Ecology, Behavior and Bionomics

Diversity and implication of symbiotic bacteria in aphids - ants relationships in Madagascar

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Abstract

Facultative bacteria in aphids provide their hosts with various physiological and ecological adaptations, such as resistance to thermal stress, parasitoids, and entomopathogenic fungi. Furthermore, these symbionts possess the capacity to modulate the composition of honeydew, a substance that is particularly favored by numerous ant species. That's why we were interested in determining whether the presence of facultative bacteria in myrmecophilic aphids influences their relationship with mutualistic ants. In the vicinity of Antananarivo (Madagascar), the objectives of the study were to (i) determine the diversity of ants, aphids and symbionts and, to (ii) assess the impacts of the identified symbiont on behavioral interactions of ants and aphids. We identified a total of four species (*Serratia symbiotica, Rickettsia, Spiroplasma, Hamiltonella defensa*) of facultative symbionts present in the three ant species (*Camponotus maculate, Monomorium madecassum, Nylanderia gracilis*) we tested and a total of six facultative symbionts (*Serratia symbiotica, Rickettsia, Spiroplasma, Hamiltonella defensa, Regiella insecticola, Rickettsiella*) among three of the five aphid species (*Aphis citricidus, Aphis fabae, Aphis spiraecola, Macrosiphum euphorbiae, Rhopalosiphum maidis*) we tested. Although our results did not show the involvement of symbionts on ant behavior or their association with aphids, our study showed that the number of

ants increases with the number of interactions, that *Nylanderia gracilis* are the ants with the most interactions, and finally that ants observed on pesticide-treated plots have fewer interactions with aphids.

Keywords: Multitrophic interaction, Entomology, Africa, Mutualism.

Introduction

With around 5,000 described species, aphids represent a significant group of insects (Foottit et al., 2008). Aphids are known to have a close relationship with ants, with approximately 30% of aphid species being obligate myrmecophiles, and another 30% being facultative myrmecophiles (Buckley, 1987; Stadler, 1997). By providing ants with a sugary liquid called honeydew, aphids receive protection and numerous ecological benefits from the worker ants (Way, 1963). Ants not only defend aphids against attacks from various predators such as hoverflies, ladybugs and parasitoids (El-Ziady & Kennedy, 1956), but they also clean aphids of pathogenic fungi (Rice & Eubanks, 2013; Nielsen et al., 2009), remove sick and dead aphids (Rice & Eubanks, 2013; Flatt & Weisser, 2000) and enhance aphid reproduction rates through the release of pheromones such as Z,E-α-farnesene (Flatt & Weisser, 2000; Xu et al., 2021). However, ants can regulate aphid populations by predation or by displaying aggression towards them to encourage increased honeydew production (Sakata, 1994; Schwartzberg et al., 2010).

Scientific studies revealed a crucial link between ants and aphids, emphasizing the symbiotic bacteria in aphids as a key element. Aphids rely on obligatory symbiotic bacteria, such as *Buchnera aphidicola* and, in rare instances, *Serratia symbiotica*, for their survival. (Oliver et al., 2010; Shigenobu et al., 2000; Braendle et al., 2003). However, the origin of symbiotic bacteria acquisition in aphids is not yet established. Some studies have suggested that this transfer can occur through their host plants or through certain parasites such as mites or parasitoid wasps (Gehrer & Vorburger, 2012; Henry et al., 2015). The bacterium *S. symbiotica* is common in ants, and its presence in free form in honeydew has led some authors to speculate that the transmission of symbiont could occur when ants collect honeydew from aphids (Pons et al., 2019, 2022; Renoz et al., 2019). Aphids also host facultative bacteria (*Serratia symbiotica, Hamiltonella defensa, Rickettsia* ...) that confer various ecological adaptations such as resistance to heat shock (Guo et al., 2017; Tsuchida et al., 2010). Some bacteria, such as *S. symbiotica*, can provide resistance to parasitoids. *Regiella insecticola* also offers protection to aphids against entomopathogenic fungi (Chen et al., 2000; Scarborough et al., 2005; Oliver et al., 2010; Guo et al., 2022). Certain bacteria associated with aphids, like *Staphylococcus sciuri*, also have the ability to alter the attraction of honeydew and related volatile organic compunds (VOCs) (Leroy et al, 2011; Fischer et al., 2015, 2017). The differences between the VOCs allows ants to discriminate between different aphid species over long distances. Arguments suggesting that facultative bacteria have the capacity to alter the behavior of ants towards aphids is that these symbionts have the ability to modify the amino acid composition of honeydew (Sabri et al., 2013; Schillewaert et al., 2017).

