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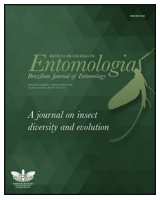

Bacterial community associated with *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) from an urban area in the Amazon, Brazil

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ABSTRACT

The emergence of insecticide resistance in different mosquito populations underscores the pressing need for alternative approaches to control vector-borne diseases. Among several technological strategies, the employment of bacterial symbionts, such as the *Wolbachia pipientis* strains *w*Mel and *w*AlbB to inhibit the ability of *Aedes aegypti* (Linnaeus, 1762) to transmit dengue and Zika viruses in endemic regions worldwide is promising. This investigation examines both the bacterial diversity associated with *Culex quinquefasciatus* and the genetic diversity of *Wolbachia* in females collected in Coari, Amazonas State, Brazil. Both 16S rRNA and *Wolbachia* surface protein (*wsp*) gene sequences were generated and examined. Proteobacteria was the dominant phylum. *Wolbachia* was the predominant genus, followed by *Providencia*, unclassified Erwiniaceae, and *Acinetobacter*. The presence of *Delftia* in *Cx. quinquefasciatus* need further investigations to identify the strains and if any of them can inhibit the transmission of arboviruses by this mosquito. *Wolbachia* 16S rRNA sequences were detected in all samples analyzed. The *wsp* sequences from Coari specimens were identified as *Wolbachia w*Pip strain of the supergroup B. These sequences are identical and share 100% similarity with those of other *Cx. quinquefasciatus* populations from Brazil. Our findings suggest the hypothesis of previous studies that the *Wolbachia* invasion in *Cx. quinquefasciatus* was recent.

Introduction

The Pipiens Complex of the genus *Culex* Linnaeus, 1758 comprises *Culex pipiens* Linnaeus, 1758, *Culex quinquefasciatus,* and *Culex molestus* Forskall, 1775. *Culex pipiens* is found in the temperate region, whereas *Cx. quinquefasciatus* occurs in tropical regions, with a hybrid zone where both species coexist and hybrid specimens are present. *Culex molestus* occurs in underground habitats across urban areas in the temperate region (Forattini, 2002).

The frequency and distribution of *Cx. quinquefasciatus* are linked to human-dominated environments. Thus, the potential of this mosquito to adapt to anthropic environments, combined with its opportunistic feeding behavior, blood feeding on humans, birds, reptiles, rodents, and other vertebrates, makes it an efficient carrier of several arboviruses in urban landscapes. *Culex quinquefasciatus* is the major vector of *Wuchereria bancrofti* (Cobbold, 1877) in Brazil (Consoli and Oliveira, 1994), West Nile virus in the USA (Murray et al., 2010), and is considered vector of St. Louis virus in the central and southern United States (Savage et al., 1993). Despite the results of previous studies (Guedes et al., 2017; Ayres et al., 2019), the association of *Cx. quinquefasciatus* in the Zika virus transmission was challenged by the findings of a vector competence study performed under laboratory conditions (Lourenço-de-Oliveira et al., 2018).

The escalation of artificial breeding sites caused, for instance, by disordered urbanization, ultimately aids the dissemination of *Cx. quinquefasciatus* populations (Wilke et al., 2021). The combination of this with the absence of vaccines and drugs to treat arbovirus infections can promote the dissemination of the vector-borne diseases. The management of vector mosquitoes through insecticides is one strategy used to decrease the number of cases of arboviruses, but the intensive use of these chemical compounds has selected for resistant populations of *Cx. quinquafasciatus* worldwide (Lopes et al., 2019). Among the alternative technologies for vector control, the use of *Wolbachia* is promising (Ant et al., 2020).

Wolbachia is an intracellular endosymbiont bacterium of maternal inheritance, and it is found in over 60% of arthropod species (Jeyaprakash and Hoy, 2000; Hilgenboecker et al., 2008). They affect the blood feeding, pathogen transmission, and host mosquito reproduction. These microorganisms can cause reproductive abnormalities, including parthenogenesis, male killing, feminization, and problems with cytoplasmic incompatibility (CI) (Rousset et al., 1992; Stouthamer et al.,

