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Assessment of Mound Soils Bacterial Community of the Red Imported Fire Ant, *Solenopsis invicta* across Guangdong Province of China

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Abstract: Soil microbes have a wide range of distribution across the world and can be found in different agricultural and forest systems including cultivated soils, ant mounds, decaying trees, leaves, roots, and on insect bodies. Across five counties of Guangdong province of China, the assemblage of bacterial associates of red imported fire ant (RIFA) were examined. The locations were selected based on evidence of high presence of RIFA mounds in these regions. Samples were analyzed from mound soils, plant debris within mounds, and the ant body. The current study analyzed bacterial species composition and richness patterns, where 525 isolates were recovered in total, comprising 44 bacterial taxa. Taxa abundance was highest in the ant body at 35 taxa, while the values were relatively similar across soil substrate and plant debris, where 3 and 6 taxa, respectively, were recorded. The highest bacterial taxa recovery rate was recorded in Guangzhou, where a total of 17 taxa were isolated. *Myroides odoratimimus* was the most common across all substrates and locations among the bacterial taxa. Others with the highest isolation frequencies includes, *Enterobacter cloacae*, *Vagococcus fluvialis*, and *Myroides odoratus*. The understanding of the bacterial community composition of RIFA is crucial for the development of successful management techniques for these notorious social ants. In order to expand on the findings of the current study, it is imperative to understand if the associated microbial communities of the RIFA form a parasitic, antagonistic, or mutualistic relationship with their host. In this vein, further studies would examine the influence of the characterized bacterial associates of the RIFA on the social behavior, physiology, and the host response to foreign pathogens.

Keywords: cuticular symbionts; environmental protection; microbial communities; phylogenetic analysis; soil nutrition and fertility; soil organisms

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1. Introduction

Social insects have been demonstrated to harbor and exhibit close associations with diverse assemblages of bacterial microbes [1]. The bacterial communities associated with many insects possess the capability of reducing the effectiveness of a biological control system, as well as determining the success or failure of biological invasions [2]. Insects rely heavily on their bacterial symbionts for vital biological functions including fecundity, fitness, or survival [3]. Bacterial symbionts are able to support host insects' immune responses and xenobiotic detoxification, these physiological functions in turn directly affect various insect biological control mechanisms or any other strategies targeted at maintaining insect population thresholds [4,5].

The red imported fire ant (RIFA), *Solenopsis invicta* Buren (Hymenoptera: Formicidae), can serve as a model system to better understand the dynamics of invasive social ants and the techniques targeted for their control. The majority of the previous studies have aimed at isolating and characterizing RIFA symbionts have targeted the midgut microbes of the instar larvae, where important bacterial endosymbionts such as *Achromobacter*, *Bacillus*, *Enterobacter*, *Kluyvera*, *Listeria*, *Pseudomonas*, *Serratia* [6–8], *Enterococcus*, *Lactococcus*, and *Staphylococcus* [9], etc., have been isolated. However, it is important to also focus our interest on the external bacterial symbionts of RIFA. It is general knowledge that a baseline analysis of fire ant mound soils would reveal unique naturally occurring microbial associates of RIFA. It is therefore imperative to establish the specific roles played by the bacterial associates of the RIFA in relation to host survival, invasion, and other ecological functions.

It is generally unknown if the diversity and density of the mound soils-associated bacteria form a mutual, parasitic, or antagonistic relationships with the hosts [1,2]. Similar to the endophytic fungal species, isolated bacterial associates can also be explored as potential biological control agents for the management of RIFA and other insect pests [10,11]. The adoption of pathogens for potential suppression of insect populations and further research into other potential biological control strategies have been documented [11–13]. Several survey studies aimed at identifying microbial associates of RIFA, such as bacteria, fungi, or virus, are available [2,4,14–19]. However, there is a need to expand on the available data, as studies conducted in China are very limited.

The isolation, identification, and characterization of the RIFA bacterial communities would be important for future studies, evaluation, and trials for the successful management of the red imported fire ants. In lieu of this, the current study examined the diversity, richness, and densities of the culturable bacteria associated with the fire ants, mound soils, and plant debris within the mound soils collected from five counties located in Guangdong province of China.

