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16S rDNA metabarcoding of the bacterial community associated with workers of *Pheidole rugaticeps* Emery (Hymenoptera: Formicidae)

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ABSTRACT

Insect microbiota are receiving increasing attention from researchers, particularly with the continued advances in next generation sequencing (NGS) techniques. However, there is a paucity of data on the microbiota of ants that scavenge around human settlements. In this study, we characterized the bacterial communities of *Pheidole rugaticeps* Emery that were collected scavenging on other household insects using Illumina MiSeq high-throughput sequencing of the bacterial 16S ribosomal DNA gene. *P. rugaticeps* DNA was extracted from the insect samples using a HiYield™ Genomic DNA isolation kit according to the manufacturer's protocols and amplified using polymerase chain reaction (PCR). The PCR products were sequenced with the Illumina MiSeq platform according to the standard protocols to amplify the V3–V4 of the 16S rDNA gene. The results for the 16S rDNA genes were analysed using QIIME 2 Core – 2020.6, and a 16S rDNA metabarcoding dataset was presented. A total of 46,651 reads were obtained from three genomic samples. A total of 368 amplicon sequence variants (ASV) comprising 165 genera were revealed and classified into 17 phyla. Proteobacteria (57.47%) and Firmicutes (33.14%) were the most abundant taxa, while *Acinetobacter* (37.10%) was the most abundant genus in all three sampling groups. Pathogenic bacteria species, such as *Acinetobacter baumannii* (15%) and *Pseudomonas aeruginosa* (2.92%), were identified from *P. rugaticeps* samples collected from a hospital environment. However, this study recommends more studies on the microbiota of *Pheidole* ants with different feeding habits and habitats to establish their core microbiome.

Introduction

Recent studies have been focused on insect microbiota in particular with the continued advances in next generation sequencing (NGS) techniques (Feldhaar, 2011; Russell et al., 2017). Ants, like many other insects, have a varying composition of microbiota ranging from a gut microbiome that aids in food processing and nutrient enrichment (Feldhaar et al., 2007; Feldhaar, 2011; Hu et al., 2018) to opportunistic bacteria that resides on the body cuticles (Birer et al., 2017). This may add to the variation in the microbial density of ants with some, such as *Crematogaster* (Rubin et al., 2014), *Solenopsis* (Ishak et al., 2011), *Linepithema* (Hu et al., 2017), and several other ant genera (Sanders et al., 2017a; 2017b), having low bacteria densities, while *Cephalotes* (Hu et al., 2014; Lanan et al., 2016) and many others (Sanders et al., 2017a; 2017b) have a high diversity of bacteria. However, *Pheidole* were recently revealed to have a comparatively stable microbiota across its species (Martins and Moreau, 2020). The study also found that food resources and geographical location influenced the microbiota of *Pheidole* ants.

Generally, *Pheidole* ants are a hyper-diverse group of ants with worldwide distribution (Fowler, 1994; Wilson, 1976) and a high species richness globally (Bolton, 2018; Longino, 2009; Wilson, 2003). They are most abundant and diverse in the tropics (Ward, 2000; Wilson, 2003). They have wide-ranging feeding habits (Fowler et al., 1991) with several studies indicating them as seed harvesters (Martins and Moreau, 2020; Thomson et al., 2016), predators of insects (Dejean et al., 2007), and scavengers of arthropods and animal carcasses (Cruz and Vasconcelos, 2006; Monteiro-Filho and Penereiro, 1987; Watson and Carlton, 2005). Studies also showed that they exhibit a discriminating capability for collecting corpses and invading colonies of other ants (Dejean et al., 2007). Hence, their multiple feeding habits, diet, microhabitat, species, and geography may influence their microbiota (Anderson et al., 2012;

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Kelly et al., 2019; Lanan et al., 2016; Martins and Moreau, 2020; Moreau and Rubin, 2017; Ramalho et al., 2017a, 2017b; Reeves et al., 2020; Sanders et al., 2014, 2017a, 2017b).