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1993; Sinkins, 2004; Arai et al., 2020). In CI, no viable descendants are produced by crossing between female mosquitoes that are *Wolbachia*(-) with *Wolbachia*(+) males (Unidirectional CI) or between male and female mosquitoes infected with different strains of *Wolbachia* (Bidirectional CI). Hence, cross sterility generated by CI is likely to diminish the mosquito population, leading to a decrease in both transmission of vector-borne pathogens and mosquito-borne diseases (Sinkins, 2004). The Incompatible Insect Technique has been employed in experimental studies focused on decreasing the population of arbovirus-carrying mosquitoes by releasing *Wolbachia*-infected males that are not compatible with females (Zheng et al., 2019). Although this bacterial genus has only one recognized species, *Wolbachia pipientis*, there are various genetic strains of *Wolbachia* within the supergroups A-U (Lo et al., 2007; Baimai et al., 2021). *Culex quinquefasciatus* is one species where *Wolbachia w*Pip is found among other mosquito species (Zhou et al., 1998).

Comprehending the bacterial diversity associated with mosquitoes can aid in controlling the vector population and advancing research focused on insecticide resistance, transmission dynamics of a particular pathogen, and other related subjects (Gao et al., 2020; Pelloquin et al., 2021). Hence, this study aims to (1) assess the bacterial diversity present in *Cx. quinquefasciatus* from Coari, Amazonas State, Brazil, and (2) establish the genetic diversity of the *wsp* gene in *Wolbachia* in the mosquitoes analyzed.

Materials and methods

Sample collection and identification

Mosquito specimens were gathered from Coari municipality, Amazonas State, Brazil, during November and December 2022 (Table S1). After each mosquito collection, mosquitoes were killed and put immediately in silica gel. Morphological identification of the specimens at the species level was performed using Forattini's identification key (Forattini, 2002).

Library preparation and 16S sequencing

Specimens were surface disinfected with 70% ethanol and rinsed with ultra-pure water. Genomic DNA was extracted from each specimen using the Quick-DNA Fungal/Bacterial Mini-prep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The DNA extracted was stored at -20 ºC until further processing. The V4 region of the bacterial 16S rRNA gene was amplified with primers (Caporaso et al., 2011) associated with Illumina adapter sequences (16S-V4 Forward 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA 3' and 16S- V4 Reverse 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGGACTACHVGGGTWTCTAAT 3'). Each reaction was carried out with 1 X GoTaq® Master Mix (Promega, Madison, WI, USA), 0.3 µM of each primer, 8 µL of genomic DNA and ultra-pure water to the volume of 20 µL. The thermal cycling conditions comprised a cycle of 94 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA) and indexed using the Nextera XT Index kit (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations. After indexing, the products were purified and quantified by real-time PCR (qPCR) using a KAPA-KK4824 kit (Library Quantification kit, Illumina/Universal), following the manufacturer's instructions. All samples were normalized to 3 nM and an equimolar pool of DNA was prepared. Sequencing was performed using a MiSeq Reagent Micro v2 kit (300 cycles: 2 × 150 base pairs) on a MiSeq sequencer (Illumina).

Quality control analysis and taxonomy

Illumina paired-end reads were joined with a minimum overlap of six base pairs using FLASH v. 1.2.11 (Magoč and Salzberg, 2011). Low-quality and chimeras sequences were removed using Deblur in QIIME2 v.2021-11 software (Bolyen et al., 2019). Sequences of mitochondria, chloroplasts, and archaea were discarded using *qiime taxa filter-seqs* in QIIME2. The taxonomy was assigned to QIIME2 using the SILVA 138 database (Quast et al., 2013; Robeson 2nd et al., 2021).

Alpha diversity and heatmap

A rarefaction curve was generated for each sample to obtain the expected number of ASVs (Amplicon Sequence Variants) according to a determined number of DNA sequences. The rarefaction curve was visualized with *Ampvis2* package (Andersen et al., 2018) of Rstudio v.1.4.1106. *Alpha* diversity index was generated with *qiime diversity core-metrics-phylogenetic* and refers to bacterial diversity (number and abundance) in each specimen. Heatmap was generated with *qiime2R* package of Rstudio v.1.4.1106 using ASV and taxonomy tables.