In this study, we aimed to determine the prevalence of different symbiotic bacteria in ants and in aphids and to investigate whether facultative symbiotic bacteria in aphids influence their association with ants.

Materials and methods

Experimental design

As described in Luttenschlager et al. (2024), we carried out our study in agricultural plots of at least one hectare on the outskirts of the city of Antananarivo in Madagascar: Amboanjobe (-19.021942, 47.536976), Ankatso (-18.911917, 47.557158), Ambohijafy (-18.923381, 47.409676), Ambohimanambola (-18.959919, 47.598944), Ambohipeno (-18.904464, 47.568790). For a plot to be selected as a study plot, it had to contain at least three plants of the same species (*Phaseolus vulgaris* L., 1753; *Citrus limon* (L.) Burm. f., 1768; *Solanum nigrum* L., 1753; *Zea mays* L., 1753) on which myrmecophilous aphids frequented by ants were present.

During 10-minute intervals, ant behaviors were recorded towards aphids using behavior grids inspired by the literature (Endo & Itino, 2012; Sakata et al., 2017). In details, behaviour observations were recorded as factor variable which influence ant behaviour in aggressive (biting or carrying aphids / bending abdomen / opening mandibles) or non-aggressive manner (touching aphid / honeydew). These observations covered the entire plant for small plants like green beans. For larger plants such as lemon trees, black nightshades and corn, ant behaviors were observed on single branches. When presented with multiple branches containing aphids and ants, the ones with the highest numbers of individuals were selected to maximize data collection. Each of the three plants per plot was observed three times.

In addition to behavioral data, covariates were recorded. For pesticide use, we asked the plot owner whether the plot was treated. Temperature was recorded at the time of each observation. To define sunlight exposure, we noted whether the sky was clear or overcast during the different observations. Finally, when a hoverfly, ladybug, or parasitoid wasp was spotted near the aphids, we considered a predator to be present during that observation.

Collection and identification of organisms

After behavioral observations, ants and aphids from each plant were harvested and stored in separate 2ml Eppendorf tubes containing 70 % ethanol. The ants were morphologically identified by specialists from the

California Academy of Sciences laboratory in Antananarivo. Regarding the aphids, following the protocol outlined by Luttenschlager et al. (2024), they were identified through the sequencing of the mitochondrial gene encoding COI (Cytochrome Oxidase I) using the Sanger technique. We used Serial Cloner 2.6.1 to align the sequences. The aligned nucleotide sequences were compared with those of other insects in the NCBI Blast database. We selected the first species with 100% similarity for the COI gene.

Symbiont DNA extraction and identification by PCR

For DNA extractions, the QIAamp PowerFecal Pro DNA kit (Reference: 51804) (QIAGEN, Hilden, Germany) was used. For symbiont DNA extraction, aphids were pooled in groups of 20 due to their low weight, whereas only a single ant was selected. Afterward the primers LCO (5'- GGTCAACAAATCATAAAGATATTGG -3') and HCO (5'- TAAACTTCAGGGTGACCAAAAAATCA -3') were selected to amplify the mitochondrial gene encoding COI (Folmer et al., 1994; Wilson et al., 2022).

