Sociobiology
An international journal on social insects

RESEARCH ARTICLE - ANTS

Bacterial Communities in the Midgut of Ponerine Ants (Hymenoptera: Formicidae: Ponerinae)

TB de Oliveira[2], M Ferro[2], M Bacci Júnior[2], DJ de Souza[3], R Fontana[1], JHC Delabie[4], A Silva[1]

1 - Universidade Estadual de Santa Cruz (UESC), Ilhéus-BA, Brazil
2 - Universidade Estadual Paulista (UNESP), Rio Claro-SP, Brazil
3 - Universidade Federal do Tocantins (UFT), Gurupi-TO, Brazil
4 - UESC/Laboratório de Mirmecologia, CEPLAC, Ilhéus-BA, Brazil

Article History

Edited by
Evandro Nascimento Silva, UEFS, Brazil
Received 04 August 2015
Initial acceptance 06 March 2016
Final acceptance 11 March 2016
Publication date 29 April 2016

Keywords
16S rRNA, *Dinoponera, Odontomachus, Pachycondyla*, symbionts.

Corresponding author
Aline Silva
Universidade Estadual de Santa Cruz
Departamento de Ciências Biológicas
Laboratório de Microbiologia
Rodovia Jorge Amado, Km 16, Salobrinho
CEP 45662-900, Ilhéus-BA, Brazil
E-Mail: asilva1@uesc.br

Abstract

Symbiotic microorganisms are directly related to the ecological success of host insects, influencing many aspects of their biology. The present study is the first to investigate the microbiota associated with ants of the subfamily Ponerinae and aims to identify the bacterial midgut communities of *Dinoponera lucida, Neoponera curvinodis*, *Pachycondyla striata*, *Odontomachus brunneus* and *Odontomachus bauri* relied on culture-dependent technique, particularly 16S rRNA sequencing. The greatest species richness was observed in *O. bauri*, with 15 OTUs, followed by *D. lucida* with five OTUs, *O. brunneus*, with four OTUs, and *N. curvinodis* and *P. striata*, both with three OTUs. There were representatives of the phyla Actinobacteria, Proteobacteria, Tenericutes and Firmicutes, including the genera *Bartonella, Mesoplasma*, *Mesorhizobium, Spiroplasma*, *Wolbachia* and *Serratia* in the guts of the studied Ponerine ants. *Spiroplasma* and *Mesoplasma* were found to be prevalent in the studied ants and they were the only genera of bacteria found in more than one of the analyzed ant species suggesting they might be beneficial symbionts. The low microbial diversity observed given the predatory trophic habits of the species studied suggests that there is selection for these microorganisms, predominantly preserving symbionts with functional roles that are able to colonize this environment. It is also valid to infer that the identified bacteria are predominant in the gut and exhibit mutualistic functions that are important mainly for immunity, but also to reproduction and nutrition; moreover, a subset may be parasites that could have considerable impacts on the studied ants.

Introduction

Insects act as hosts for microorganisms, with which they share a wide range of interactions. The orders Blattaria, Hymenoptera, Coleoptera and Hemiptera are typically involved in symbiotic relationships with microorganisms (Boursaux-Eude & Gross, 2000). Among the symbionts of these species, bacteria have received the most attention because they determine the ecological success of the insect host, influencing insect development and the immune response (Shoemaker et al., 2000), reproduction (Giorgini et al., 2010), behavior (Dillon et al., 2002) and particularly nutrition (Eilmus & Heil, 2009; Feldhaar et al., 2007; Jaenike et al., 2010; van Borm et al., 2002).

The gut is an environment with a high incidence of symbiotic partnerships. There are reports of the existence of endosymbionts throughout the gastrointestinal tracts of insects (Dillon & Dillon, 2004; Dunn & Stabb, 2005). The bacterial diversity in these environments is related to the pH, redox potential, digestive enzymes in the gut and type of food, among other factors (Dillon & Dillon, 2004).

Social insects, such as ants (Hymenoptera: Formicidae), are interesting models for studies involving symbiotic relationships with microorganisms and for studies on

coevolution. However, little is currently known about these interactions. In the tribe Camponotini, the omnivorous ants of the genus *Camponotus* have established an association with the intracellular bacteria *Blochmannia* and it has been a fundamental partner by improving colony growth and the host immune system (Souza et al., 2009). The congruence in the topology of bacteria and ant phylogeny suggests it has initiated in a common ancestral followed by the coevolution between the partners (Sameshima et al., 1999; Sauer et al., 2000). Moreover, genomic analysis of *Blochmannia* from host divergent lineages has showed differential loss of genes that affect cellular functions and metabolic pathways and this variation have been linked to distinct host-associated pressures (Williams and Wernegreen, 2015).

It was also observed the symbiosis between *Tetraponera* ants and nitrogen-fixing root-nodule bacteria which might be related to recycling nitrogen-rich metabolic waste (Borm et al., 2002). Nitrogen-fixing bacteria were also observed in a great diversity of ants and it is has been linked with the evolution of herbivory in ants (Russel et al., 2009). In this sense, many researches have attempted to characterize microbial communities associated to ants and their putative role to improve host fitness (Eilmus & Heil, 2009; Ishak et al., 2011; Anderson et al., 2012; He et al., 2014).