Amplification and sequencing of the wsp gene

Amplification and sequencing of the gene fragment of *Wolbachia* surface protein (*wsp*) was carried out to analyze the genetic diversity of this gene in *Cx. quinquefasciatus* females collected in Coari, Amazonas state, Brazil. The primers used to amplify were 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and 691R (5'-AAAAATTAAACGCTACTCCA-3') (Zhou et al., 1998). Each reaction was carried out with 1 X GoTaq® Master Mix (Promega, Madison, WI, USA), 0.2 µM of each primer, 1-4 µL of genomic DNA, and ultra-pure water to the volume of 20 µL. The thermal cycling conditions comprised a cycle of 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and 30 s, and a final extension of 72 °C for 10 min. PCR products were purified by polyethylene glycol (PEG) precipitation (Silva-do-Nascimento et al., 2021). Sequencing was performed in the forward direction using the same forward PCR primer and the Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA). Products of the sequencing were purified using Sephadex G50 columns (GE Healthcare, Chicago, IL, USA) and analyzed in an Applied Biosystems 3130 DNA Analyzer (PE Applied Biosystems).

Wsp analyses

Sequences of *wsp* were edited in BioEdit version 7.2.5 and the R primer region was removed. The following *wsp* sequences from Genbank were used as references in the analyses: AF020061, AM999887, AF020072, AF020070, AF020068. The sequences were aligned by nucleotide using the muscle algorithm, implemented in MEGA version 11.0.13 (Tamura et al., 2021). Maximum likelihood tree was constructed using the Kimura 2-parameter model and bootstrap support (1000 replicates) implemented in MEGA version 11.0.13.

Results

16S sequences data

Thirty-six specimens were identified as *Cx. quinquefasciatus* females and used to generate the 16S rRNA sequences (Table S1). Next Generation Sequencing (NGS) generated 2,820,148 (R1 or R2) raw reads,

with variation between 64,318 and 113,037 in the samples (Table S2). After joining reads and filtering steps, 771,494 sequences were used to analyses (Table S2).

Taxonomy, heatmap and alpha diversity

Overall, 661 ASVs were identified in the samples (Table S3). Proteobacteria was the dominant phylum, succeeding by Firmicutes (Fig. S1). Anaplasmataceae family was abundant in most samples, with *Wolbachia* being the predominant genus, followed by *Providencia*, unclassified Erwiniaceae, and *Acinetobacter* (Figs. 1 and S2, Table S4). The abundance of bacterial genera present in each sample can be observed in the heatmap (Fig. 2). Rarefaction curves showed that the sequencing depth sufficed to infer the bacterial community abundance in *Cx. quinquefasciatus* (Fig. S3). Shannon-Weaver index was used to measure alpha diversity. Sequences were normalized to calculate α diversity using as cut-off the lowest number of sequences found in all samples. This value corresponds to 8993 sequences, as shown in Table S2. Shannon-Weaver indices varied between 1.10 and 4.33 (Fig. S4 and Table S5).

Wsp analyses

Except for two specimens, the *wsp* gene fragment was amplified from 34 females of *Cx. quinquefasciatus*. After the edition and elimination of the R primer region, 31 sequences of 530 base pairs (bp) were used

