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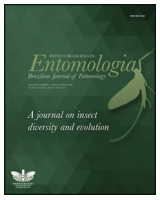

Asaia (Rhodospirillales: Acetobacteraceae) and *Serratia* (Enterobacterales: Yersiniaceae) associated with *Nyssorhynchus braziliensis* and *Nyssorhynchus darlingi* (Diptera: Culicidae)

Tatiane M. P. Oliveira[1]*, Sabri S. Sanabani[2], Maria Anice M. Sallum[1]

[1]*Universidade de São Paulo, Faculdade de Saúde Pública, Departamento de Epidemiologia, São Paulo, SP, Brasil.*
[2]*Universidade de São Paulo, Faculdade de Medicina da São Paulo (FMUSP), Hospital das Clínicas (HCFMUSP), Brasil.*

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ABSTRACT

Midgut transgenic bacteria can be used to express and deliver anti-parasite molecules in malaria vector mosquitoes to reduce transmission. Hence, it is necessary to know the symbiotic bacteria of the microbiota of the midgut to identify those that can be used to interfering in the vector competence of a target mosquito population. The bacterial communities associated with the abdomen of *Nyssorhynchus braziliensis* (Chagas) (Diptera: Culicidae) and *Nyssorhynchus darlingi* (Root) (Diptera: Culicidae) were identified using Illumina NGS sequencing of the V4 region of the 16S rRNA gene. Wild females were collected in rural and periurban communities in the Brazilian Amazon. Proteobacteria was the most abundant group identified in both species. *Asaia* (Rhodospirillales: Acetobacteraceae) and *Serratia* (Enterobacterales: Yersiniaceae) were detected in *Ny. braziliensis* for the first time and its presence was confirmed in *Ny. darlingi*.

Although the malaria burden has decreased worldwide, the disease still imposes enormous suffering for human populations in the majority of endemic countries. Also, the disease continuously threatens the public health, and have a negative impact on the socioeconomic growth of poor communities (Gallup and Sachs, 2001; Shretta et al., 2017). In addition, a recent study by Haakenstad et al. (2019) demonstrated that in 2016, US$ 4.3 billion was spent on malaria worldwide, and that will reach US$ 6.6 billion annually in 2020. Although the intensive worldwide controlling effort, sustaining achievement in malaria control will require an enormous effort from endemic countries, and international funding support for the programs (Shretta et al., 2017; Haakenstad et al., 2019).

Currently, the main pillars for malaria control rely on the commodities targeting anopheline vector species, and the detection and treatment of *Plasmodium* spp. human infection (Baird, 2017; Shretta et al., 2017). However, increasing resistance to artemisinin combination therapies (ACTs) threatens *Plasmodium falciparum* Welch malaria control (Menard and Dondorp, 2017). Controlling strategies targeting Anophelinae vector species are primarily focused on decreasing human exposure to mosquito bites by the use of insecticide-treated bed nets, and insecticide indoor residual spraying (Baird, 2017; Shretta et al., 2017; WHO, 2018). The effectivity of vector control technologies is threatened by the emergence of mosquito resistance to insecticides (Baird, 2017).

New technological commodities, such as the genetic manipulation of organisms (Bilgo et al., 2018), are being developed for controlling vector-borne diseases. The employment of transgenic bacteria from the adult mosquito midgut is a potential tool to be employed for decreasing vector competence and vectorial capacity of vector species involved in a pathogen transmission (Villegas and Pimenta, 2014; Kotnis and Kuri, 2016). The symbiotic bacteria (Damiani et al., 2010), viruses (Ren et al., 2008), and fungi (Fang et al., 2011), which are present in a large array of mosquito species, are potential tools for blocking a pathogen dispersion into a mosquito. In this context, the paratransgenesis of microbial organisms by genetically manipulating the insect endosymbiotic bacteria is a promising approach (Durvasula et al., 1997; Wang and Jacobs-Lorena, 2013; Bilgo et al., 2018). Among the symbiotic bacteria found in anopheline vector species, the *Asaia* (Rhodospirillales: Acetobacteraceae), *Pantoea* (Enterobacterales: Erwiniaceae), *Serratia* (Enterobacterales: Yersiniaceae), *Pseudomonas* (Pseudomonadales: Pseudomonadaceae) and *Thorsellia* (Enterobacterales: Thorselliaceae)

* Corresponding author:
Email: porangaba@usp.br (Tatiane M. P. Oliveira).