2. Materials and Methods

2.1. Sampling Location

The sampling location includes five counties within Guangdong province of China: Dongguan, Guangzhou, Huizhou, Jiangmen, and Zhuhai (Figure 1). From each city, soil samples were collected from three randomly selected fire ant mounds, where about 500 g of mound soil was dug at approximately 10–15 cm below ground surface. For each sampling, a combination of soil and roots were collected with the help of a hand shovel that was disinfected with 70% ethanol between each sampling. Samples were transported to the laboratory in a zip-locked plastic bags, and soil samples that were not used immediately were stored at 4 °C.

2.2. Media Preparation

Isolation of bacterial isolates from insect bodies, plant debris, and soil samples was achieved using selective media method. Luria–Bertani (LB) solid media consisting of 5 g yeast extract, 10 g tryptone, 10 g sodium chloride, and 15 g of agar added to 1 L of distilled water, was used for bacteria isolation. In order to inhibit fungi growth, the LB media was supplemented with 50 mg/L nystatin (Sangon Biotech, Shanghai, China).

2.3. Procedure for Bacteria Isolation from Soil Samples

Soil samples were sieved using a 2 mm pore sieve to remove existing clumps and stones. For each sampling location/colony, a representative sample of 50 g soil was suspended in 500 mL of sterile distilled water contained in a 1 l flat-bottomed conical flask (Shuniu, Chengdu, China). Following the procedure of Dhar et al. [20], the flasks were vortexed on a rotary shaker (ZD-85, GTCS, Shanghai, China) at 200 rpm for about 30 min at room temperature in order to dislodge the existing microbial cells in the soil particles. Thereafter, soil particles were allowed to settle for 15 min, and 100 µL of a 10^{-3} dilution of the supernatant was evenly spread across solid media in Petri plates (90 mm) with a

sterile disposable cell spreader (Changde Bkmam Biotech, Changde, China). The plates were then incubated at 25 °C in a BOD incubator (BS-1E, China) for about 48–72 h, until bacterial growth was evident. This procedure was followed by multiple sub-culturing into new LB media plates until distinct monocultures were obtained for all isolates.

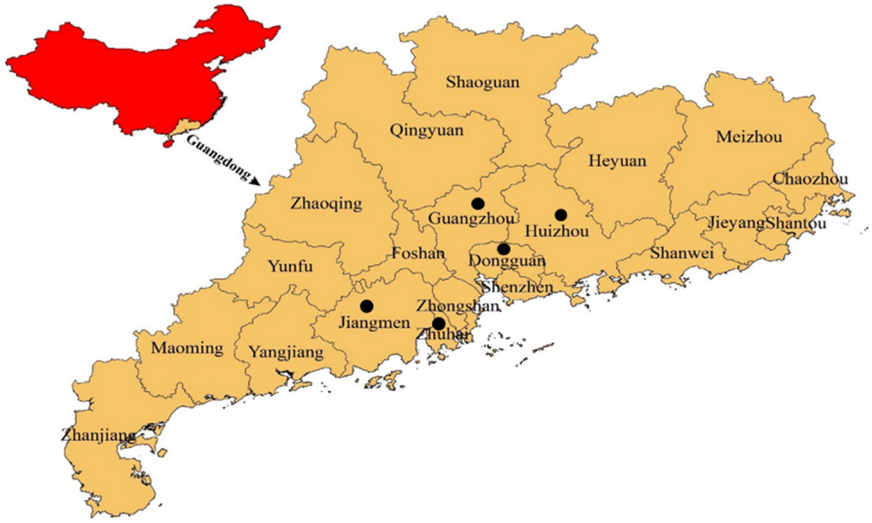

Figure 1. Diagrammatical representation of locations where mound soil samples were collected for baseline analysis of bacterial communities of RIFA. The excerpt map (in red) is showing the China map, while the main map is showing the map of Guangdong province, with the location sites highlighted with black spots.

2.4. Bacteria Isolation from Mound Plant Debris

Plant tissues contained in the soil samples were carefully removed and washed in sterile distilled water for about 1 min to remove existing soil clogs. Plant tissues were allowed to dry in a sterile laminar flow hood for about 2–3 min, and thereafter trimmed into smaller pieces of about 8–10 mm long, and plated in 9.0 cm Petri dishes containing freshly prepared LB media. For each colony, 24 plant tissues were plated, where 4 tissue pieces were plated per LB plate. Plates were supplemented with nystatin to minimize contamination, and incubated in similar conditions as previously described.