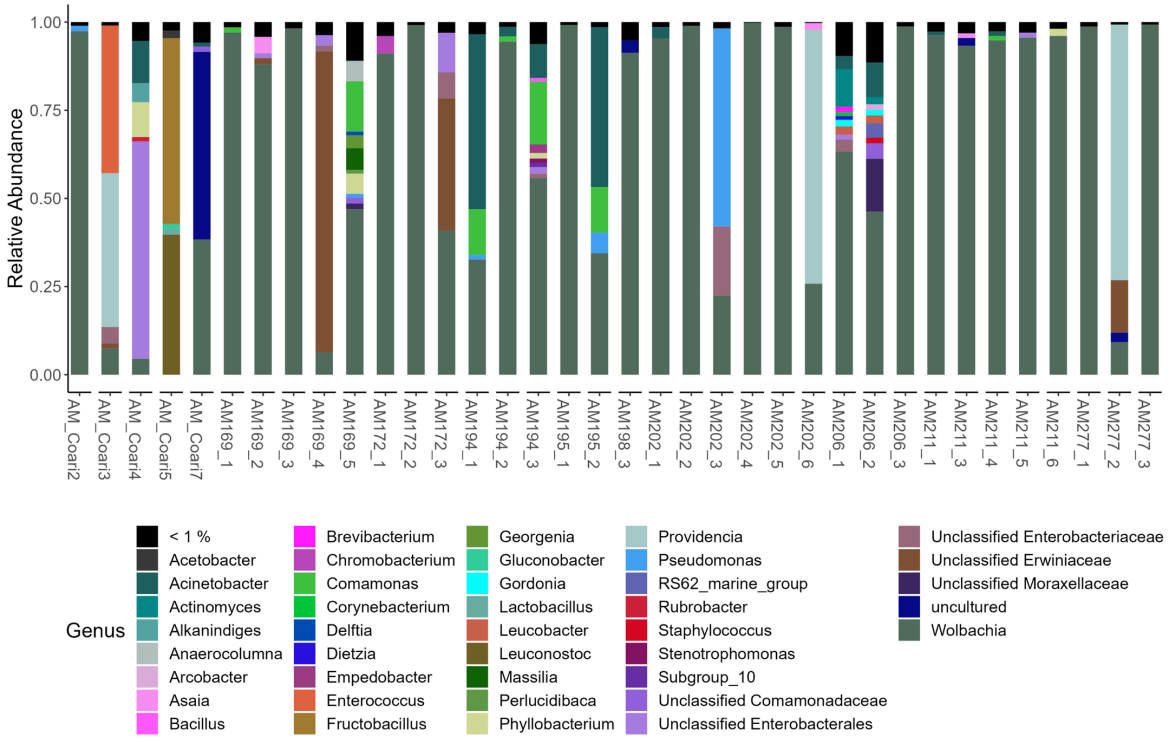

Figure 1 Bar chart of the relative abundance of each bacterial genus per sample. The black bar comprises the genera that show relative abundance of less than 1%.

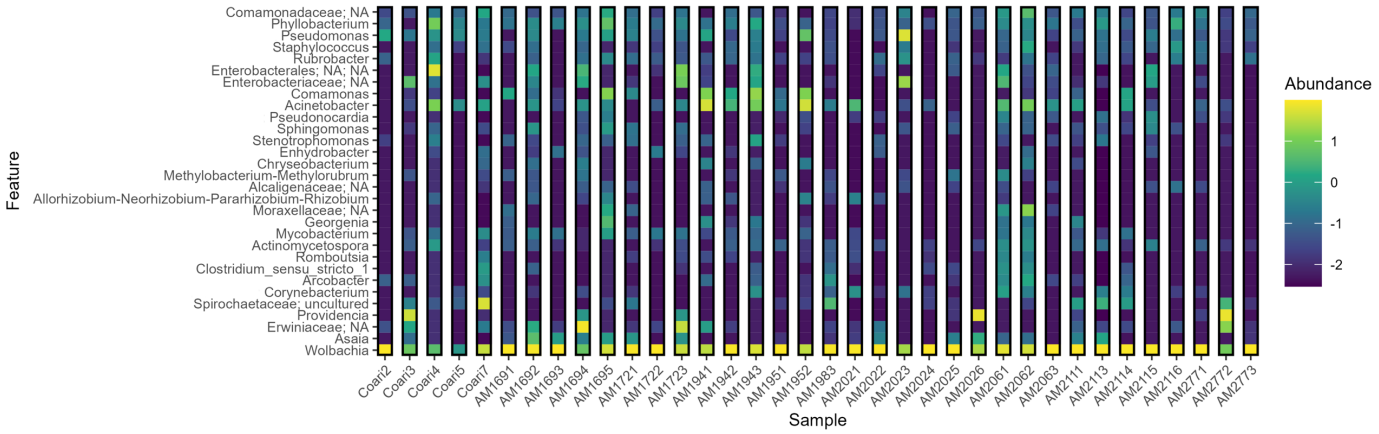

Figure 2 Heatmap of sequences with taxonomic assignment to genus level. The color gradient (yellow to purple) represents abundance. Yellow: higher bacterial abundance. Purple: lowest bacterial abundance. Abundance legend corresponds log10(%).

for the analysis. The segment scrutinized corresponds to the interval 1,005,952–1,006,481 bp of the genome of *Wolbachia* endosymbiont of *Cx. quinquefasciatus* (Genbank: AM999887).

All the *wsp* sequences obtained from specimens collected in Coari shared 100% similarity. Identical percentage of similarity was shared with specimens of *Cx. quinquefasciatus* collected in Cuiabá, Mato Grosso state and Rio Branco, Acre state, Brazil (Genbank: MK614790 and HM563686). The maximum likelihood tree showed that the sequences were consistent with the *Wolbachia w*Pip strain of the supergroup B (Fig. 3).