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bacteria are candidates for paratransgenesis (Villegas and Pimenta, 2014; Mancini et al., 2016; Raharimalala et al., 2016).

Asaia bacteria was found in field-collected specimens of *Anopheles stephensi* Liston, *Anopheles gambiae* Giles, *Anopheles funestus* Giles, *Anopheles coustani* Laveran, *Anopheles maculipennis* Meigen, *Anopheles superpictus* Grassi, *Anopheles fluviatilis* James, *Anopheles dthali* Patton, *Aedes albopictus* (Skuse), *Aedes aegypti* (Linnaeus), species of the *Culex pipiens* complex (Favia et al., 2007; Crotti et al., 2009; Manguin et al., 2013; Rami et al., 2018), and recently in *Nyssorhynchus darlingi* (Root) (Alonso et al., 2019). Species of the *Asaia* can colonize mosquito salivary gland, midgut, and male and female reproductive apparatus (Favia et al., 2007). Bacteria of the genus *Serratia* can be employed for malaria control (Koosha et al., 2018). The genetically modified AS1 isolate of the *Serratia* was able to inhibit the development of *P. falciparum* in *An. gambiae* through the secretion of anti-*Plasmodium* protein molecules. Currently, the *Serratia* was found in *Anopheles albimanus* Wiedemann (Gonzalez-Ceron et al., 2003), *Anopheles stephensi* (Rani et al., 2009), and *Ny. darlingi* (Arruda et al., 2017).

Recent findings clearly show the importance of mosquito microbiota and the potential of the organisms to reduce the vector competence of a mosquito population by interfering in the sexual life cycle of *P. falciparum* and *Plasmodium vivax* Grassi and Feletti (Gonzalez-Ceron et al., 2003; Cirimotich et al., 2011). However, the bacterial diversity of the microbiota of a few species has been investigated. Additional studies can provide important information regarding microbiota of primary and secondary Anophelinae species, including those of the Neotropical Region (Terenius et al., 2008; Arruda et al., 2017; Bascuñán et al., 2018; Alonso et al., 2019). This study aims to provide further information about the bacteria associated with the abdomen of field-collected females of *Nyssorhynchus darlingi*, the primary vector, and *Ny. braziliensis* (Chagas), the secondary vector species involved in the malaria transmission cycle in the Amazon river basin.

Mosquito female of *Ny. braziliensis* was collected in the municipality of Humaitá (-63.285549, -7.887513; Amazonas State) in July of 2016, and *Ny. darlingi* was collected in Cruzeiro do Sul (-72.688722, -7.631889; Acre State) in April of 2015. Mosquitoes were killed with ethyl acetate ($C_4H_8O_2$) and immediately preserved in silica gel until species identification by morphological characteristics. After identification, females were preserved at -80 °C. Females were bisected in the head/thorax and abdomen. DNA extractions were performed at different times. Genomic DNA of the abdomen of the female of *Ny. braziliensis* was extracted employing Laporta et al. (2015) protocol. The PowerSoil DNA kit (MO BIO Laboratories, Carlsbad, CA, USA) was employed for DNA extraction of *Ny. darlingi* abdomen, following the manufacturer's instructions.

The V4 hypervariable region of the 16S rRNA gene was amplified according to Caporaso et al. (2011). Sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, EUA) with MiSeq Reagent Kit v2 (300 cycles), according to the manufacturer's instructions. The PANDAseq v.2.9 software (Masella et al., 2012) was used to assemble the forward and reverse reads using default parameters. The UCHIME algorithm (Edgar et al., 2011) was employed to detect and remove any recombinant sequences from the Illumina sequence data. A minimum of 97% cutoff of sequence similarity identity was used to define the taxonomic classification of each read. The EzBioCloud (Yoon et al., 2017), with the Mothur algorithm, was used to calculate the diversity indices of bacterial communities, whereas the Shannon diversity index was employed to characterize species diversity in the Illumina sequence data set.

