtain on their own. The Beltian food bodies produced by acacias have, in fact, been shown to include all essential amino acids (Heil et al., 2004). Alternatively, a more significant transition in diet and gut microbiota may have occurred at an earlier stage of evolution when Pseudomyrmecines initially transitioned to an arboreal lifestyle. A variety of arboreal ant taxa are known to have tight and often obligate relationships with symbiotic bacteria (Borm et al., 2002; Feldhaar et al., 2007; Hu et al., 2014; Russell et al., 2009; Sanders et al., 2014, 2017) and, given that all *Pseudomyrmex* species are arboreal, they may already share the requisite bacterial taxa to thrive in this type of environment. The symbionts that are present, including *Ca. Tokpelaia* and the clearly dominant Acetobacteraceae, may play a role in the enrichment of ant diet across *Pseudomyrmex* mutualists and generalists and, potentially, the ant family as a whole. Even without changing in relative abundance, these bacteria may readily compensate for dietary differences between ant groups.

4.2 | Potential nitrogen recycling by *Tokpelaia*

The bacterial communities of *Cephalotes* ants serve to recycle nitrogenous waste into a form usable by their hosts (Hu et al., 2018). A complete urea recycling pathway is also present in *Ca. Tokpelaia* symbionts associated with both the carnivorous ant *H. saltator* and herbivorous species in the genus *Dolichoderus* (Bisch et al., 2018; Neuvonen et al., 2016). In addition to possible nitrogen recycling, the presence of complete pathways for all essential amino acids in the *Ca. Tokpelaia* symbionts of *Dolichoderus* but not *Harpegnathos* is suggestive of a role in amino acid enrichment of herbivorous ant diets (Bisch et al., 2018). Here, we find a related bacterial taxon widespread across *Pseudomyrmex*, also with a functional urease pathway, but lacking pathways for all essential amino acids. The differences in amino acid pathway completeness among *Ca. Tokpelaia* symbionts of different ant groups indicate that these bacteria may play different roles depending on host, even among those with similar dietary habits (e.g., the largely herbivorous *Pseudomyrmex* and *Dolichoderus*). The universal presence of the urease pathway in all *Ca. Tokpelaia* lineages, however, suggests that nitrogen recycling may be key to the widespread symbiosis between ants and these bacteria, regardless of other possible nutritive benefits that may occur. We also find signatures of genomewide relaxed selection in the *Ca. Tokpelaia* lineage associated with *Pseudomyrmex* (Figure 6), a pattern symptomatic of obligately symbiotic taxa (Clayton et al., 2012; Oakeson et al., 2014), indicating a tight relationship between ants and *Ca. Tokpelaia*. The widespread presence of these bacteria across both

herbivorous and predatory ants could help to explain the flexibility of ant diets generally.

We were also able to assemble the genome of a *Frischella*-related taxon present in *Pseudomyrmex*, but this species is present in a small number of individuals so is unlikely to play a substantial role in *Pseudomyrmex* biology. The best known *Frischella* species, *F. perrara*, appears to incur an immune cost to its honeybee hosts without providing any known benefit (Emery, Schmidt, & Engel, 2017; Engel, Bartlett, & Moran, 2015).

4.3 | Acetobacteraceae

Acetic acid bacteria are common inhabitants of insect guts, particularly among those that feed largely on nectar (Crotti et al., 2010, 2016) and including two widespread groups of ants, *Camponotus* (Brown & Wernegreen, 2016) and *Linepithema* (Hu et al., 2017). *Parasaccharibacter apium*, an Acetobacteraceae lineage associated with honeybees, appears to contribute to larval survival and immune function (Corby-Harris et al., 2014, 2016). Other lineages contribute to development in mosquitoes and *Drosophila* (Mitraka, Stathopoulos, Siden-Kiamos, Christophides, & Louis, 2013; Shin et al., 2011). Unfortunately, our attempts to characterize the metabolic capacities of *Pseudomyrmex*-associated Acetobacteraceae through metagenomics were largely unsuccessful due to the presence of multiple related strains and a resulting inability to assemble large genomic scaffolds. Additional work on this taxon in *Pseudomyrmex* is clearly needed.