2.5. Bacteria Isolation from Ant Bodies

The guidelines of Parks et al. [21] were followed for bacteria isolation from ant bodies, with adaptation for RIFA in the current study. From each colony, a total of 12 ants were selected at random and transferred in pairs into a sterile 15 mL conical tube containing 1 mL sterile freshly prepared LB broth. The tubes were shaken by a vortex at 700× *g* for 10 s. Thereafter, three 10-fold serial dilutions were formed from the stock solution and 100 µL of the 10^{-3} dilution was evenly spread on LB media with a sterile disposable cell spreader under aerobic conditions. Plates were incubated following similar conditions as previously described. All emerging bacteria colonies were subsequently sub-cultured into freshly prepared LB media up to four weeks until a monoculture was obtained for all isolates.

2.6. Morphological Characterization

Morphological characterization was performed to identify different or distinct bacterial colonies by analyzing their color, size, shape, height, edge (margin), texture, etc. Thereafter, phenotypically distinct monocultures were immediately utilized for DNA extraction procedures or stored in 60% sterile glycerol (ratio 1:1) at −80 °C for further experimentation.

2.7. Molecular Identification

For DNA extraction, total genomic DNA was obtained from 4 day-old bacterial cultures using genomic DNA extraction kits provided by the manufacturer: TIANamp Bacteria

DNA kit, Tiangen, Beijing, China. The extracted DNA fragments were amplified using primers pairs (27F/1492R) targeting the full-length of the bacterial small subunit 16S rRNA and had the following sequences: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTAACTTGTTACGACT-3′).

PCR was performed in a 50 µL reaction mix containing 3.0 µL DNA template, 2.0 µL of each forward and reverse primers, 25.0 µL 2 × High Fidelity PCR MasterMix (Sangon Biotech, Shanghai, China) and 18.0 µL PCR-grade water. The procedures applied for DNA amplification include: 5 min of pre-denaturation at 95 °C, 30 cycles denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, followed by primer extension and final extension at 72 °C for 90 s and 5 min, respectively. Amplified DNA was visualized on 1.0% *m*/*v* agarose gel, and Sanger-sequencing was carried out by Sangon Biotech Co., Ltd., Guangzhou, China.

2.8. Sequence Alignments and Phylogenetic Analysis

The obtained bacterial sequence traces were manually edited using BioEdit v 7.1.9. [22]. BLASTn was used to retrieve reference sequences from the GenBank database of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/, accessed on 15 November 2022). Manual editing of sequences and alignment was performed using reference sequences obtained from GenBank. Phylogenetic analysis including the reference sequences and the sequences produced for the present study was performed using Neighbor-Joining method (NJ) analysis, based on Kimura 2-parameter model, via MEGA v. 11. The robustness of branches was estimated by bootstrap analysis with 1000 repeated samples from the data.

2.9. Statistical Analysis

Statistical analyses were carried out to determine taxa abundance, species richness (n), evenness (J′), diversity index (H′), and Coefficient of Community (CC), where the biodiversity indices of isolated bacterial samples from various substrates were based on the isolation frequencies. Consequently, bacterial species diversity was computed using Shannon–Wiener index (H′), which was calculated as follows: $H' = \sum PiInPi$, $Pi = Ni/Nt$, where Ni represents the number of isolates that belong to the i-th genus and Nt is the total number of isolates per location or environmental samples examined. Whereas, the Coefficient of Community (CC) was calculated as: $CC = C/(S\_1 + S\_2 - C)$, where C is the total number of species common in both communities under study, i.e., community 1 and 2, while S_1 and S_2 represent the total number of species in community 1 and 2, respectively.

Where applicable, the relative frequency values were subjected to one-way analysis of variance (ANOVA) to detect the differences among substrates and sampling locations, as appropriate. Multiple comparison among treatment means was performed using the Least Significance Difference (LSD) test at $p < 0.05$. All the statistical analyses were performed using IBM SPSS statistical software v22.0 (SPSS Inc., Chicago, IL, USA) and Statistix 8.1 (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Morphological Characterization of Isolated Bacterial Strains

All bacterial isolates were subjected to multiple sub-culturing until monocultures were cultivated (Supplementary Figure S1). Thereafter, morphological characterization based on the evidence of their phenotypic distinctiveness was carried out.