In the subfamily Ponerinae, although the existence of bacteria in the midgut of *Odontomachus bauri* Emery 1892 has been reported using an ultrastructural approach (Caetano et al., 2008; Caetano et al., 2010), the identity of these microorganisms remains to be determined. Although these ants exhibit plesiomorphic behavioral and morphological characteristics, such as very small colonies, reduced fertility rates and a poor capacity for dispersal and colonizing new areas, they present a wide geographical distribution (Bolton, 2003; Martins et al., 2007; Werren et al., 2008), which could be related to the presence of symbiotic microorganisms.

As a first step toward understanding the mutual benefits of symbiotic microorganisms in these ants, the purpose of the present study was to describe the bacterial communities in the midguts of the ants *Dinoponera lucida* (currently included in the red list of endangered species in Brazil (Ministério do Meio Ambiente, 2003), *Neoponera curvinodis*, *Pachycondyla striata*, *Odontomachus brunneus*, *Odontomachus bauri* and to discuss the putative functional roles of bacteria-host interactions.

Materials and Methods

Biological material and collection

Individuals of the Ponerine species *D. lucida*, *N. curvinodis, P. striata, O. brunneus* and *O. bauri*. For each species, approximately 15 individuals were collected from a single nest. The individuals collected were processed immediately to avoid the possible alteration of their intestinal microbiota. The collected specimens were identified and deposited in the collection of the Myrmecology Laboratory of the Cocoa Research Center (Centro de Pesquisas do Cacau – CEPEC) under accession number # 5676.

DNA extraction and amplification of 16S rRNA

A total of 8 to 13 individuals of each species were used to increase the amount of DNA extracted. The ants were anesthetized by freezing at -20°C for 2 min and then immersed in 70% ethanol, followed by washing in 0.9% NaCl for external disinfection. The dissection was conducted under a dissecting microscope, with the materials placed on a glass slide containing a drop of 0.9% NaCl. Then, the midgut was washed three times in 0.9% NaCl, and DNA extraction was performed using standard proteinase K digestion in TNES buffer (250 mM Tris, 2 M NaCl, 100 mM EDTA and 2.5% SDS, pH 7.5) and incubated in 1.5 ml tubes for 3 h at 55°C with 5 µL of proteinase K (20 mg/mL); then incubated at 37°C for 30 min after addition of 3 µL of RNAse A (4 mg/mL). Proteins were precipitated with 200 µL of 5-molar NaCl and centrifugation; DNA was precipitated from the supernatant using 600 µL of isopropanol and washing with 70% ethanol. Samples were re-suspended with 30 µL of TE (10 mM Tris, pH 8; 1 mM EDTA pH 8) and extraction was confirmed in 1% agarose gel.

For *N. curvinodis,* the amplification of the bacterial 16S rRNA region was conducted via PCR, which was performed using the PuReTaq Ready-To-Go PCR Beads kit (GE Healthcare). Each reaction contained 1.0 µL of DNA (~ 30 ng) and 5 pmol of each of the universal bacterial primers 27F (5′-AGAGTTTGATCA/CTGGCTCAG) and 1492R (5′-TACGGT/CTACCTTGTTACGACTT) (Lane, 1991) in a final reaction volume of 25 mL. For all of the other species, the reactions consisted of 3 µL of 1X buffer ($(NH_4)_2SO_4$), 2.4 µL of $MgCl_2$ (2 mM), 1.5 µL of dNTPs (0.2 mM), 0.3 µL of each primer (10 pmol), 1 µL of DNA (~ 30 ng) and 0.2 µL of Taq polymerase (1 U) (Fermentas) in a final reaction volume of 30 µL. The PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 3 min for *N. curvinodis*; there was an additional final extension at 72°C for 10 min for the other species. The primers amplified a region of approximately 1.5 kb. The PCR products obtained from *N. curvinodis* were purified using the GFX PCR Gel Band and DNA Purification Kit (GE Healthcare). For the other species, the purification was performed using a Nucleic Acid and Protein Purification kit (Macherey-Nagel).

Cloning and sequencing

Purified PCR products from *P. curvinodis* were ligated into pJET vectors using the Clone JET PCR Cloning Kit (Fermentas). Competent *E. coli* DH10β cells were transformed via heat shock. Subsequently, 100 µL of cells was inoculated into Petri dishes containing LB agar and ampicillin (20 µg/mL), then incubated overnight at 37°C. For all of the other species, the purified PCR products were ligated into pGEM vectors using