Discussion

Despite the significance of *Cx. quinquefasciatus* for public health (Ministério da Saúde, 2011; Serra et al., 2016; Reis et al., 2023), there are few studies regarding the microbiota of populations of this species of different regions worldwide (Ramos-Nino et al., 2020; Wang et al., 2021; Nourani et al., 2023), including Brazil (Gonçalves et al., 2019). The resistance of *Cx. quinquefasciatus* populations to some insecticide classes is hindering the effectiveness of this tool for vector control (Lopes et al., 2019). Alternatively, the potential of *Wolbachia* strains for population suppression and to reduce vector-borne disease transmission is promising (Sinkins, 2004; Dennison et al., 2014). *Serratia marcescens* has potential to suppress *Anopheles dirus* Peyton & Harrison, 1979 population (Jupatanakul et al., 2020) and reduce *Plasmodium falciparum* burden in *Anopheles gambiae* Giles, 1902 (Akorli et al., 2022) and therefore it is an endosymbiont bacterium candidate for vector and vector-borne diseases control. Recently, *Delftia tsuruhatensis* TC1 was shown to inhibit the early stages of *Plasmodium* in the mosquito vector by secreting the hydrophobic molecule harmane (Huang et al., 2023), and *Wolbachia* strains *w*Mel or *w*AlbB that have potential for reducing the transmission of positive-sense RNA viruses by *Ae. aegypti* (Fraser et al., 2020). In addition, paratransgenesis approaches employ genetically manipulated symbiotic bacteria to express negative effect molecules on a pathogen of a specific disease (Wilke and Marrelli, 2015; Ratcliffe et al., 2022). The results of the current study showed that several bacterial genera found in *Cx. quinquefasciatus* population from Coari, Amazon state, Brazil, have been reported in other populations of the species (Wang et al., 2021; Nourani et al., 2023). Among the bacterial core we found, it is worthy to note that some are deemed potential candidates for paratransgenesis in culicids, such as *Asaia* and *Pseudomonas* (Chavshin et al., 2012).

Asaia is found in several mosquito species and can colonize different tissues (Favia et al., 2007; De Freece et al., 2014; Nourani et al., 2023). In addition, this bacterium can spread vertically and horizontally across mosquito populations (Mancini et al., 2016). For these and other characteristics, *Asaia* is a promising candidate for malaria vector control by paratransgenesis. Genetically modified *Asaia* strains capable of releasing antiplasmodial effector molecules could significantly reduce the number of oocysts of *Plasmodium berghei* in the midgut of female *Anopheles stephensi* Liston, 1901 (Bongio and Lampe, 2015). *Providencia* and *Acinetobacter* genera were predominant in the analyzed samples, and they have been associated with different mosquito species, such as *Anopheles coluzzii* Coetzee & Wilkerson, 2013 (Chen et al., 2022), *Aedes albopictus* (Skuse, 1895) (Minard et al., 2013; Tuanudom et al., 2021), and *Anopheles darlingi* Root, 1926 (Santos et al., 2023). *Acinetobacter baumannii* and *Acinetobacter johnsonii* were isolated from field-caught *Ae. albopictus* and they appear to improve blood digestion and nectar assimilation, respectively, by the mosquito host (Minard et al., 2013).

Four ASVs were identified as genus *Deftia* in *Cx. quinquefasciatus*. After alignment with sequences available in Genbank, sequences corresponding to two of these ASVs showed 100% similarity with different species of *Deftia*, including *Deftia tsuruhatensis*. Further studies need to be carried out to verify which *Deftia* species is associated with *Cx. quinquefasciatus* and whether it is capable of inhibit the transmission of arbovirus in these mosquitoes.

The accomplishment of the *Wolbachia* technique for dengue control in different parts of the world (WMP, 2022) highlights the potential of these bacteria to regulate vector-borne diseases. *Wolbachia* infection rate varies between different populations of *Cx. quinquefasciatus* (Carvajal et al., 2018; Shih et al., 2021). In the present study, this bacterium was found predominant in most of the mosquitoes analysed. The *wsp* gene was not PCR-amplified in two mosquito specimens, but a low number of *Wolbachia* 16S reads were identified in the Illumina