The diversity of insect-associated Acetobacteraceae and their likely diversity of functions makes it difficult to speculate on the role played in *Pseudomyrmex* mutualists and generalists. Possibly, the different strains of acetic acid bacteria in *Pseudomyrmex* influence their hosts in different ways, but it is also possible that these bacteria are simply colonizing the environments to which they are best adapted, environments that likely differ predominantly in the relative amount and types of sugars present. Mutualistic acacias provide only glucose and fructose in their extrafloral nectar, failing to supply the sucrose typically provided by other plants (Heil, Rattke, & Boland, 2005; Kautz, Lumbsch, Ward, & Heil, 2009). Regardless, it is obvious that Acetobacteraceae is a core member of the gut community present in a wide diversity of ants and insects, including *Pseudomyrmex* (Crotti et al., 2010, 2016; Kautz, Rubin, & Moreau, 2013).

4.4 | Nocardiaceae

In addition to an Acetobacteraceae taxon, a single lineage of Nocardiaceae in the order Actinomycetales is present in greater abundance in acacia-ants than in the congeneric generalist *P. gracilis*. Although there are a few examples of Actinomycetales likely providing nutritional assistance to their insect hosts (Ben-Yakir, 1987; Durvasula et al., 2008; Salem et al., 2014), defensive mutualisms are much more common (Kaltenpoth, 2009). The leaf-cutter ants engage in a well-studied defensive mutualism with bacteria (including taxa within Nocardiaceae), using the compounds produced by their symbionts to eliminate a parasitic fungus from their gardens (Barke et al., 2010; Currie, Poulsen, Mendenhall, Boomsma, & Billen, 2006; Currie, Scott, Summerbell, & Malloch, 1999; Haeder, Wirth, Herz, & Spiteller, 2009; Mattoso, Moreira, & Samuels, 2012). Another genus of obligate plant-ants, *Allomerus*, may use the antibiotic compounds of Actinobacteria symbionts in a similar way (Ruiz-Gonzalez et al., 2011; Seipke et al., 2012), and several other obligate plant-nesting ant species are known to host related lineages, including another acacia-ant (Hanshew et al., 2015). Although associations with fungus have not been reported in *Pseudomyrmex*, Nocardiaceae clearly makes up a core component of the *Pseudomyrmex* microbiome. Given the much greater abundance of Nocardiaceae taxa in larvae than adults, these bacteria may be helping to defend juveniles from pathogens as occurs in beewolf wasps (Kaltenpoth, Göttler, Herzner, & Strohm, 2005). As yet, it is not clear why *Pseudomyrmex* in particular would require such protections or how this relationship may differ in acacia-ants.

5 | CONCLUSIONS

We find little support for the hypothesis that specialized bacterial partners are required for the evolution of the strict diets of acacia-ants. Instead, we show that widespread bacterial symbionts of ants change in relative abundance in association with shifts in trophic level. In addition, a bacterial lineage with genomic signatures of obligate host association and the ability to recycle urea is present across diverse ants, including both *Pseudomyrmex* mutualists and generalists. Ant bacterial communities appear to adjust to the diets of their hosts and may serve to improve the nutritional value of these diets, potentially contributing to the impressive flexibility of ant feeding habits.

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DATA ACCESSIBILITY

All bacterial 16S rRNA amplicon and metagenomic sequencing data have been deposited in NCBI's Short Read Archive under Accession no. SRP128534. ZOTU sequences, metagenomic assemblies and stable isotope data have been deposited in the Dryad Digital Repository at https://doi.org/10.5061/dryad.gg6n17s.

AUTHOR CONTRIBUTION

B.E.R.R., S.K. and C.S.M. conceived of and designed this study. B.D.W. performed the laboratory work. B.E.R.R. analysed the data. All authors contributed to the interpretation of results and writing of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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