the pGEM-T Easy Vector Systems kit (Promega). Competent *E. coli* JM109 cells were transformed via thermal shock. Subsequently, 100 µL of cells was inoculated into Petri dishes containing LB agar, ampicillin (20 µg/mL) and X-Gal (45 µL/plate) and incubated overnight at 37°C. The obtained recombinant colonies were transferred to 96-well plates containing LB agar (1 mL) and ampicillin (20 µg/mL) and grown at 37°C for approximately 22 h. The extraction of the plasmid DNA was performed following the protocol described by Vettore et al. (2001). The results of the extraction were visualized on 2% agarose gels, and only those clones that contained an insert of the expected size were used in the subsequent stages. The sequencing reactions were performed on microplates using the ABI BigDye Terminator Cycle Sequencing kit (version 3.1), 0.5 to 1 µg of DNA and 5 pmol of the pJET1.2*_forward* primer (5'-CGACTCACTATAGGGAGAGCGGC) for *N. curvinodis* and the M13*_forward* primer (5'-GTTTTCCCAGTCACGAC) for the other species. After purification, the samples were run in an ABI 3500 automated sequencer (Applied Biosystems).

Analysis of 16S rRNA sequences

The obtained 16S rRNA sequences were pre-processed using the EGene automated pipeline generation system (Durham et al., 2005). At this stage, the sequences were screened for primer and vector sequences (pJET1.2 and M13), which were then removed. The base quality was also checked, with 90% considered to be the threshold for good bases within a window (Phred value > 20), using a window size of 200 bp. Subsequently, the sequences were filtered based on a minimum size of 200 bp and aligned using the ClustalW tool (Thompson et al., 1994), followed by manual refinement. Using the distance matrix generated in DNAdist, the sequences were assigned to operational taxonomic units (OTUs) using MOTHUR (version 1.8.0) (Sanchez-Contretas & Vlisidou, 2008). The obtained frequency data were used to construct rarefaction curves and to calculate the Chao1 richness estimator and the Shannon and Simpson diversity indices. The sequences were assigned to phylogenetic classes using the Classifier tool of the Ribosomal Database Project (RDP) (Xie et al., 2010) and compared with related sequences in the GenBank database using the BLASTN tool (http://www.ncbi.nlm.nih.gov/genbank/index.html).

Results

After pre-processing, 603 high-quality sequences were obtained; 129 were from *D. lucida*, 76 from *P. curvinodis*, 159 from *P. striata*, 125 from *O. brunneus* and 116 from *O. bauri*. These sequences were classified into known bacterial phyla using RDPClassifier from the Ribosomal Database Project public database. Using the 97% similarity criterion, the sequences were grouped into OTUs, and a representative sequence for each OTU was compared with sequences deposited in GenBank.

The following phyla were detected: Proteobacteria (in *D. lucida, N. curvinodis*, *P. striata* and *O. bauri*), Tenericutes (in *D. lucida, N. curvinodis*, *P. striata* and *O. brunneus*), Actinobacteria (in *N. curvinodis* and *O. bauri*) and Firmicutes (in *O. bauri*) (Tables 1, 2 and 3).

Table 1. The phylogenetic grouping of the 16S rRNA sequences from the midguts of the ant *Dinoponera lucida.*

Phylum	Sequence database match (organism; % identity; GenBank accession No.)	Accession No.	OTU	No. of clones
Tenericutes	*Spiroplasma velocicrescens*; 98%; NR_025713	KM503191	1	45
	Spiroplasma diminutum; 96%; CP005076	KM503192	2	72
Proteobacteria	*Mesorhizobium* sp.; 97%; FJ827045	KM503193	3	2
	Uncultured *Mesorhizobium;* 97%; DQ303307.1	KM503194	4	2
	Uncultured *Bartonella*; 97%; DQ113413	KM503195	5	8

Table 2. The phylogenetic grouping of the 16S rRNA sequences from the midguts of the ants of the genus *Neoponera* and *Pachycondyla.*

Ant Species	Phylum	Sequence database match (organism; % identity; GenBank accession No.)	Accession No.	OTU	No. of clones
Neoponera curvinodis	Proteobacteria	*Wolbachia pipientis*; AY026912; 98%	JQ957016	1	35
	Actinobacteria	*Micrococcus* sp.; JN602241; 98%	JQ957017	2	1
	Tenericutes	*Mesoplasma* sp.; GQ275130; 96%	JQ957018	3	40
Pachycondyla striata	Proteobacteria	*Bartonella vinsonii*; EU295657.1; 98%	JQ957014	1	1
	Tenericutes	*Spiroplasma velocicrescens*; NR025713.1; 97%	JQ957013	2	69
		Spiroplasma sp.; AY189317.1; 96%	JQ957015	3	89

Table 3. The phylogenetic grouping of the 16S rRNA sequences from the midguts of the ants of the genus *Odontomachus.*