The abdomen of forty-seven anopheline females were sequenced to obtain information of the V4 region of the 16S rRNA gene (unpublished data). In the current study, the microbiota associated with the abdomen of two females is reported. After quality filtering, 56,467 reads were clustered into OTUs at 97% similarity threshold. The results from the rarefaction curve analysis showed that the sequencing depth adopted was adequate to detect all bacteria OTUs in both female specimens. Fifty-nine genera of eight phyla were identified in *Ny. braziliensis* and 83 genera of six phyla were detected in *Ny.darlingi*. Proteobacteria was the phylum dominant in all samples (Table 1). At the genus level, *Escherichia* (Enterobacterales: Enterobacteriaceae) was the dominant group in the abdomen of *Ny. braziliensis* and *Ny. darlingi* (Fig. 1). *Escherichia* and *Enterobacteriaceae_uc* (Enterobacterales: Enterobacteriaceae) were the most abundant genera detected in *Ny. braziliensis*. The value of the Shannon diversity index was 3.14 for *Ny. braziliensis* and 3.96 for *Ny. darlingi*.

Our results revealed a low relative abundance of the genera *Asaia* (0.03%) and *Serratia* (0.09%) in *Ny. braziliensis*, whereas in *Ny. darlingi* both genera were found in higher abundance (*Asaia* - 3.35%, *Serratia* - 1.02%). The presence of *Asaia* and *Serratia* has been previously reported in *Ny. darlingi* (Arruda et al., 2017; Alonso et al., 2019), whereas they were identified in *Ny. braziliensis* for the first time. The presence of bacteria in other Anopheline species (Gonzalez-Ceron et al., 2003; Lindh et al., 2005; Favia et al., 2007; Rani et al., 2009; Manguin et al., 2013; Arruda et al., 2017; Rami et al., 2018) show that they are capable of colonizing a wide range of mosquito because they share stable symbiotic relationship with these vector species. However, further investigations will be necessary to fill gaps in knowledge of the relationships between mosquito species

Table 1.
Number of reads of each phylum in *Nyssorhynchus braziliensis* and *Nyssorhynchus darlingi*.

Phylum	*Nyssorhynchus braziliensis*	*Nyssorhynchus darlingi*
Acidobacteria	47	0
Actinobacteria	105	47
Bacteroidetes	12	80
Cyanobacteria	26	754
Deinococcus-Thermus	30	0
Firmicutes	189	312
Gemmatimonadetes	23	0
Proteobacteria	47699	7123
Verrucomicrobia	0	20

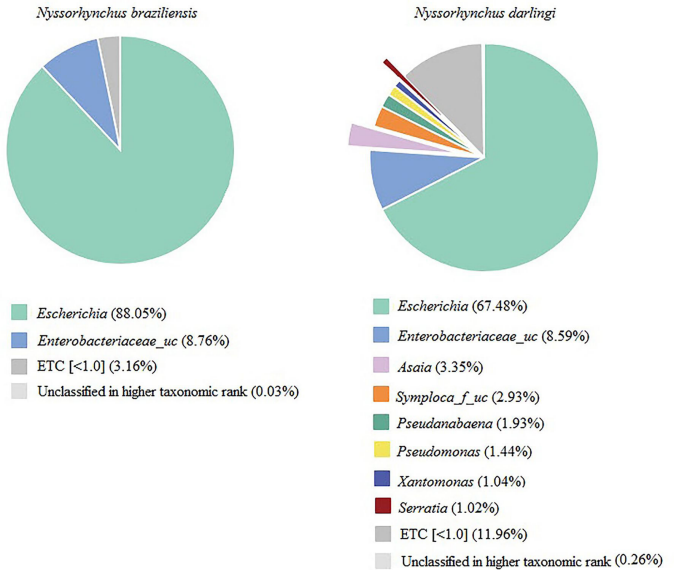

Figure 1. Composition of bacteria from the abdomen of *Nyssorhynchus braziliensis* and *Nyssorhynchus darlingi*. Only genera that had a relative abundance of 1% or greater are presented.

and their associated symbiotic bacteria before these organisms can be employed for a paratransgenic approach to control vector-borne diseases. Genetically manipulated bacteria can be employed for a distinct approach, i.e., to interfere with mosquito reproduction, and oogenesis and embryogenesis processes. In addition, they can be manipulated to express effector molecules to reduce vector competence or cause pathogenic effect in a mosquito population as discussed by Wilke and Marrelli (2015), including Neotropical vector species.

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Data Statement

Sequencing data generated for this study have been deposited in the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena/) (Project: PRJEB32570, Access numbers: ERS3411567 and ERS3411575).

Conflicts of Interest

The authors declare no conflicts of interest.

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