3.2. Molecular Identification and Phylogenetic Placement of Isolates

Molecular characterization involving primer pairs targeting regions that are specific for almost all bacterial 16S sequences was performed. Here, using 27F + 1492R primer pairs, approximately 1500 bp from the bacterial 16S rRNA gene was amplified. In addition, phylogenetic analysis of the sequence data further assisted in the successful classification

of isolates into taxa. In total, 27 bacterial taxa were identified in addition to 17 that were not identified (Table 1).

Table 1. List of bacterial taxa characterized from mound soils, plant debris within mound, and ant body across five counties of Guangdong province, China.

Source of Isolation	Taxa (Isolate Number)	Location of Isolation	GenBank Accession No.
Ant body	*Vagococcus fluvialis* D121	Dongguan	OP811464
Ant body	*Vagococcus fluvialis* D141	Dongguan	-
Ant body	D142-Unidentified bacterium sp.	Dongguan	-
Ant body	D181-Unidentified bacterium sp.	Dongguan	-
Ant body	D232-Unidentified bacterium sp.	Dongguan	-
Ant body	*Myroides odoratimimus* H231	Huizhou	OP811465
Ant body	*Lactococcus taiwanensis* H182	Huizhou	-
Ant body	*Myroides odoratus* H191	Huizhou	-
Ant body	H242-Unidentified bacterium sp.	Huizhou	-
Ant body	*Pseudomonas monteilii* G911	Guangzhou	OP811474
Ant body	*Lactococcus garvieae* G1012	Guangzhou	OP811476
Ant body	*Myroides odoratimimus* G2112	Guangzhou	OP811479
Ant body	*Pseudomonas mosselii* G2212	Guangzhou	OP811480
Ant body	*Acinetobacter calcoaceticus* G812	Guangzhou	-
Ant body	*Myroides marinus* G1113	Guangzhou	-
Ant body	G441-Unidentified bacterium sp.	Guangzhou	-
Ant body	G512-Unidentified bacterium sp.	Guangzhou	-
Ant body	G632-Unidentified bacterium sp.	Guangzhou	-
Ant body	G811-Unidentified bacterium sp.	Guangzhou	-
Ant body	G912-Unidentified bacterium sp.	Guangzhou	-
Ant body	G921-Unidentified bacterium sp.	Guangzhou	-
Ant body	G2111-Unidentified bacterium sp.	Guangzhou	-
Ant body	*Myroides odoratus* J4312	Jiangmen	OP811481
Ant body	*Enterobacter asburiae* J7312	Jiangmen	OP811482
Ant body	*Enterobacter cloacae* J7322	Jiangmen	OP811483
Ant body	*Pantoea dispersa* Z241	Zhuhai	OP811466
Ant body	*Acidovorax radicis* Z331	Zhuhai	OP811468
Ant body	*Enterococcus saccharolyticus* Z412	Zhuhai	OP811469
Ant body	*Myroides odoratus* Z421	Zhuhai	OP811470
Ant body	*Myroides odoratus* Z442	Zhuhai	OP811471
Ant body	*Providencia rettgeri* Z511	Zhuhai	OP811472
Ant body	*Enterobacter cloacae* Z721	Zhuhai	OP811473
Ant body	*Enterobacter cloacae* Z631	Zhuhai	-
Ant body	Z311-Unidentified bacterium sp.	Zhuhai	-
Ant body	Z332-Unidentified bacterium sp.	Zhuhai	-
Mound soil	*Enterobacter cloacae* G922	Guangzhou	OP811475
Mound soil	*Myroides odoratimimus* G1612	Guangzhou	OP811478
Mound soil	Z411-Unidentified bacterium sp.	Zhuhai	-
Plant debris	*Vagococcus fluvialis* G1322	Guangzhou	OP811477
Plant debris	G722-Unidentified bacterium sp.	Guangzhou	-
Plant debris	*Lysinibacillus fusiformis* J8321	Jiangmen	OP811484
Plant debris	J1011-Unidentified bacterium sp.	Jiangmen	-
Plant debris	*Myroides odoratimimus* Z312	Zhuhai	OP811467
Plant debris	Z422-Unidentified bacterium sp.	Zhuhai	-

For phylogenetic characterization, supplementary sequences available in the database of NCBI were obtained and analyzed together with the bacterial sequence data obtained in the current study using the partial sequences of the nuclear protein-encoding gene (Figure 2).

