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CHARACTERIZATION OF THE CULTURABLE GUT MICROBIOTA OF TWO COLOMBIAN MAIN MALARIA VECTORS

Caracterización de la microbiota intestinal cultivable de dos vectores principales de malaria de Colombia

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ABSTRACT

Bacteria inhabiting the gut of malaria vectors can strongly influence their biology and competence to transmit *Plasmodium* parasites and other pathogens. This study aimed to isolate and characterize the culturable gut bacterial microbiota in two main Colombian malaria vectors. *Anopheles* (Diptera: Culicidae) *darlingi* Root, 1926 and *Anopheles* (Diptera: Culicidae) *nuneztovari* Gabaldón, 1940, specimens were collected in two malaria-endemic regions and under two feedings status. The bacterial composition was compared according to mosquito species, geography, and feeding status. Bacterial isolates from homogenized mosquito guts were morphologically and biochemically characterized, and their taxonomy was determined by 16S rRNA sequencing. Results showed that Gram-negative bacilli, particularly of the Proteobacteria phylum, are predominant in the gut of the two-vector species regardless of geography and feeding status. At the genus level, *Enterobacter*, *Acinetobacter,* and *Bacillus* were common among the two-vector species and geographic sites; and some genera were locality or vector-specific. In addition, the presence of blood in the mosquito gut negatively impacted bacterial richness at the genus level. These results advanced the knowledge of mosquito-microbe interactions for these malaria vectors. In addition, the generation of a bacterial culture repertoire may allow us to investigate the potential role of some bacteria as biocontrol agents.

Keywords: Gut bacteria, anopheline mosquitos, malaria, Colombia.

RESUMEN

Las bacterias que habitan en el intestino de los vectores de la malaria pueden influir en su biología y competencia para transmitir parásitos *Plasmodium* y otros patógenos. Este estudio tuvo como objetivo aislar y caracterizar la microbiota bacteriana intestinal cultivable en dos vectores principales de malaria de Colombia. Los especímenes de *Anopheles* (Diptera: Culicidae) *darlingi* Root, 1926 y *Anopheles* (Diptera: Culicidae) *nuneztovari* Gabaldón, 1940, fueron colectados en dos regiones endémicas para malaria y bajo dos estados de alimentación. La composición bacteriana se comparó según la especie de mosquito, la geografía y el estado de alimentación. Las bacterias aisladas de intestinos de mosquitos homogeneizados se caracterizaron morfológica y bioquímicamente y se determinó su taxonomía mediante secuenciación de ARNr 16S. Los resultados mostraron que los bacilos Gram-negativos, particularmente del filo Proteobacteria, predominan en el intestino de las dos especies de vectores, independientemente de la geografía y el estado de alimentación. A nivel de género, *Enterobacter*, *Acinetobacter* y *Bacillus* fueron comunes entre las dos especies de vectores y sitios geográficos; y algunos géneros fueron específicos de la localidad o del vector. Además, la presencia de sangre

en el intestino del mosquito afectó negativamente la riqueza bacteriana a nivel de género. Estos resultados aportan al avance del conocimiento sobre las interacciones mosquito-microbio para estos vectores de la malaria; sumado a esto, la generación de un repertorio de cultivos bacterianos podrá permitir investigar el papel potencial de algunas bacterias como agentes de biocontrol.

Palabras clave: bacterias intestinales, mosquitos anofelinos, malaria, Colombia.

INTRODUCTION

Malaria is an infectious disease caused by parasites of the genus *Plasmodium* and transmitted to humans through the bite of female *Anopheles* (Diptera: Culicidae) mosquitoes (WHO, 2021). Colombia occupies third place in the number of malaria cases in the American continent (WHO, 2021). In 2021, 72,022 malaria cases were reported, with the highest transmission regions occurring in the Pacific and Bajo Cauca-Alto Sinú (INS, 2022). Current vector-control strategies in the country rely mostly on the use of Insecticide-Treated Nets (ITNs)/Long Lasting Insecticidal Nets (LLIN), Indoor Residual Spraying (IRS), and larval control (WHO, 2021). However, like in many other regions worldwide, these approaches are being threatened by the rapid adaptive capacity of vector populations to survive exposure to insecticides (Orjuela *et al*., 2018; WHO, 2021). In Colombia, the three primary malaria vectors, *Anopheles darlingi*, *Anopheles nuneztovari,* and *Anopheles albimanus* Wiedemann, 1820, have shown some degree of resistance to routinely used insecticides (Orjuela *et al*., 2018). These findings strongly call for novel tools to complement the present strategies used to control malaria in this and other countries. Previous studies have revealed that the microbiota of the mosquito gut plays important roles in several traits of its biology, such as development (Coon *et al*., 2016), blood digestion (Gaio *et al*., 2011), and reproduction (Gendrin *et al*., 2015). In addition, several studies have demonstrated that the innate gut bacteria of some malaria vectors modulate the malaria parasite's success during its passage through the mosquito gut (Bai *et al*., 2019). Similarly, field and semi-field studies have shown that variations in the gut microbiota of the African malaria vector *Anopheles gambiae*, Giles, 1902, strongly define the mosquito's capacity to transmit *Plasmodium* parasites (Boissière *et al*., 2012; Tchioffo *et al*., 2013). *A. gambiae* mosquitoes orally co-exposed to field-isolated gut bacteria and *Plasmodium* parasites showed that some members of the Enterobacteriaceae family highly reduced malaria infection rates (Tchioffo *et al*., 2013). Likewise, a gut bacterium of the genus *Chromobacterium* (Csp_P), originally isolated from field-collected *Aedes aegypti* mosquitoes, also showed *in vitro* anti-*Plasmodium* activity (Ramirez *et al*., 2014). Bacterial strains such as *Enterobacter* Esp_Z and *Asaia* sp, isolated from *Anopheles arabiensis* Patton, 1905, and *Anopheles stephensi* Liston, 1901, respectively, also have a negative effect on the parasite (Favia *et al*., 2007; Cirimotich *et al*., 2011). Thus, the idea of using mosquito gut bacteria as biocontrol agents against the malaria parasite, either directly or by genetically modifying a symbiont to then reintroduce it into the mosquito population (i.e. paratransgenesis), is gaining attention. However, this alternative requires the selection of a culturable bacterium, preferably a symbiont isolated from the disease-transmitting vector to ensure a stable symbiotic relationship (Wilke and Marrelli, 2015). For this reason, studies directed to isolate and characterize the mosquito-culturable bacterial microbiota are essential to obtain symbionts for paratransgenesis or native bacteria with biocontrol properties.