We decided to investigate the presence of six facultative symbionts of aphids that have been known for many years and have been the subject of numerous studies: *Spiroplasma sp, Regiella insecticola, Hamiltonella defensa, Rickettsiella sp, Serratia symbiotica, Rickettsia sp*. To identify the presence of six symbionts of interest, PCR were conducted using specific primers for each symbiont (Table 1).

Table 1 : Table of primers used to identify various facultative symbionts

Primer name	5' - 3' sequence of the primer	Primer specifiity	Reference
16SA1	AGAGTTTGATCMTGGCTCAG	Generic	(Fukatsu & Nikoh, 1998)
Spi1500R	ATCATCAACCCTGCCTTTGG	*Spiroplasma sp*	(Peccoud et al., 2014)
PAUS16R	TCGGACGCCATAACACTAGG	*Regiella insecticola*	(Peccoud et al., 2014)
PABS480R	GGTATTCGCATTTATCGCTTC	*Hamiltonella defensa*	(Peccoud et al., 2014)
P136Ric-470R	TGGGTACCGTCACAGTAATCGA	*Rickettsiella sp*	(Tsuchida et al., 2010)
P136F	GGGCCTTGCGCTCTAGGT	*Rickettsiella sp*	(Tsuchida et al., 2010)
PASS1140R	TTTGAGTTCCCGACTTTATCG	*Serratia symbiotica*	(Peccoud et al., 2014)
Ric600R	TTTGAAAGCAATTCCGAGGT	*Rickettsia sp*	(Peccoud et al., 2014)

For each sample, a 13.5-microliter mix was prepared and supplemented with 1.5 microliters of DNA or sterile water for the negative control (Table 2). The DNA from the positive controls comes from various strains of the aphid *Acyrthosiphon pisum* (Harris, 1776). These strains, known to possess the different symbionts of interest, originated from the Laboratory of Functional and Evolutionary Entomology at Gembloux Agro-Bio Tech (University of Liège).

Table 2 : Composition of a PCR mix to amplify sequences specific to each facultative symbiont.

Products	**Quantity**
One Taq Quick Load Reaction Buffer 5x (New England Biolabs Inc, Massachusetts, USA)	3 µl
Deoxynucleoside Triphosphates (dNTP) (New England Biolabs Inc, Massachusetts, USA)	0,3 µl
Primer Forward	0,3 µl
Primer Reverse	0,3 µl
Sterilized demineralized water	9,525 µl
One Taq Polymerase (New England Biolabs Inc, Massachusetts, USA)	0,075 µl
Total	**13,5 µl**

A 2-hour program used with the iCycler thermocycler (BioRad, California, USA) was as follow: activation of Taq Polymerase and DNA denaturation at 94 °C for 5 minutes before the start of the cycles. Each of the 38 cycles included DNA denaturation at 94 °C for 30 seconds, primer annealing at 58 °C for 30 seconds, and strand extension at 72 °C for 1 minute. A final extension phase at 72 °C for 5 minutes followed the cycles, after which the temperature was reduced to 4°C until the samples were retrieved. Electrophoresis was then performed in a gel containing 1 % agarose and SYBER Safe DNA Gel Stain (Reference: S33102) (Thermo Fisher Scientific, Massachusetts, USA) at 110V for 45 minutes. Gel visualization was performed using a Molecular Imager Gel Doc XR system (BioRad, California, USA). We assessed the presence of the symbiont by a positive revelation of the DNA amplicon in the electrophoresis gel.