Human settlements harbor many ant species, including *Pheidole* in areas, such as kitchens, restaurants, and food stores, with poor control and food storage practices (Santos et al., 2009; Zarzuela et al., 2002). These ants harbor a complex community of bacteria (Currie et al., 1999; Lanan et al., 2016) both internal (Boursaux-Eude and Gross, 2000; Zientz et al., 2005) and external (de Zarzuela and de Campos-Farinha, 2005; Hughes et al., 1989; Zurek and Gorham, 2008). Their presence can aid the spread of pathogens (Boursaux-Eude and Gross, 2000; Campos and Silva, 2010). Pathogens are typically picked up during foraging (Zurek and Gorham, 2008) and may eventually be deposited on food, utensils, and/or hospital apparatus. Several bacteria pathogens have been reported from ants collected from hospital environments (dos Lima, 2013; Fowler et al., 1993; Máximo et al., 2014; Oliveira et al., 2017) and houses (Alharbi et al., 2019; Silva et al., 2014; Simothy et al., 2018). The majority of the previous studies examined the bacteria communities using culture-dependent approaches that merely recovered 1%–10% of the actual bacterial diversity present in an environment (Pace, 1997; Hugenholtz et al., 1998).

In this study, we characterized the bacteria communities of *P. rugaticeps* collected scavenging on other household insects using Illumina MiSeq high-throughput sequencing of the bacterial 16S ribosomal DNA gene. *P. rugaticeps* was the most abundant scavenging ant where these ant samples were taken. No research has been done on the bacterial communities of *P. rugaticeps,* and little or no research has been conducted to compare the microbial communities of ants collected from hospitals and residential areas. The molecular analysis of bacterial communities of an environment allows for the identification of several low-abundance bacteria, which would not be identified by culture dependent techniques (Jackson et al., 2013). In this study, we determined the microbial composition of *P. rugaticeps* using a 16S rDNA metabarcoding approach.

Materials and methods

Ants Sample collection and processing

Household ants were baited with dead American cockroaches during night hours (20:00 h to 22:00 h). The ants were collected scavenging on *Periplaneta americana* after the cockroaches were sprayed with aerosol insecticides (Knockdown). Some of the cadavers of the cockroaches were picked with sterile tweezers and placed in and around ant nests inside houses, hospitals, and other administrative areas, and ants foraging on the bait were collected into sterile tubes. Ants are frequently seen around dead *P. americana* and other household insects, which led us to study the microbiome of these scavenging ants. The ant samples were grouped as the administrative areas: both polytechnic and federal university (BPF), hospitals: Dalhatu Specialist Hospital Lafia and a primary healthcare center in Akwanga (DPH), and residential areas: collected from residents in rural and urban areas of Nasarawa state, Nigeria (UR) according to where they were collected. The ant samples were sorted and identified using standard taxonomic keys (AntWeb, 2020; Taylor, 2012). *P. rugaticeps* Emery happened to be the most abundant species collected, which is why we selected *P. rugaticeps* Emery for the 16S rDNA metabarcoding analysis to determine the bacteria composition. Both major and minor workers of *P. rugaticeps* Emery were selected for DNA extraction. Fresh workers from each group were pooled separately and their genomic DNA was extracted.

DNA Extraction

The ant specimens were surface washed with 2 mL of sterile distilled water to remove soil and other debris from the samples prior to molecular analysis. A pool of ten minor and major workers were ground in 200 µL of phosphate-buffered saline (PBS) using a sterile pestle with adequate up and down strokes without twisting. The choice of 10 ants is in line with the procedure of the Global Ant Genomics Alliance (GAGA) to fundamentally advance the multidisciplinary genomics-based study of ant biology. According to GAGA, the minimum requirement for microbiome analyses is 3×10 worker ants regardless of individual size. The DNA extraction was performed using a HiYield™ Genomic DNA isolation kit (Real Biotech Corporation, Taiwan) according to the manufacturer's protocols with little modifications: 200 uL 1X PBS was used in lieu of QGT Buffer; PBS was simultaneously mixed with Proteinase K, RNAse A, and QGB Buffer before tissue homogenization and incubation; the incubation period was reduced to 2 h; and the solutions were gently mixed by flicking the tube not pipetting (Kingan et al., 2019). The modifications were done to achieve a high molecular weight DNA. The process was conducted without negative control but under sterile conditions to avoid possible contaminants (Moreau, 2014). The total DNA samples were submitted to MyTACG Bioscience Enterprise (Kualar Lumpur, Malaysia) for Illumina sequencing.