Ant Species	Bacterial Phylum	Sequence database match (organism; % identity; GenBank accession No.)	Accession No.	OTU	No. of clones
Odontomachus brunneus	Tenericutes	*Spiroplasma leucomae*; AB681166.1; 99%	JQ957019	1	43
		Uncultured *Mesoplasma*; GQ275130.1; 97%	JQ957020	2	22
		Spiroplasma atrichopogonis; AB681165.1; 99%	JQ957021	3	42
		Uncultured *Mesoplasma*; HM996788.1; 98%	JQ957022	4	18
Odontomachus bauri	Actinobacteria	*Brevibacterium paucivorans*; EU086796; 99%	JQ957026	1	1
		Propionibacterium acnes; JF277163.1; 99%	JQ957033	2	2
		Uncultured *Propionibacterium* sp.; JF893681.1; 99%	JQ957027	3	1
		Leifsonia xyli; DQ232616; 99%	JQ957028	4	1
		Microbacterium aurum; GU441767.1; 98%	JQ957031	5	1
		Schumannella luteola; NR041637.1; 95%	JQ957034	6	1
		Rubrobacter xylanophilus; CP000386.1; 99%	JQ957035	7	1
	Firmicutes	*Bacillus thuringiensis*; JQ004436.1; 99%	JQ957025	8	1
		Dolosigranulum pigrum; NR026098; 98%	JQ957037	9	2
	Proteobacteria	*Pseudochrobactrum kiredjianiae*; NR042519.1; 99%	JQ957024	10	1
		Agrobacterium larrymoorei; EU741094.1; 99%	JQ957036	11	1
		Serratia marcescens; AJ550467; 98%	JQ957023	12	55
		Aeromonas veronii; JF920563.1; 100%	JQ957029	13	1
		Enterobacter cancerogenus; JN644583.1; 98%	JQ957030	14	1
		Serratia marcescens; AB681729.1; 98%	JQ957032	15	46

In *D. lucida* were detected five OTUs, in which were detected two species of the genera *Spiroplasma* (Table 1). In *N. curvinodis,* three OTUs were found, with a predominance of the genera *Wolbachia* and *Mesoplasma*. Three OTUs were also detected in *P. striata*, in which two *Spiroplasma* species were predominant (Table 2). In these three species of ants, OTUs were found that showed less than 97% similarity with homologous GenBank sequences, indicating that they potentially represented new species of the genera *Spiroplasma* and *Mesoplasma*.

Four OTUs were detected in *O. brunneus* and 15 in *O. bauri* (Table 3). Bacteria of the *Mesoplasma* and *Spiroplasma* genera were found in *O. brunneus*, whereas in *O. bauri*, there was a predominance of species from the genus *Serratia* as well as a large number of OTUs represented by single or only a few sequences from other genera.

Rarefaction analysis showed no significant differences in the richness of the bacterial communities present in *D. lucida, N. curvinodis, O. brunneus* and *P. striata* whereas *O. bauri* showed a significantly greater richness compared with the other species (Figure 1). The curves reached plateaus at 3% difference, except for *O. bauri*, between sequences (95% confidence), indicating that the number of clones was sufficient to cover the diversity of bacteria in the midgut of these ants. In addition, according to the Chao1 values, the highest species richness is expected in the bacterial community in *O. bauri*,

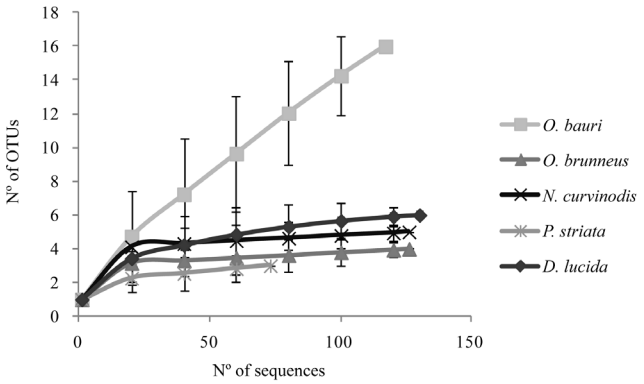

Fig 1. Rarefaction analyses of the *Dinoponera lucida, Neoponera curvinodis, Pachycondyla striata, Odontomachus bauri* and *O. brunneus* midgut 16S rRNA clone libraries. The predicted numbers of OTU's were calculated at the 3% level of sequence divergence.

followed by *D. lucida* and *O. brunneus*, and there was no difference in the bacterial species richness between the two *Pachycondyla* and *Neoponera* (Table 4).

In terms of diversity, according to the Shannon index, the *Odontomachus* and *Dinoponera* samples differed significantly from the *Pachycondyla* samples. However, using the Simpson index, differences were detected both between genera and between the *Odontomachus* species studied, but there was no significant difference when compared with *Dinoponera*.

Table 4. The richness of OTUs (*S*), estimated richness of OTUs and diversity indices for the 16S rRNA clone libraries.

Sample	*S*	Richness estimate	Diversity indices	
		Chao	Shannon	Simpson
D. lucida	5	6 (±0)	1.03 (±0.15)^{a}	0.43 (±0.15)^{a,b,c}
O. bauri	15	33 (±13.86)	1.31 (±0.23)^{a}	0.37 (±0.05)^{b}
O. brunneus	4	4 (±0)	1.33 (±0.06)^{a}	0.27 (±0.03)^{a}
P. striata	3	3 (±0)	0.72 (±0.06)^{b}	0.51 (±0.02)^{c}
N. curvinodis	3	3 (±0)	0.76 (±0.01)^{b}	0.51 (±0.03)^{c}

The values that are followed by different letters are significantly different (95% confidence interval).