Statistical Analysis

Statistical analyses were performed using RStudio Version 1.4.1103 (©2009-2021 RStudio, PBC). We opted for Generalized Linear Mixed Models (GLMM) to explore and deeply analyze the complex relationships and interactions within our data. This approach allows us to capture the joint variations between variables and account for both random and fixed effects. To determine which of our variables influence ant behaviors, whether aggressive or non-aggressive towards aphids, a GLMM model of the negative binomial family (nbinom) was used with the glmmTMB R package (Brooks et al., 2017). The day and the observer were specified as random effects (1| day), (1| observer). The selected predictors were specified as fixed effects as a function of the aphid species, the ant species, the host plant species, the number of aphids, the number of ants, the order of which plant was observed, the presence/absence of symbiont, the plot type (garden or agricultural plot), the pesticide usage or not, the presence of predators, the temperature, the time of day, the and sunlight. The selection of explanatory variables were selected based on the Akaike information criterion.

Results

Diversity of facultative symbionts

The presence of four facultative symbionts was identified in the ant *Nylanderia gracilis* (Forel, 1892), three in the ant *Monomorium madecassum* Forel, 1892 and none of the six symbionts tested were found in the ant *Camponotus maculatus* (Fabricius, 1782). The presence of the six symbionts tested was identified in the aphid *Aphis fabae* Scopoli, 1763, two in the aphid *Aphis citricidus* (Kirkaldy, 1907) and just one in the aphid *Aphis spiraecola* Patch, 1914. Finally, none of the six symbionts tested were found in the aphids *Macrosiphum euphorbiae* (Thomas, 1878) and *Rhopalosiphum maidis* (Fitch, 1856) (Table 3).

Table 3 : Diversity of bacterial symbionts in aphids and ants. The green dots correspond to present symbiont while grey dots correspond to undetected symbiont.

		Serratia. symbiotica	*Rickettsia sp*	*Regiella. insecticola*	*Ricketsiella*	*Spiroplasma sp*	*Hamiltonella. defensa*
Formicidae	*Camponotus maculatus*	absent	absent	absent	absent	absent	absent
	Monomorium madecassum	present	present	absent	absent	absent	present
	Nylanderia gracilis	present	present	absent	absent	present	present
Aphididae	*Aphis citricidus*	present	absent	absent	absent	absent	present
	Aphis fabae	present	present	present	present	present	present
	Aphis spiraecola	present	absent	absent	absent	absent	absent
	Macrosiphum euphorbiae	absent	absent	absent	absent	absent	absent
	Rhopalosiphum maidis	absent	absent	absent	absent	absent	absent

Legend : (grey dot) = absent (green dot) = present

The prevalence of symbionts among the tested aphid species was dominated by *S. symbiotica* (60% of occurrence). The pea aphid, *A. fabae*, presented all the tested symbionts, while none were found in *M. euphorbia* and *R. maidis*. In aphid species with symbionts, *S. symbiotica* was always identified beside other symbionts, except in *A. spiraecola*.

Influence of symbionts on ant behavior

Based on the Akaike information criterion, ant species, the number of ants, and the use of pesticides in the study plot were selected as explanatory variables that influence non-aggressive behaviors of ants towards aphids. Non-aggressive behaviors (touching aphids and collecting honeydew) increase with the number of ants p-value < 0,01. This result is clearly illustrated by a positive trend on the prediction graph, where we observe a progressive increase in non-aggressive behavior as a function of ant density (Figure 1).