16S Illumina Library and Sequencing

The 16S rDNA amplicon library was constructed using the primer set 341F and 806R to amplify the V3–V4 region of the 16S rDNA gene (Zhang et al., 2018). Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.) prior to sequencing. Sample libraries were pooled in equimolar with paired-end sequences ($2 \times 250/300$ bp) on an Illumina MiSeq platform according to the standard protocols.

16S rDNA Metabarcoding data analysis

The raw fastq files were analysed with the QIIME 2 software package (q2cli version 2020.6.0) (https://qiime2.org.) (Bolyen et al., 2019) and its associated plugins. The demux emp-paired command was used to demultiplex the paired-end reads using the demux plugin (https://github.com/qiime2/q2-demux). Quality control, chimeric sequence filtering, and feature table construction were performed using the q2-dada2 plugin (Callahan et al., 2016) and the trimming parameters were done based on the demux visualization. Taxonomic classification, clustering, and dereplicating with 99% similarity were done using the Feature-classifier plugin (Bokulich et al., 2018) and VSEARCH plugin (https://github.com/qiime2/q2-vsearch) (Rognes et al., 2016) against the SILVA 132 database (Quast et al., 2013). The phylogenetic tree was reconstructed using the q2-phylogeny plugin (https://github.com/qiime2/q2-phylogeny) while the alpha and beta diversity analysis were done via the q2-diversity plugin (https://github.com/qiime2/q2-diversity).

Deposition of nucleotide sequences

All sequences obtained were deposited at National Centre for Biotechnology Information (NCBI) Sequences Read Archive (SRA) databases with the bioproject accession number: PRJNA609841 (Runs: SRR11235855, SRR11235854, and SRR11235853).

Results

Data Summary from Illumina Sequencing

Following the quality filtering and removal of chimeric sequences using dada2 on the QIIME 2 platform, a total of 46,651 reads were produced from all the groups (BPF, DPH, and UR). The number of reads per sample and other alpha diversity analyses, such as Chao 1 and Shannon of the results are presented in Table 1. The Good's coverage

Table 1
Results from the alpha diversity analysis of the three samples

Sample ID*	Reads	0.97				
		ASVs	Chao 1	Coverage	Shannon	Simpson
BPF	14293	202	202 (202,202)	1.000000	4.2 (4.18,4.23)	0.0347 (0.0334,0.0361)
DPH	14073	111	111 (111,111)	1.000000	2.73 (2.7,2.76)	0.2012 (0.1949,0.2074)
UR	18285	55	55 (55,55)	1.000000	1.66 (1.63,1.69)	0.4478 (0.4392,0.4565)
Total	46651	368				

*BPF represents samples collected from Both Polytechnic Nasarawa and Federal University Lafia, DPH represents Dalhatu Specialist Hospital Lafia and a Primary Healthcare Center in Akwanga, and UR represents samples collected from residents in Urban Residential areas in Nasarawa state, Nigeria.

estimates showed that the sequencing coverage sufficiently secured the microbial diversity of *P. rugaticeps* (Table 1). A rarefaction curve of the *P. rugaticeps* sample groups showing the species richness of each sample is also shown in Fig. 1.

Taxonomic classification

The majority of the reads from the *Pheidole* samples groups were taxonomically classified down to the species level. The total number of bacterial taxonomic units (ASVs) identified was 368 defined at a 99% sequence similarity. Of the total number of 368 ASVs, 202 were from the BPF sample, 111 were from the DPH sample, and 55 were from the UR sample. We found that 99.96% of these were classified as bacteria. These 368 ASVs were assigned to 17 phyla, 28 classes, 53 orders, 94 families, and 165 genera. Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes were the four of the most predominant phyla with percentage frequencies of 57.47%, 33.14%, 6.77%, and 1.90%, respectively. Of all the sequences, 99.28% belonged to these four phyla and the rest (0.72%), with less than 1%, were classified as others. At the class level, Gammaproteobacteria (54.74%) was the most abundant followed by Bacilli (29.80%), Actinobacteria (6.45%), Clostridia (2.96%) Alphaproteobacteria (2.70%), and Bacteroidia (1.90%). The remaining classes with less than 1% occurrence were represented as Others (1.45%) as presented in Fig. 2.