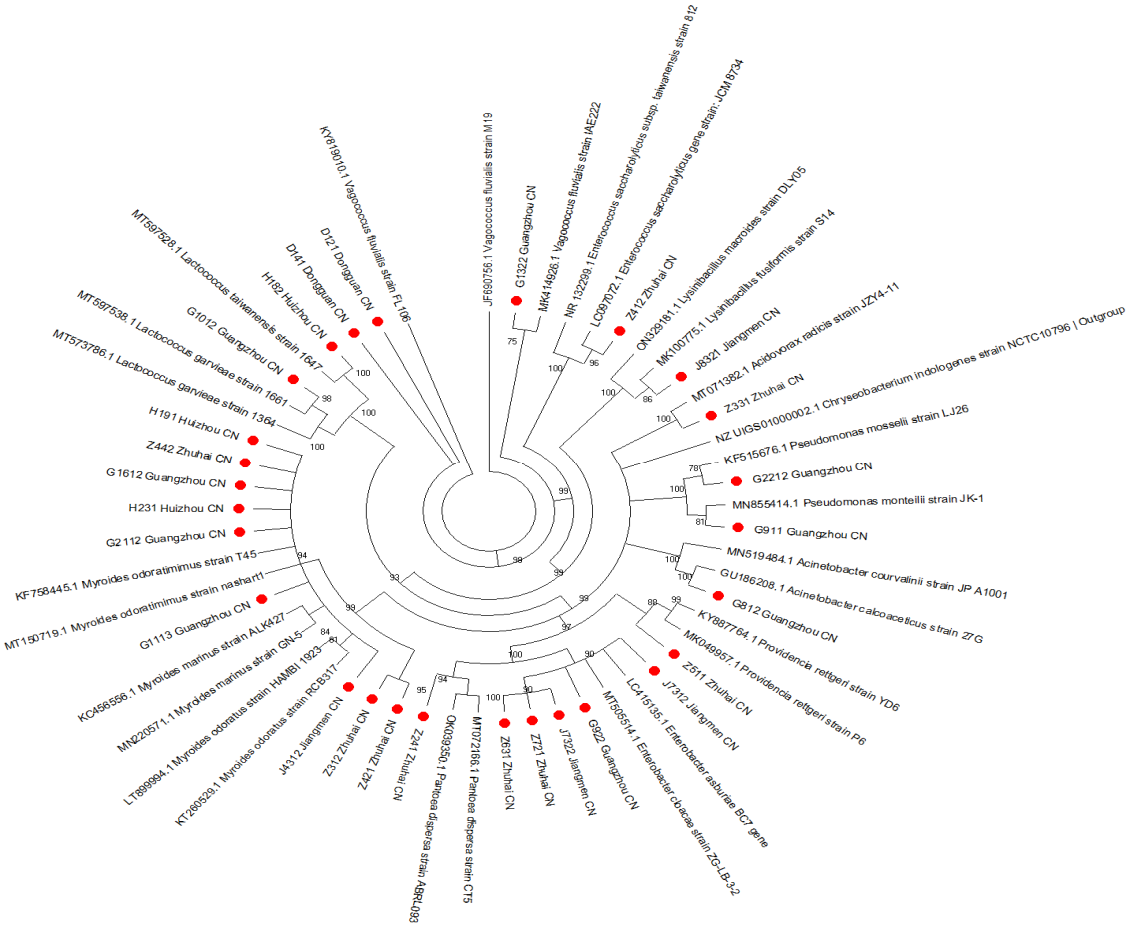

Figure 2. Phylogenetic analysis based on the alignment of 16S rRNA partial sequences of the characterized bacterial isolates. The evolutionary history was inferred using the Neighbor-Joining method, and the distances were computed using the Kimura 2-parameter method. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 3336 positions in the final dataset. The bacterial taxa used in this study are highlighted with red dots.

3.3. Analysis of the Diversity, Richness, and Densities of the Culturable Bacterial Species

For the analysis of the diversity, richness, and densities of the culturable bacterial species, isolates were sub-classed based on substrates: mound soils, plant debris within mound, and ant body; as well as the five isolation sites: Dongguan, Huizhou, Guangzhou, Zhuhai, and Jiangmen. Overall, isolation frequencies among taxa ranged from 1.3% to 12.6%, while 29.1% were unidentified. The taxa with the highest percent isolation across various cities and substrates was *M. odoratimimus* (12.6%). In addition, *E. cloacae* (9.5%), *V. fluvialis* (8.4%), *M. odoratus* (8.2%), *M. marinus* (5.7%), and *P. dispersa* (3.4%), were the other five taxa with the highest values. The identified taxa were unevenly distributed across substrates (Figure 3), where 79.5% was recorded in the ant body, 6.8% across the soil samples, and 13.6% was isolated from the plant debris within the mound.