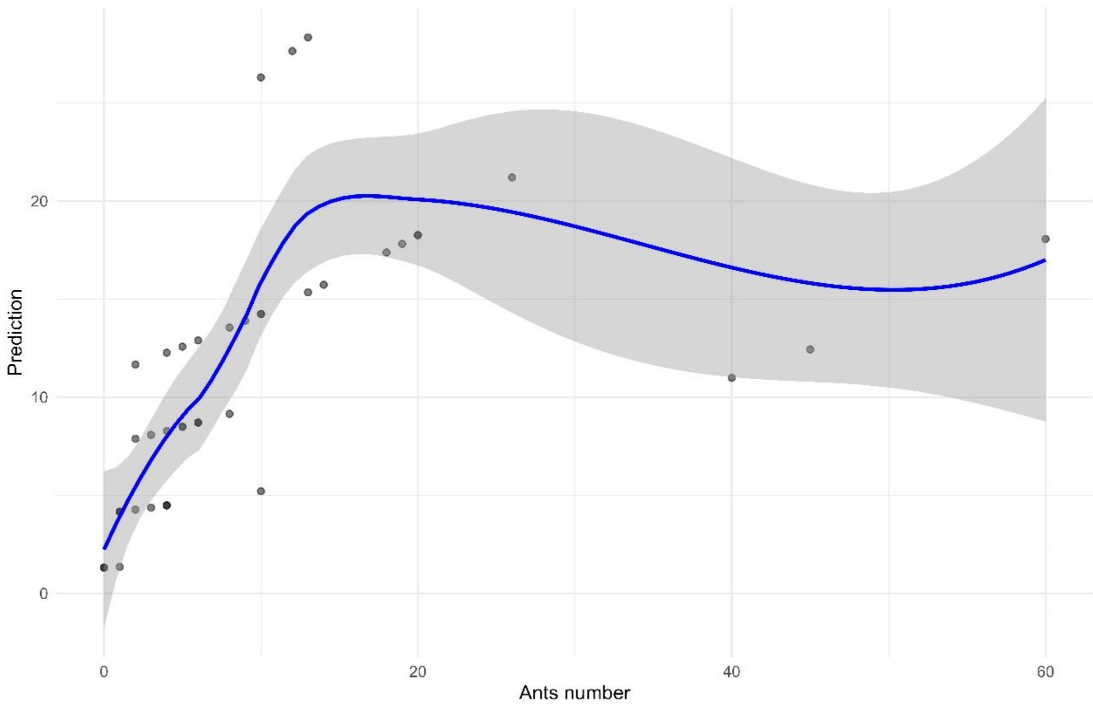

Figure 1 : Impact of ant number on non-aggressive interactions with aphids.

Additionally, the ant species *N. gracilis* displays more non-aggressive behaviors compared to *C. maculatus* and *M. madecassum* (p-value < 0,001) (Figure 2).