Bacterial genera with percentage occurrence of more than 1% in *P. rugaticeps* samples are presented in Fig. 3. *Acinetobacter* (37.10%) was the most abundant genus followed by *Weissella* (15.94%). Other genera with a high percentage occurrence included *Pseudomonas* (5.87%), *Enterococcus* (3.07%), *Bacillus* (3.02%), *Escherichia-Shigella* (2.80%), *Lysinibacillus* (2.77%), and *Gilliamella* (2.13%). All the other remaining

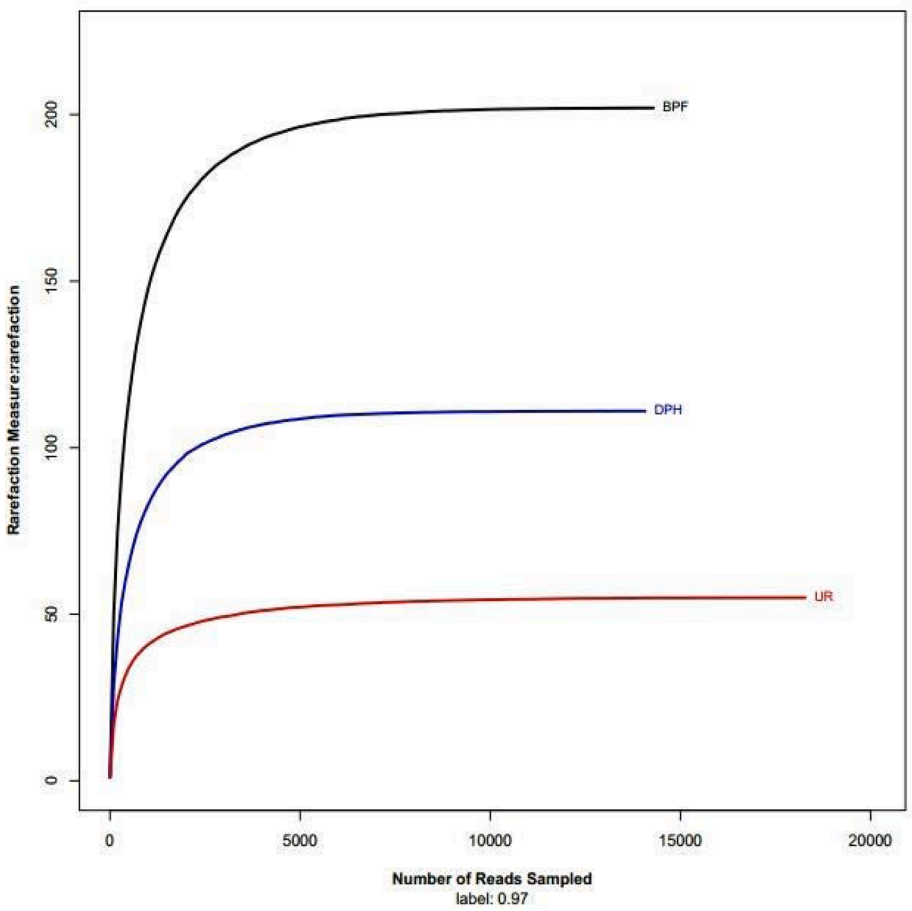

Fig. 1. Rarefaction curves showing species richness of the microbial communities of *P. rugaticeps* Emely. Samples BPF (black curve), DPH (blue curve) and UR (green curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