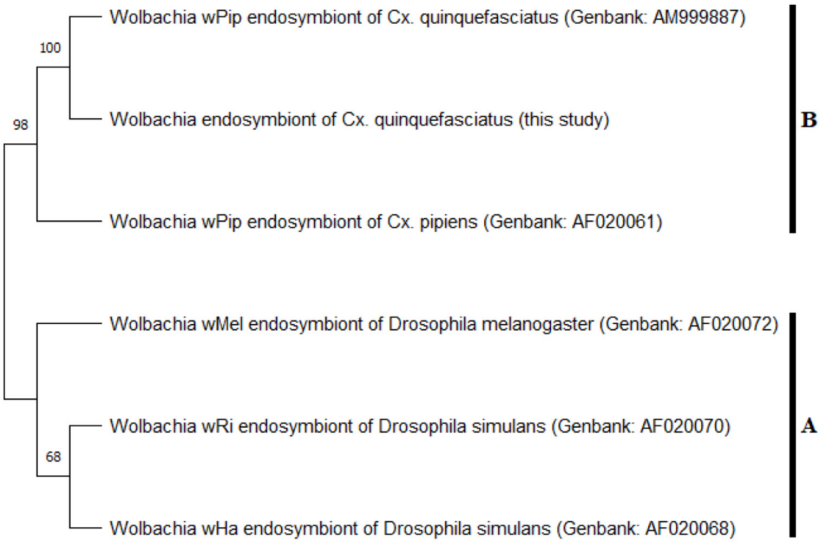

Figure 3 Phylogenetic tree based on maximum likelihood method using MEGA 11. The numbers shown next to the branches correspond to the percentage of replicate trees that the taxa were clustered together in the bootstrap test (1000 replicates).

sequencing analysis of the same specimens. This difference may have occurred because the *wsp* fragment was not re-amplified in a nested-PCR (Wong et al., 2020), thus the amount of DNA generated in the standard PCR was not enough to be visualized in the agarose gel.

Wolbachia surface protein gene encodes the largest surface protein that has four hypervariable regions (Baldo et al., 2005). Sequences from this gene are widely used to identify the strains within *Wolbachia* supergroups A and B (Zhou et al., 1998). *Wolbachia w*PiP of the supergroup B is commonly found in *Cx. quinquefasciatus*, whereas *Wolbachia* strains of the supergroup A were identified in specimens collected in Indonesia (Shih et al., 2021).

The *Wolbachia w*Pip identified in the females analysed for this study is a non-antiviral strain, as shown in *Ae. aegypti* (Fraser et al., 2020). The *wsp* sequences got from *Cx. quinquefasciatus* are identical and they share 100% genetic similarity with two sequences generated from specimens collected in another region in Brazil (Genbank: MK614790 and HM563686). The high similarity indicates, according to Morais et al. (2012), that the invasion of *Wolbachia* in this mosquito species is a recent event. In addition, the likelihood of cytoplasmatic incompatible caused by bidirectionally incompatible is low in *Cx. quinquefasciatus* in Brazil.

Conclusion

This study verified the bacterial composition of samples collected in Coari, Brazil. *Wolbachia* was the predominant genus in most samples and bacterial genera considered potential candidates for paratransgenesis were found, such as *Asaia* and *Pseudomonas*. *Delftia* was found in *Cx. quinquefasciatus* and other studies are needed to verify whether these bacteria can inhibit the transmission of arboviruses in these mosquitoes. There was no variation in the *wsp* sequences of the analyzed samples or with others of *Cx. quinquefasciatus* from Brazil, showing a low diversity of this gene in *Wolbachia* strain *w*Pip.

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Data statement

The unprocessed 16S sequences generated in this study are available from the NCBI Sequence Read Archive under accession PRJNA1015571. All *wsp* sequences generated in this study are available on GenBank with the following accession numbers: OR455003–OR455036.

Conflicts of interest

The authors declare that they have no competing interests.

Author contribution statement

TMPO and MAMS conceived the study; TMPO and HS conducted the analyses; TMPO and MAMS wrote the manuscript. All authors approved the final version of the manuscript.

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Supplementary material

The following online material is available for this article:

Figure S1 - Bar chart of the relative abundance of each phylum per sample. The black bar comprises the phyla that show relative abundance of less than 1%.

Figure S2 - Bar chart of the relative abundance of each family per sample. The black bar comprises the families that show relative abundance of less than 1%.

Figure S3 - Rarefaction curve. ASVs count per sequencing depth in each sample.

Figure S4 - Shannon diversity index in each female sampled.

Table S1 - Collection information of each specimen sequenced for 16S rRNA.

Table S2 - Count of reads obtained by Illumina sequencing (R1 and R2) and of contigs after join and filtering steps in each sample.

Table S3 - Count of each ASVs per sample.

Table S4 - Count of 16S rRNA contigs of each bacterial genus in each sample.

Table S5 - Shannon index in each sample.