Discussion

Studies on the occurrence and functional role of bacterial symbionts in ants are still very scarce, making it difficult to understand how these bacteria affect their hosts or how host diet and intestinal physiology and structure affect the bacterial community.

A predominance of species from the genera *Mesoplasma*, *Spiroplasma*, *Wolbachia* and *Serratia* was observed in the guts of the Ponerine ants studied. *Mesoplasma* and *Spiroplasma* are genera of intracellular bacteria from the order Entomoplasmatales (phylum Tenericutes; class Mollicutes) that are commonly found in the guts of insects and are known to be present in a number of ant species (Funaro et al., 2011; Ishak et al., 2011; Sapountzis et al., 2015). However, previous studies (Funaro et al., 2011) have found that these bacteria are not always present in different subcastes within a single ant colony or different colonies from the same population, indicating that these microorganisms are not essential for the development of the host species. Nevertheless, in certain cases, there is specificity between associated bacteria and ant species. Sapountzis et al. (2015) suggest that their function might be related to the processing of chitin, the main component of the cuticles of insect prey.

In the present study, *Spiroplasma* and *Mesoplasma* were found to be prevalent in the studied ants and they were the only genera of bacteria found in more than one of the ant species analyzed, and more than one species from the same genus was present in several cases, indicating that ants of this tribe is a natural reservoir of this group of microorganisms. It was observed that in the ant species in which both of these genera occurred (*N. curvinodi*s, *P. striata* and *O. brunneus*), the diversity of other phyla was reduced. In the presence of these bacteria, there is likely a mechanism for the inhibition of other prokaryotes.

Spiroplasma has been reported to be responsible for causing the death of males originating from infected females (Anbutsu & Fukatsu, 2003; Hurst & Jiggins, 2000). This effect distorts the sex ratio, increasing the number of females in the population (Wang et al., 2007). On the other hand, *Spiroplasma* was reported as a mutualistic symbiont in *Drosophila* associated with an increased tolerance against infection by nematodes and parasitic attack. Moreover, no other symbiont bacteria was detected, and possibly this is the only symbiont responsible for defense against parasites (Jaenike et al, 2010; Xie et al, 2010) what could be essential for these ants considering their predatory habits which can bring several entomopathogenic microorganisms.

Another relatively common bacterium found in *O. bauri* in the present study was the enteropathogenic species *Serratia marcescens*. This bacterium has been used as a model in studies addressing the biological control of insects due to its low to moderate pathogenicity and its ability to infect a wide variety of insects (Connick et al., 2001; Dillon et al., 2005).

Bacteria from the genus *Wolbachia* were found in *N. curvinodis*. This genus includes intracellular bacteria that are widely distributed in arthropods. It is estimated that more than 20% of insect species are infected by a strain of *Wolbachia* (Russel et al., 2009). This occurrence is even more widespread in ants and may be related to their mode of colony formation, as *Wolbachia* are more common in species in which the queen depends on workers to found the colony (Watts et al., 2009).

Wolbachia belongs to the order Rickettsiales (phylum Proteobacteria, class Alphaproteobacteria), and its members are considered to be reproductive parasites because they induce reproductive changes in their hosts, such as the death of males, the feminization of males, thelytokous parthenogenesis

and cytoplasmic incompatibility (Wenseleers & Billen, 2000). In ants, cytoplasmic incompatibility is likely the main mode of action of these bacteria (Wenseleers et al., 1998; Wenseleers et al., 2002; Watts et al., 2009).

In other insects, it has been observed that *Wolbachia* interfere with the expression of ferritin and with iron metabolism. These bacteria reduce the concentration of iron, protecting the cell from oxidative stress and apoptosis. This phenomenon has been observed both in species in which these bacteria occur as mutualists and in species in which they occur as facultative parasites (Keller et al., 2001). The occurrence of *Wolbachia* has also been related to supplementation with vitamin B (Hosokawa et al., 2010). Despite the high incidence of these bacteria in ants, there are no conclusive studies addressing their effects, and little is known about this topic (Wenseleers et al., 1998; Jaffe et al., 2001; Rani et al., 2009; Schloss et al., 2009).

Interestingly, the three species of ants found in this study to harbor bacteria that alter the reproduction or sex ratio, such as *Wolbachia* and *Spiroplasma*, are polygynous. This characteristic of their colonies could be evolved in the ants to reduce the effects of these bacteria by allowing uninfected queens to maintain the production of males in the nest. However, the opposite was observed to the ant *Solenopsis*, where *Wolbachia* infection was prevalent in monogynous colonies (Ishak et al., 2011).