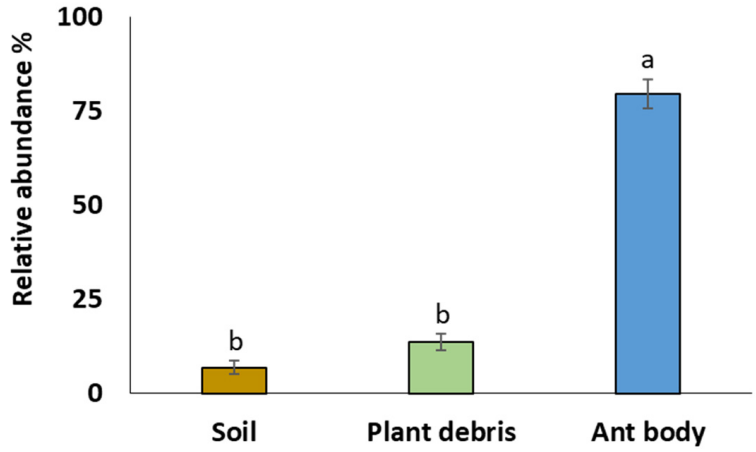

Figure 3. Distribution of bacterial taxa across substrates: mound soils, plant debris within mound, and fire ant body. Bars (±SE) with different letters indicate significant differences at $p < 0.05$ (LSD after One-way ANOVA).

Among the bacterial taxa, *M. odoratus* at 10.5% and *M. odoratimimus* at 35.4% were the dominant species from the ant body and plant debris within mound soils, respectively, while *E. cloacae* at 47.4% was the most common in the soil samples. Following the analysis of environmental samples and sampling locations, the results show that the highest taxa abundance (total isolations) and species richness values were from the ant body (35 taxa, 15 species) and Guangzhou (17 taxa, 8 species), across substrates and locations, respectively. On the other hand, the lowest bacterial taxa abundance was recorded in Huizhou samples at 4 taxa, while the lowest species richness was from the samples collected from Dongguan, where only *V. fluvialis* was recovered. For the substrates, the lowest species richness at 2, and abundance values at 3, were recorded in the mound soils (Figure 4; Table 2). Among the species, only *M. odoratimimus* was isolated across all substrates, where 28.9%, 35.4%, and 6.6% isolation was recorded from soil, plant debris, and ant body, respectively. In contrast, aside *Lysinibacillus fusiformis*, all other species were successfully recovered from the ant body.

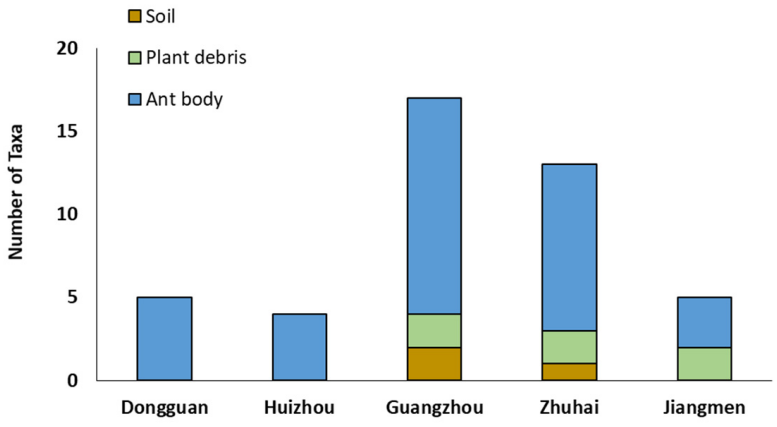

Figure 4. Culturable bacterial taxa extracted from mound soils, plant debris within mounds, and fire ant body across various counties.

Table 2. Bacterial abundance, richness, evenness, and diversity index across various substrates and counties.