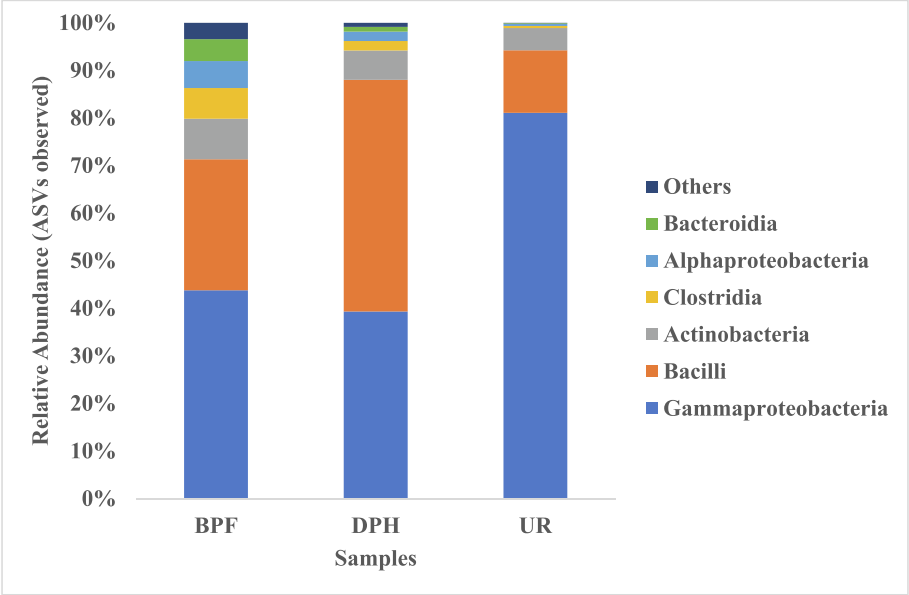

Fig. 2. A summary of the bacterial classes identified from *P. rugaticeps* collected from Administrative area (BPF), Hospitals (DPH) and Residential areas (UR).

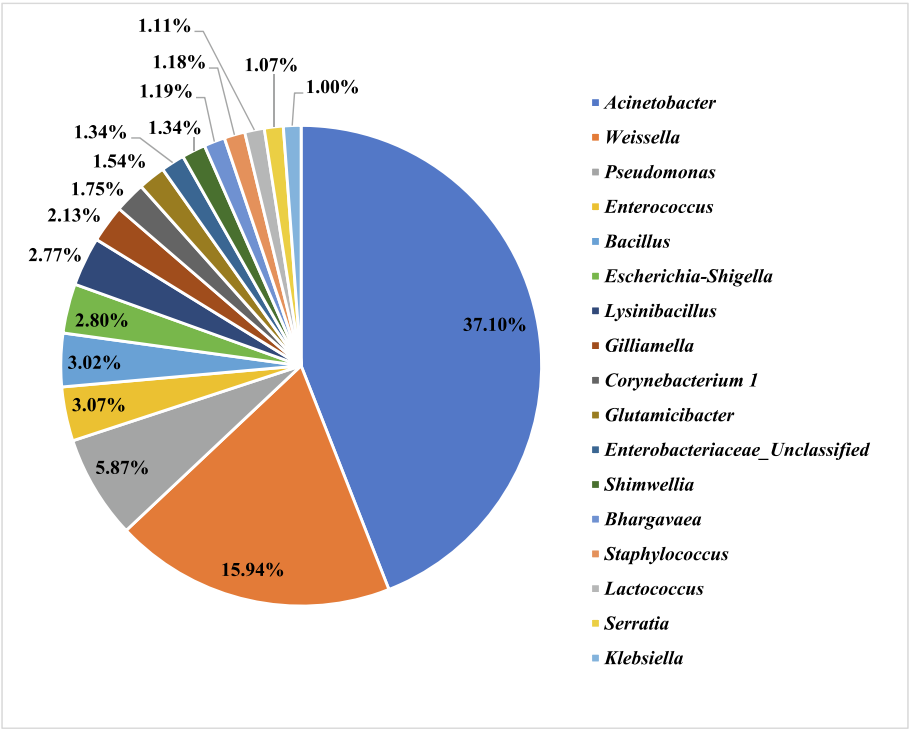

Fig. 3. A pie chart of the top microorganisms at genera from the four (4) of the most abundant bacteria phyla.

genera that had at least 1% occurrence are presented in Fig. 3. All these genera were from one of the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes.