Species belonging to the order Rhizobiales (class Alphaproteobacteria, phylum Proteobacteria) were found in the present analysis, such as *Mesorhizobium* and *Bartonella* in *D. lucida*, *Pseudochrobactrum* and *Agrobacterium* in *O. bauri* and *Bartonella* in *P. striata*. Rhizobiales includes known nitrogen-fixing species. The presence of these bacteria has been reported in the guts of other ants, and it has been suggested that these microorganisms are responsible for dietary supplementation through nitrogen fixation or possibly through the recycling of nitrogen (Reuter et al., 2005).

The occurrence of *Micrococcus*, which was found in *N. curvinodis*, has also been reported in the midgut of other insects based on dependent and independent culture methods (Broderick et al., 2004; Kremer et al., 2009; Rafagopal, 2009). These bacteria are not pathogenic. Their presence is related to the immune response via the production of compounds that act against fungal antagonists (Cardoza et al., 2006; Hillesland et al., 2008).

Overall, there was a low diversity of bacteria in the midguts of *N. curvinodis, P. striata* and *O. brunneus*; the genera *Spiroplasma* and *Mesoplasma* were always present in these species but not necessarily together. In contrast, the bacterial diversity found in *O. bauri* was high (13 genera) and included *S. marcescens*, an entomopathogenic species. In *O. bauri,* neither of the two bacterial genera mentioned above were detected.

Ishak et al. (2011) explored the microbiome of the ant *Solenopsis geminata*, which has a more granivorous diet, using a massive parallel sequencing, and they found a high abundance of *Spiroplasma* in workers and that is why they suggested it to be an important partner for this ant. Therefore, considering that these bacteria infect ants with different diet it is possible to predict that *Spiroplasma* and *Mesoplasma* are likely mutualists and have more immunologic instead of nutritional role by inhibiting the occurrence of a greater diversity of bacteria, including potential pathogens, protecting the host ants against massive infection by other prokaryotic microorganisms.

Taking into account the predatory trophic habits of the studied species, the low microbial diversity observed suggests that there is selection of these microorganisms by the host ants, which primarily maintain symbionts that play functional roles and that are able to colonize this environment. Feeding from other insects may bring entomopathogenic bacteria to the ant gut, thus it can also be concluded that the predominant identified bacterial species might exhibit mutualistic functions that are important mainly for immunity, but also to reproduction and nutrition.

Acknowledgements

This work was supported by SECTI/FAPESB/CNPq (Process: FAPESB/CNPq nº. 020/2009-PRONEX MBJ and JHCD acknowledge their CNPq research grants).

References

Anbutsu, H., Fukatsu, T. (2003) Population dynamics of male-killing and non-male-killing *Spiroplasma* in *Drosophila melanogaster*. Applied and Environmental Microbiology, 69: 1428-1434. doi: 10.1128/AEM.69.3.1428-1434.2003

Anderson, E. A., Russel, J. A., Moreau, C. S., Kautz, S., Sullam, K. E., Basinger, U., Mott, B. M., Buck, N., Wheeler, E. Highly similar microbial communities are shared among related and trophically similar ant species. Molecular Ecology, 21: 2282-2296. doi: 10.1111/j.1365-294X.2011.05464.x

Bolton, B. (2003) Synopsis and classification of Formicidae. Memoirs of the American Entomological Institute. Gainesville, Florida.

Borm, S. van, Buschinger, A., Boomsma, J. J., Billen, J. (2002) *Tetraponera* ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings of the Royal Society B, 269: 2023-2027. doi: 10.1098/rspb.2002.2101

Boursaux-Eude, C., Gross, R. (2000) New insights into symbiotic associations between ants and bacteria. Research in Microbiology, 151: 513-519. doi:10.1016/S0923-2508(00)00221-7

Broderick, N.A., Raffa, K.F., Goodman, R.M., Handelsman, J. (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Applied and Environmental Microbiology, 70: 293-300.

Caetano, F.H., Bution, M.L., Zara, F.J. (2008) First report of endocytobionts in the digestive tract of ponerine ants. Micron 40: 194-197. doi: 10.1016/j.micron.2008.09.004

Caetano, F.H., Zara, F.J., Bution, M.L. (2010) A new strategy of endosymbiont midgut bacteria in ant (Ponerinae). Micron 41: 183-186. doi: 10.1016/j.micron.2009.11.007

Cardoza, Y.J., Klepzig, K.D., Raffa, K.F. (2006) Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. Molecular Ecology, 31: 636-645. doi: 10.1111/j.1365-2311. 2006.00829.x

Connick, W.J., Osbrink, W.L.A., Wright, M.S., Williams, K.S., Daigle, D.J., Boykin, D.L., Lax, A.R. (2001) Increased mortality of *Coptotermes formosanus* (Isoptera: Thinotermitidae) exposed to eicosanoid biosynthesis inhibitors and *Serratia marcescens* (Eubacteriales: Enterobacteriaceae). Environmental Entomology, 30: 449-455. doi: 10.1603/0046-225X-30.2.449

Dillon, R.J., Dillon, V.M. (2004) The gut bacteria of insects: nonpathogenic interactions. Annual Review of Entomology, 49: 71-92. doi: 10.1146/annurev.ento.49.061802.123416