Location	Dongguan 11.4 (1)	Huizhou 9.1 (3)	Guangzhou 38.6 (8)	Zhuhai 29.5 (7)	Jiangmen 11.4 (4)	H′ 1.44	J′ 0.89
Substrates	Soil 6.8 (2)	Plant debris 13.6 (3)	Ant body 79.5 (15)			H′ 0.64	J′ 0.58

Data presented under each location or substrate refer to the total number (percentage) of unique taxa, while the numbers in parentheses represent the species richness (S) values. H′ and J′ represent Shannon Diversity Index and evenness of species, across location or substrates, respectively.

Analysis of species diversity using Shannon's index of diversity revealed the overall species diversity across locations was 1.44 (H′) and 0.64 (H′) across substrates. Similarly, species evenness was 0.89 (J′) and 0.58 (J′) across locations and substrates, respectively (Table 2). The CC values calculated based on data obtained from different locations and substrates ranged from 0.12 to 0.25 for ant body–mound soil–plant debris across all five locations. The Coefficient of Community value was higher when comparing ant body to plant debris, while the values were relatively similar among ant body versus mound soils, and plant debris versus mound soil (Table 3). For most locations, Dongguan, Huizhou and Jiangmen, bacterial species recorded across different substrates were dissimilar, hence CC = 0.0; while for Guangzhou and Zhuhai CC values ranged from 0.17 to 0.5 and 0.14, respectively (Table 4).

Table 3. Coefficient of Community (CC) of all bacterial taxa isolated from mound soils, plant debris, and ant body across five counties of Guangdong province, China.

Substrates	CC
Ant body–Mound soil	0.13
Ant body–Plant debris	0.25
Plant debris–Mound soil	0.12

Table 4. Coefficient of Community (CC) of all bacterial taxa isolated from mound soils, plant debris, and ant body within individual counties of Guangdong province, China.

Location	Substrates	CC
Dongguan	Ant body–Mound soil	-
	Ant body–Plant debris	-
	Plant debris–Mound soil	-
Guangzhou	Ant body–Mound soil	0.17
	Ant body–Plant debris	0.33
	Plant debris–Mound soil	0.5
Huizhou	Ant body–Mound soil	-
	Ant body–Plant debris	-
	Plant debris–Mound soil	-
Zhuhai	Ant body–Mound soil	0.14
	Ant body–Plant debris	0.14
	Plant debris–Mound soil	-
Jiangmen	Ant body–Mound soil	-
	Ant body–Plant debris	-
	Plant debris–Mound soil	-

(-) was ascribed for locations where there was no bacterial species in common among the two substrates examined, i.e., CC was not calculated.

4. Discussion

Social insects' association with microbes evolve in various forms, ranging from mutualism, commensalism, antagonistic, parasitic, or pathogenic [2]. It has been found that microbial organisms can vary from beneficial symbionts to pathogens within a vast microbial community existing in a given host or environment [23,24]. Understanding the specific roles played by the microbial associates of the ant communities is relevant for identifying potential pathogens of RIFA, and this has been the motivation for conducting most baseline analysis studies. Characterization of microbial communities associated with RIFA in some of these studies heavily relied on culture-dependent techniques. These studies are nevertheless limited in scope, as the true bacterial diversity are underestimated by the culture-dependent survey trials. A more suitable approach as demonstrated in the current study, similar with other related studies, is no doubt the application of high-throughput sequencing technology that provides the ability to process the massively parallel reads as required [25,26]. The technology is applied in addition to culture-based isolation, and other morphological characterization techniques. In order to conduct a more comprehensive sampling of RIFA-associated bacteria, a procedure involving field collection of environmental samples supported with 454 pyrosequencing of PCR products of the 16S rRNA gene was proposed [27]. This approach would generally increase the measure of relative bacterial abundance, and the classification of unculturable bacteria species [2].