Bacterial Communities Associated with P. rugaticeps Collected from the Administrative (BPF), Hospitals (DPH), and Residential (UR) Areas

At the phylum level, Proteobacteria had a relative percentage of 49.48% in BPF, 41.40% in DPH, and 81.54% in UR. Firmicutes was the second most abundant phylum with 35.12%, 50.72%, and 13.58% in BPF, DPH, and UR, respectively. The phylum, Actinobacteria had 9.32%, 6.31%, and 4.68% while Bacteroidetes had 4.59%, 0.98%, and 0.13% in BPF, DPH, and UR, respectively. The remaining phyla, having less than 1% in all the locations, were grouped as Others and had 1.49%, 0.60%, and 0.07% in BPF, DPH, and UR, respectively.

Gammaproteobacteria, being the most abundant class, had a relative frequency of 43.79% in BPF, 39.33% in DPH, and 81.11% in UR. This was followed by the class Bacilli with a relative abundance of 27.54% in BPF, 48.7% in DPH, and 13.15% in UR. The third most abundant class was Actinobacteria with a relative frequency of 8.52% in BPF, 6.15% in DPH, and 4.68% in UR. The relative frequency of the remaining bacterial classes identified from *P. rugaticeps* are summarized in Fig. 2.

Of the total of 165 genera, only *Acinetobacter* and *Enterococcus* were shared by all the three groups at a relative frequency of more than 1% (Fig. 4). *Acinetobacter* had the highest frequency in the ant samples collected from the administrative (15.01%) and residential areas (68.99%). In addition, *Acinetobacter* was the second most abundant genera in the sample from the hospital environment (27.29%). While the genus *Enterococcus* recorded relative frequencies of 3.03%, 2.32%, and 3.86% in BPF, DPH, and UR, respectively. *Weissella* (43.25%) had the highest percentage of the microbial community identified from the sample collected from the hospital environment. This was also recorded in the administrative area (BPF) (4.57%). Other genera, such as *Pseudomonas* (13.15% and 4.32%), *Escherichia-Shigella* (7.34% and 1.07%), *Lactococcus* (2.23% and 1.03%), and *Corynebacterium* 1 (2.88% and 2.36%), were shared only by the administrative (BPF) and hospitals (DPH) samples, respectively. *Glutamicibacter* was the only genus shared by BPF (1.52%) and UR (2.79%). All the other genera were unique to each location at greater than 1% relative frequency (Fig. 4).

Discussion

This study presents the microbiota of *Pheidole* ants (*P. rugaticeps*) collected from Administrative, Hospital, and Residential areas using Illumina MiSeq high-throughput sequencing of the bacterial 16S rDNA gene. A previous study investigated the influence of phylogeny, geographic locations, and a seed harvesting diet on the bacteria communities of this globally distributed ant genus, *Pheidole* (Martins and Moreau, 2020). The study revealed that geographic locations and diets could influence the bacterial community of *Pheidole* ants (Martins and Moreau, 2020). Several other studies investigated the microbial composition of ants and revealed diet, geography, phylogeny, and species as factors influencing the ant microbiome (Anderson et al., 2012; Kelly et al., 2019; Lanan et al., 2016; Martins and Moreau, 2020; Moreau and Rubin, 2017; Ramalho et al., 2017a, 2017b; Reeves et al., 2020; Sanders et al., 2014, 2017). Many bacteria pathogens were identified as associated with ants collected in hospital environments (Fowler et al., 1993; dos Lima et al., 2013; Máximo et al., 2014; Oliveira et al., 2017) and residential areas (Alharbi et al., 2019; Silva et al., 2014; Simothy et al., 2018).

From the findings of this study, Proteobacteria and Firmicutes were the most predominant phyla and this is consistent with previous studies conducted on *Pheidole* ants (Martins and Moreau, 2020), and the guts of other ants (Brown and Wernegreen, 2016; Li et al., 2012), the *P. americana* gut (Bertino-Grimaldi et al., 2013; Fang et al., 2013), as well as several other insects (Colman et al., 2012; Esposti and Romero, 2017; Jones et al., 2013; Lim and Ab Majid, 2020; Yun et al., 2014). Likewise, other findings on the microbiota of cadavers and associated insects also revealed Proteobacteria and Firmicutes as the two predominant phyla (Hyde et al., 2017; Pechal and Benbow, 2016). The predominance of this group of bacteria in *Pheidole*, ants and other insects suggests that they are an important part of an ant's microbiome.