Dillon, R.J. Vennard, C.T., Charnley, A.K. (2002) A note: gut bacteria produce components of a locust cohesion pheromone. Journal of Applied Microbiology, 92: 759-763. doi: 10.1046/j. 1365-2672.2002.01581.x

Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. Ecology Letters, 8: 1291-1298. doi: 10.1111/j.1461-0248.2005.00828.x

Dunn, A.K., Stabb, E.V. (2005) Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis* (Neuroptera: Myrmeleontidae). Applied and Environmental Microbiology, 71: 8784-8794.doi: 10.1128/AEM.71.12.8784-8794.2005

Durham, A.M., Kashiwabara, A.Y., Matsunaga, F.T., Ahagon, P.H., Rainone, F. Varuzza, L., Gruber, A. (2005) EGene: a configurable pipeline generation system for automated sequence analysis. Bioinformatics 21: 2812-2813. doi: 10.1093/bioinformatics/bti424

Eilmus, S., Heil, M. (2009) Bacterial associates of arboreal ants and their putative functions in an obligate ant-plant mutualism. Applied and Environmental Microbiology, 75: 4324-4332. doi: 10.1128/AEM.00455-09

Feldhaar, H., Straka, J., Krischke, M., Berthold, S.S., Mueller, M.J., Gross, R. (2007) Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. BMC Biology, 5: 1-11. doi: 10.1186/1741-7007-5-48

Funaro, C.F., Kronauer, D.J.C., Moreau, C.S., Goldman-Huertas, B., Pierce, N.E., Russell, J.A. (2011) Army ants harbor a host-specific clade of *Entomoplasmatales* bacteria. Applied and Environmental Microbiology, 77: 346-350. doi: 10.1128/AEM.01896-10

Giorgini, M., Bernardo, U., Monti, M.M., Nappo, A.G., Gebiola, M. (2010) *Rickettsia* symbionts cause parthenogenetic reproduction in the parasitoid wasp *Pnigalio soemius* (Hymenoptera: Eulophidae). Applied and Environmental Microbiology, 76: 2589-2599. doi: 10.1128/AEM.03154-09

He, H., Wei, C., Wheeler, D. E. (2014) The gut bacterial communities associated with lab-raised and field-collected ants of *Camponotus fragilis* (Formicidae: Formicinae). Current Microbiology, 69: 292-302. doi: 10.1007/s00284-014-0586-8

Hillesland, H., Read, A., Subhadra, B., Hurwitz, I., Mckelvey, R., Ghosh, K., Das, P., Durvasula, R. (2008) Identification of aerobic gut bacteria from kala azar vector, *Phlebotomus argentipes*: a platform for potential paratransgenic manipulation of sand flies. American Journal of Tropical Medicine and Hygiene, 79: 881-886.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y., Fukatsu, T. (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proceedings of the National Academy of Sciences of the United States of America, 107: 769-774. doi: 10.1073/pnas.0911476107

Hurst, G.D.D., Jiggins, F.M. (2000) Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerging Infectious Diseases, 6: 329-336. doi: 10.3201/eid0604.000402

Jaenike, J.J., Unckless, R., Cockburn, S.N., Boelio, L.M., Perlman, S.J. (2010) Adaptation via Symbiosis: Recent Spread of a *Drosophila* Defensive Symbiont. Science, 329: 212-215. doi: 10.1126/science.1188235

Ishak, H. D., Plowes, R., Sen, R., Kellner, K., Meyer, E., Estrada, D. A., Dowd, S. E., Mueller, U. G. (2011) Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminate* ant colonies characterized by 16S amplicon 454 pyrosequencing. Environmental Microbiology, 61: 821-831. doi: 10.1007/s00248-010-9793-4

Jaffe, K., Caetano, F.H., Sánchez, P., Hernández, J.V., Caraballo, L., Vitelli-Flores, J., Monsalve, W., Dorta, B., Lemoine, V.R. (2001) Sensitivity of ant (*Cephalotes*) colonies and individuals to antibiotic implies feeding symbiosis with gut microorganims. Canadian Journal of Zoology, 79: 1120-1124. doi: 10.1139/z01-079

Keller, L., Liautard, C., Reuter, M., Brown, W.D., Sundström, L., Chapuisat, M. (2001) Sex ratio and *Wolbachia* infection in the ant *Formica exsecta*. Heredity, 87: 227-233. doi:10.1046/j.1365-2540.2001.00918.x

Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B., Vavre, F. (2009) *Wolbachia* interferes with ferritin expression and iron metabolism in insects. PLOS Pathogens, 5: 1-12.

Lane, D. J. (1991) 16S/23S rRNA sequencing, p. 115-147. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acids techniques in bacterial systematics. John Wiley & Sons, Chichester, United Kingdom.