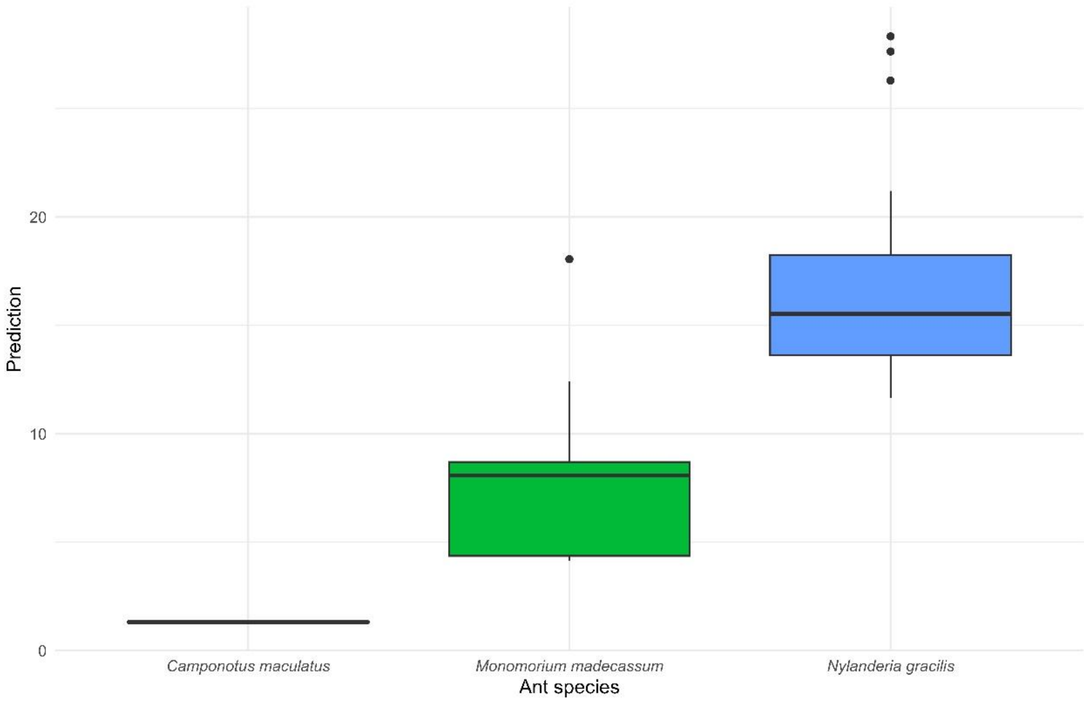

Figure 2 : Variation in non-aggressive behaviors across ant species.

Finally, the use of pesticides in study plots reduced the number of non-aggressive behaviors of ants towards aphids (p-value < 0,01) (Figure 3).

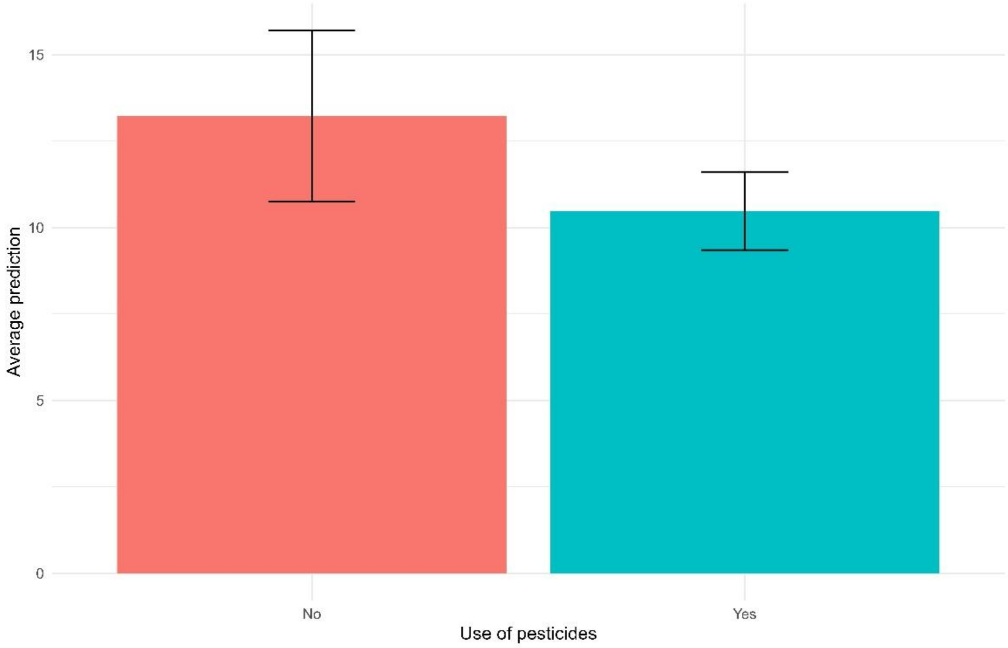

Figure 3 : Impact of pesticide use on non-aggressive interactions with aphids.