At the genus level, the most prevalent bacteria was *Acinetobacter*, with a relative abundance of 37.10%, and this was previously recorded as the second highest genus in *Pheidole* ants (Martins and Moreau, 2020). The genus *Acinetobacter* has been reported to be widespread in arthropod microbiota (Esposti and Romero, 2017), in ants (Ishak et al., 2011; Meirelles et al., 2016), and in other insects (Brucker and Bordenstein, 2012; Hussin et al., 2018; Paulson et al., 2014). *Acinetobacter* is a highly diverse bacterial genus generally found in wet environments. The majority are nonpathogenic environmental organisms. However, *Acinetobacter baumannii* with a prevalence of 15.39% from the sample group collected in hospital environments, is a common species that causes infections, such as bacteremia and nosocomial infections (Wong et al., 2017). This antibiotic-resistant pathogen (Lopez-Otsoa et al., 2002; Cisneros and Rodríguez-Baño, 2002), has previously been isolated from ants collected in hospital (Moreira et al., 2005) and houses (Alharbi, 2019) using culture dependent techniques.

The genus *Weissella* was the second most prevalent genus with a 15.94% relative frequency, and was previously identified from ants (Chua et al., 2018; Fhoula et al., 2018); house flies (Park et al., 2019); pine weevils (Berasategui et al., 2016); from the gastrointestinal tract, vagina, and feces of humans (Fusco et al., 2015); other animal feces; soil; plants; and fermented plant-based foods (Bjorkroth et al., 2002; Fhoula et al., 2018; Lee et al., 2012; Lee, 2005). Some specific strains of *Weissella* have been studied for use as probiotics, for combatting diseases, like periodontal disease, and for potential prebiotic activity (Fusco et al., 2015). Conversely, species, such as *W. cibaria*, which is the most prevalent in the samples we collected in the hospital environments (14.17%) were previously recognized as a potential source of human opportunistic infections, like bacteremia and endocarditis (Fusco et al., 2015).

The genus *Pseudomonas* was another predominant genus identified with a relative abundance of 5.87%. Though the genus *Pseudomonas* was identified as a contaminant in early studies (Salter et al., 2014), it was

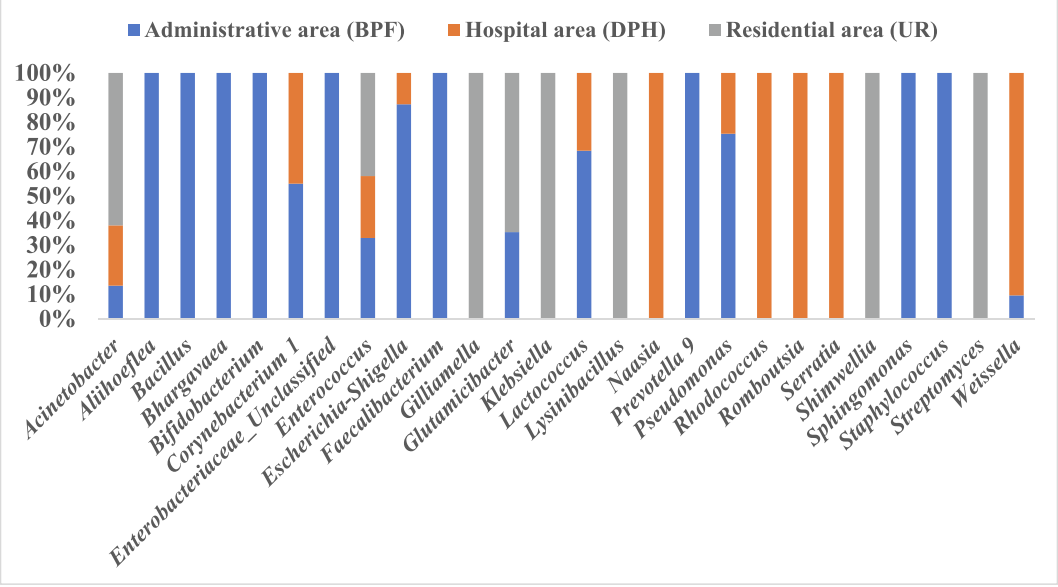

Fig. 4. Composition of bacterial genera (less than1%) in workers of *P. rugaticeps* from Administrative (BPF), Hospital (DPH) and Residential (UR) Areas.