Martins, J., Solomon, S.E., Mikheyev, A.S., Mueller, U.G., Ortiz, A., Bacci, M. (2007) Nuclear mitochondrial-like sequences in ants: evidence from *Atta cephalotes* (Formicidae: Attini). Insect Molecular Biology, 16: 777-784. doi: 10.1111/j.1365-2583.2007.00771.x

Rajagopal, R (2009) Beneficial interactions between insects and gut bacteria. Indian Journal of Microbiology, 49: 114-119. doi: 10.1007/s12088-009-0023-z

Rani, A., Sharma, A., Rajagopal, R., Adak, T., Bhatnagar, R.K. (2009) Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. BMC Microbiology, 9: 1471-2180. doi: 10.1186/1471-2180-9-96

Reuter, M., Pedersen, J.S., Keller, L. (2005) Loss of *Wolbachia* infection during colonisation in the invasive Argentine ant *Linepithema humile*. Heredity, 94: 364-369. doi: 10.1038/sj.hdy.6800601

Russell, J.A., Moreau, C.S., Goldman-Huertas, B., Fujiwara, M., Lohman, D.J., Pierce, N.E. (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proceedings of the National Academy of Sciences of the United States of America, 106: 21236-21241. doi: 10.1073/pnas.0907926106

Sameshima, S., Hasegawa, E., Kitade, O., Minaka, N., Matsumoto, T. (1999) Phylogenetic comparison of endosymbionts with their host ants based on molecular evidence. Zoological Science, 16:993-1000. doi: 10.2108/zsj.16.993

Sanchez-Contreras, M., Vlisidou, I. (2008) The diversity of insect-bacteria interactions and its applications for disease control. Biotechnology and Genetic Engineering Reviews, 25: 203-244.

Sanpountzis, P., Zhukova, M., Hansen, L.H., Sørensen, S.J., Schiøtt, M., Boomsma, J.J. (2015) *Acromyrmex* leaf-cutting ants have simple gut microbiota with nitrogen-fixing potential. Applied and Environmental Biology, 81: 5527-5537. doi: 10.1128/AEM.00961-15

Sauer, C., Stackebrandt, E., Gadau, J., Holldobler, B., Gross, R. (2000) Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus* Blochmannia gen. nov. International Journal of Systematic and Evolutionary Microbiology, 50: 1877-1886. doi: 10.1099/00207713-50-5-1877

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartimann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology, 75: 7537-7541. doi: 10.1128/AEM.01541-09

Shoemaker, D.D., Ross, K.G., Keller, L., Vargo, E.L., Werren, J.H. (2000) *Wolbachia* infections in native and introduced populations of fire ants (*Solenopsis* spp.). Insect Molecular Biology, 9: 661-673. doi: 10.1046/j.1365-2583.2000.00233.x

Souza, D.J., Bézier, A., Depoix, D., Drezen, M., Lenoir, A. (2009) *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus Fellah*. BMC Microbiology 9: 1-8. doi:10.1186/1471-2180-9-29

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

van Borm, S., Buschinger, A., Boomsma, J.J., Billen, J. (2002) Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings of the Royal Society B, 269: 2023-2027. doi: 10.1098/rspb.2002.2101

Vettore, A.L., Silva, F.R., Kemper, E.L., Arruda, P. (2001) The libraries that made SUCEST. Genetics and Molecular Biology, 24: 1-7. doi: 10.1590/S1415-47572001000100002

Xie, J., Vilchez, I., Mateos, M. (2010) *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. PLoS ONE, 5: 1-7. doi: 10.1371/journal.pone.0012149

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. (2007) Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology, 73: 5261-5267. doi: 10.1128/AEM.00062-07

Watts, T., Haselkorn, T.S., Moran, N.A., Markow, T.A. (2009) Variable incidence of *spiroplasma* infection in natural populations of *Drosophila* Species. PLoS One, 4: 1-6. doi: 10.1371/journal.pone.0005703

Wenseleers, T., Ito, F., Van Borm, S., Huybrechts, R., Volckaert, F., Billen, J. (1998) Widespread occurrence of the micro-organism *Wolbachia* in ants. Proceedings of the Royal Society B, 265: 1447-1452. doi: 10.1098/rspb.1998.0456

Wenseleers, T., Sundström, L., Billen, T. (2002) Deleterious *Wolbachia* in the ant *Formica truncorum*. Proceedings of the Royal Society B, 269: 623-629. doi: 10.1098/rspb.2001.1927

Wenseleers, T., Billen, J. (2000) No evidence for *Wolbachia*-induced parthenogenesis in the social Hymenoptera. Journal of Evolutionary Biology, 13: 277-280. doi: 10.1046/j.1420-9101.2000.00168.x

Werren, J.H., Baldo, L., Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews Microbiology, 6: 741-751. doi:10.1038/nrmicro1969

William, L. E., Wernegreen, J. J. (2015) Genome evolution in an ancient bacteria-ant symbiosis: parallel gene loss among *Blocmannia* spanning the origin of the ant tribe Camponotini. PeerJ, 3:e881. doi: 10.7717/peerj.881

Wilson, E.O., Hölldobler, B. (2005) The rise of the ants: a phylogenetic and ecological explanation. Proceedings of the National Academy of Sciences of the United States of America, 102:7411-7414. doi: 10.1073/pnas.0502264102