Discussion

In our study, we aimed to explore the diversity of facultative symbiotic bacteria in aphids and ants associated on the outskirts of Antananarivo. The presence of four symbiont species (*S. symbiotica, Rickettsia, Spiroplasma* and *H. defensa*) was identified in three mutualistic ant species (*C. maculatus, M. madecassum, N. gracilis*), which is the first report for ants originating from Madagascar. Furthermore, this study is the first to have highlighted the presence of *Spiroplasma sp, R. insecticola, H. defensa, Rickettsiella sp, S. symbiotica, and Rickettsia sp* in aphids from Madagascar. Since we did not test the insects individually, we cannot definitively determine cohabitation of symbionts within the same organism. However, within a single aphid's colony, we found up to four different facultative symbionts. Nevertheless, some uncertainty remains about the cohabitation of multiple aphid species on the same plant. Our identifications were based on COI identification through Sanger sequencing, which implied that we obtained a single sequence even in the case of cohabitation among multiple organisms. Then, two assumptions can be proposed. The first is that multiple aphid species coexist on the same plant (Dugravot et al., 2007). However, we were careful to pool aphids of the same species for our analyses. Alternatively, facultative bacteria may not be evenly distributed within the same aphid population (Brady et al., 2014). We ruled out a third assumption that all four symbiont species coexist within the same aphid because it is rare for facultative symbionts to cohabit in more than two species in the same host (Koga et al., 2003; Tsuchida et al., 2002). In cases of symbiont cohabitation in the same aphid colony, *S. symbiotica* was consistently present. This might be explained by the fact that symbiont was the most prevalent in our study. The literature suggested that *S. symbiotica* was not often associated with other symbionts especially within the same host *Acyrthosiphon pisum* (Harris, 1776) especially for *H. defensa* and *R. insecticola* (Zytynska, 2019). Comparing the prevalence of symbionts in our study to literature prevalence (Oliver et al., 2010; Zheng et al., 2022) indicated some differences. For example, we detected *S. symbiotica* in 40% of cases and *H. defensa* in 30% of cases, whereas in the article by Oliver et al., (2010), these prevalences are 15% and 14%, respectively. These findings support those of Henry et al. (2015) who suggested that the presence of ants can influence the distribution of facultative symbiont populations in aphids.

This pioneering study firstly investigates the impact of facultative symbiotic bacteria on the ecological associations between aphids and ants in Madagascar, concurrently assessing the influence of aphid symbionts on ant behaviors, encompassing both aggressive and non-aggressive responses. However, no significant effect of symbiont presence on ant behaviour was observed. We observed a behaviour that could modify our understanding of ant attraction to aphids, or at least to their honeydew. Some *M. madecassum* individuals, instead of directly collecting honeydew from the aphid abdomen, move to leaves beneath aphid groups to gather honeydew that has fallen on the leaf surfaces. It would be interesting, in future experiments, to consider this behaviour when characterizing interactions between ants and aphids. This study was conducted in an uncontrolled natural environment, we cannot guarantee that the observed insects exclusively interact with each other, excluding potential associations with other aphids and ants. Several authors have suggested that ants could be a pathway for the transmission of facultative bacteria from aphids (Pons et al., 2019, 2022; Renoz et al., 2019). It would be interesting to investigate in a controlled environment whether ants or parasitoids can transmit their symbionts or serve as intermediate hosts in the transmission of symbionts between two aphid populations (Gehrer & Vorburger, 2012). This would help in gaining a better understanding of the various transmission sources, although some are already known: via the host plant, through stings or bites by parasitoids and parasites, respectively. Nevertheless, three parameters that influence non-attack behaviours of ants towards aphids were identified. Our results showed that the number of non-attack behaviours increased with the number of ants, that the ant *N. gracilis* exhibited more non-attack behaviours, and finally, the use of pesticides in the plot negatively affected ant non-attack behaviours. The work of Lapolla et al., 2011, describing *Nylanderia* ants as fast and efficient foragers with the ability to quickly recruit many workers, could explain why we observed increased interactions of *N. gracilis* with aphids. Even though some studies have focused on the effect of pesticides on the foraging behavior of insects (Kwon 2010; Haynes, 1988), it would be interesting to study their effect on the mutualistic behavior between aphids and ants.

Conclusion

This study identified which populations of symbiotic bacteria can be found among different species of mutualistic ants and myrmecophilous aphids around Antananarivo, Madagascar. Additionally, this study highlighted that certain factors, such as the ant species, the number of ants, and the use of pesticides on the host plant, influence the interactions between ants and aphids. The presence of certain symbiont did not influence the ant behavior on aphids in the field.

Conflict of interest

The authors declare no competing interests.

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