identified as part of the microbiome in a recent study on *Pheidole* ants (Martins and Moreau, 2020) that involved negative control. A study on the microbiome of animal carcasses revealed *Pseudomonas* was the dominant bacterial community (Hyde et al., 2017). The study further hinted at the possibility of interaction between insects and cadaver microbiomes. The *Pheidole* ants used in this study were observed and collected scavenging on cadavers of other cockroaches and other insects. Previously, *Pseudomonas* were thoroughly represented as a highly abundant part of the community metagenomes of ants and beetles (Aylward et al., 2014). However, the presence or absence of *Pseudomonas* in the microbiome of ants, and in particular those that scavenge on other insects, will remain open to future studies that involve the use of the Decontam package (https://github.com/benjjneb/decontam) (Davis et al., 2018) to illuminate contaminants using a negative control.

Along with *Bacillus, Pseudomonas* was also reported from the guts of gypsy moth caterpillars with bacterial communities that are highly dependent on their diet (Broderick et al., 2004). *Bacillus* is another genus identified from the *Pheidole* samples with a relative frequency of 6%. The genus Bacillus has been isolated from dried human feces (Hoyles et al., 2012).

The genera *Escherichia-Shigella* and *Lysinibacillus* have relative frequencies of 2.80% and 2.77%, respectively. The genus *Escherichia-Shigella* was identified in a relatively high abundance in potato tuber moths (Zheng et al., 2020), in the feces of animals (Flannigan et al., 2018), and the feces of patients of kidney diseases (Hu et al., 2020). The genus *Escherichia-Shigella* was suggested to play a potential role as a biomarker for kidney illness (Hu et al., 2020). *Escherichia-Shigella* is correlated with classical renal damage markers (Hu et al., 2020). The genus *Lysinibacillus,* was also isolated from the guts of *Anopheles albimanus* larvae (Galeano-Castañeda et al., 2019) and dried human feces (Hoyles et al., 2012).

Other genera identified from the samples were *Staphylococcus, Enterococcus,* and *Gilliamella*. All these genera are commonly associated with insects, animals, and human surroundings. *Staphylococcus* and *Enterococcus* were recently reported in *Pheidole* ants (Martins and Moreau, 2020; Silva et al., 2014). *Enterococcus* together with *Gilliamella* were also identified in Apis mellifera (Engel et al., 2012), and they function in digestion and protection against parasites (Engel et al., 2012; Koch and Schmid-Hempel, 2011b; Martinson et al., 2011). *Enterococcus* is possibly an important part of the microbial community of *Pheidole* as it has been isolated from all ant samples collected from different locations in this study and previous studies (Martins and Moreau, 2020; Silva et al., 2014). Similarly, *Staphylococcus* has also been reported from the guts of *Acromyrmex echinatior* workers ants (Zhukova et al., 2017), house flies (Park et al., 2019) and termite spp. (Eutick et al., 1978).

Shimwellia is bacteria genus that had a relative frequency of 1.34%. This bacteria genus was previously isolated from the hind-gut of cockroaches (Brzuszkiewicz et al., 2012), as well as moths and beetles (Ignasiak and Maxwell, 2017). *Shimwellia blattae* DSM 4481 = NBRC is a bacterium that was first isolated from the hindgut of cockroaches and was also identified in our samples.

Though it is difficult to make conclusions from a sample size of three, the findings of this study and other related studies suggest that *Acinetobacter* and *Enterococcus* might be vital parts of the microbiota of *Pheidole*. This study has some limitations, which include the lack of a negative control and the absence of the Decontam platform to eliminate possible contaminants from the extraction kits and reagents. However, the study was conducted under sterile conditions and analyzed using the qiime2/dada2 platform, which reduced the impurities to the barest minimum. Therefore, studies with larger biological replicates and negative controls are recommended to complement our results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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