In the current study, bacterial associates of *S. invicta* were characterized using the 16S rRNA gene. This 16S rRNA region is conserved enough for primer design, whereas it is variable enough for identification and was documented to favor the species of bacteria that are difficult or impossible to culture or clone [28,29]. In total, 44 bacterial taxa were obtained, comprising 27 characterized taxa, and 17 additional taxa that are yet to be identified. The most commonly isolated bacterial taxa across locations and substrates include, *M. odoratimimus*, *E. cloacae*, *V. fluvialis*, *M. odoratus*, *M. marinus*, and *P. dispersa*. The majority of the taxa characterized are similar to the documented taxa in previous studies: for instance, *Enterococcus*, *Pantoea*, *Pseudomonas*, and *Vagococcus* in Ishak et al. [2]; *Enterobacter* in Enagbonma and Babalola [30]; *Acinetobacter* and *Lactococcus* in Powell et al. [4]. In contrast, none of the bacterial taxa obtained in this study was reported in a related study conducted by Baird et al. [14]. The study surveyed the bacterial and fungal communities within fire ant mounds across four counties in Mississippi, USA, where the most common bacterial species were *Actinomadura yumaensis*, *Arcanobacterium haemolyticum*, *Chryseobacterium indolegenes*, and *Stenotrophomonas maltophilia*. In a related study by Woolfolk et al. [18], the most common taxa include *Achromobacter xylosoxidans*, *Bacillus cereus*, *Lysininibacillus boronitolerans*, *Lysinibacillus sphaericus*, *Pseudomonas protegens*, and *Serratia liquefaciens*. Moreover, none of these taxa were obtained in the current study.

The bacterial taxa were unevenly distributed across substrates and locations. The highest species richness and abundance values across locations and substrates were recorded in the samples collected from Guangzhou and the ant body, respectively. This outcome contradicts the findings of the aforementioned studies of Baird et al. [14] and Woolfolk et al. [18], where species richness and diversity were highest in the mound soils in comparison to the ant body and plant debris within mound. A number of biotic and abiotic factors are capable of influencing the diversity of microorganisms in the soil. For instance, in a study conducted by Enagbonma et al. [31], the high nutrient concentrations recorded in termite mound soils was believed to be responsible for the observed complex diversity of microorganisms in mound soils. The authors observed similarities within mound soils and the surrounding soils in respect of the obtained bacterial taxa. However, none of the reported taxa was successfully isolated in the current study. Environmental factors such as precipitation and temperature can as well-affect the diversity and densities of microorganisms [14]. Another determinant is the competition with other existing soil-inhabiting microbes, including fungi.

Overall, the current study, similar with few previous studies, demonstrated the fire ant mound soils as a potential reservoir for beneficial bacterial associates of the RIFA. Nevertheless, some other related studies argued that the internal tissues (midguts) are the

more likely sites for bacteria isolation rather than the ant external tissues, plant debris, or the mound soils [9,32,33]. This explains why the majority of the previous studies have been bias and narrowly targeted the midgut microbes of the instar larvae of RIFA [6–9,34]. The diversity, richness, and abundance of the bacterial communities existing on the outside or within the habitat cannot be overlooked, hence, it is important that we also focus our attention on the external bacterial symbionts of RIFA. Although the specific roles played by these bacterial symbionts in regulating insect populations, and in other ecological functions remain unclear. Notably, the ability of some ant-associated bacterial strains to induce resistance against dicistrovirus, a single-stranded RNA virus with a promising prospect for use as biological control agent against RIFA, was documented [4]. Similarly, a bacterial symbiont of *Drosophila melanogaster*, *Wolbachia pipientis*, have been reported to induce resistance of the host against *Drosophila* C virus [3]. Aside offering protection to their insect hosts against foreign pathogens, others biological functions have been proposed for bacterial associates of social insects, including serving as survival, fitness, fecundity, foraging, and other social behavior mediators [2–4,14]. Notably, some of the bacteria characterized in this study, for instance, *Acinetobacter* sp., *Enterococcus* sp., *Enterobacter* sp., etc., have been reported to mediate several of the aforementioned functions [2,7,9].

5. Conclusions

Some aspects of bacterial symbionts acquisition and transmission efficiency in social ants, the RIFA specifically, are yet undefined. It is generally unknown whether, or to what extent, the already existing or indigenous microbial associates deter the successful colonization or growth of the newly acquired symbionts. It is noteworthy that there are some available reports on hosts' responses to artificial introduction of microbes in many social insects, although the documented responses significantly varies and is often complex across different insects. To this end, the specific roles of the bacterial associates of RIFA identified in this study and the mechanisms underlying the beneficial effects have not been explored at the current stage of the study. It is imperative that future studies should be targeted towards unveiling the importance of bacterial symbionts of RIFA in mediating host survival, tolerance to foreign pathogens, and other physiological functions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15021350/s1. The data supporting the results are included in this article as supporting information—Supplementary Figure S1.

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