To ensure the success of the development and implementation of vector biocontrol strategies based on paratransgenesis, it is essential to first understand the culturable bacterial composition of the mosquito populations and the factors that shape their structure (Wilke and Marrelli, 2015). For instance, it is known that the gut bacterial community of many mosquitoes greatly varies according to geography (Minard *et al.*, 2015; Akorli *et al*., 2016), mosquito species (Muturi *et al*., 2016), habitat (Bascuñán *et al*., 2018), diet of the adult (consumption of nectar or blood) (Müller *et al*., 2010; Singh *et al.*, 2022) and even mosquito stages (Galeano *et al.*, 2020). To our knowledge, only a limited number of studies have analyzed the bacterial microbiota composition of Latin American malaria vectors (Gonzalez-Ceron *et al*., 2003; Bascuñán *et al.*, 2018; Galeano-Castañeda *et al.*, 2020; Galeano *et al*., 2019); one of these characterized the culturable gut microbiota of the main vector *A. albimanus* (Galeano *et al.*, 2019), but no study has yet analyzed the culturable gut microbiota of the other two Colombian main malaria vectors *A. darlingi* and *A. nuneztovari*. Studies using traditional culture-based methods are essential for the collection of live bacterial specimens as well as to gain an understanding of their characteristics and properties; for instance, bacterial metabolites, antiparasitic activity, or pathogenic activity against the mosquito host. In this study, the culturable gut microbiota of two of the three main Colombian malaria vectors, *A. darlingi,* and *A. nuneztovari,* was isolated and characterized according to species, geography, and feeding status. In addition, a bacterial culture collection was generated to encourage further investigation of the role of the bacteria as potential biocontrol agents.

MATERIALS AND METHODS

MOSQUITO COLLECTION AND SPECIES IDENTIFICATION

Female mosquitoes were collected in January and September 2015 in two localities of two Colombian regions: San Antonio (5°7′ N 76°41′ W), located in the municipality of Istmina in the Department of Chocó, Pacific Coast, W Colombia. La Capilla (7°31′ N 74°43′ W), in El Bagre municipality, Department of Antioquia, Urabá Bajo Cauca Alto-Sinú Region, NW Colombia. Mosquitoes were collected during three nights in each locality, from 18:00-24:00 h, using 70 % barrier screens (Burkot *et al*., 2013) located between houses and a river/forest. Collections were performed under an informed consent agreement and collection protocol approved by a Review Board of Sede de Investigación Universitaria-SIU, University of Antioquia (Number: 15-41-580). The workflow of the study is described (Fig. 1). Gut dissections were performed in the field, in mosquitoes collected during the last night, to be able to remove intact guts to be preserved avoiding random bacterial proliferation and therefore, changes in bacterial composition. The dissections were performed under sterile conditions, specimens were surface-sterilized in 70 % ethanol for 2-3 min and rinsed three times in sterile 1X phosphate-buffered saline (PBS). In addition, 70 % ethanol tool cleansing was performed between sample dissections, and a Bunsen burner was placed close to the dissecting area. This methodology was previously implemented and it is validated by a study that demonstrated that it can be adopted to avoid sample contamination (Galeano *et al*., 2019). Dissected guts were stored individually in 50 µl of 1X PBS and kept on ice or fridge to be transported to the laboratory. A posterior leg and the wings were mounted on a glass slide for morphological species identification, following a morphological key (Gonzalez and Carrejo, 2018). Also, a leg of each specimen was individually preserved in microcentrifuge tubes with 50 µL of a grinding buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl) for rapid DNA extraction as previously described (Shaw *et al*., 2016), and molecular species identification using a PCR-RFLP of the Internal Transcribed Spacer 2-ITS2 protocol (Cienfuegos *et al*., 2011). In addition, the *Plasmodium* infection status of the mosquitoes was evaluated by a nested PCR assay (Singh *et al*., 1999); but, given that only one specimen was found infected, no analysis of the microbiota about the infection status could be performed.

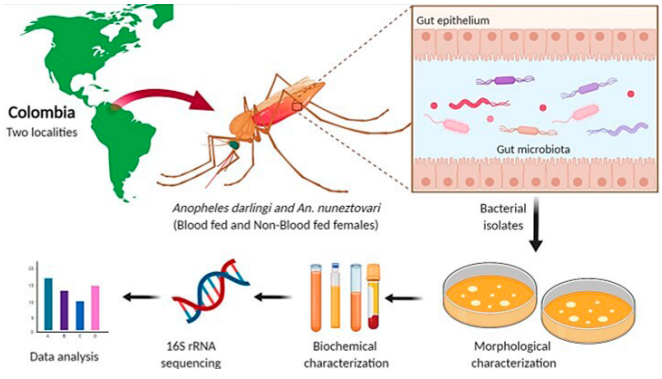

Figure 1. Experimental design flowchart. General study methodology for the analysis of the cultivable intestinal microbiota of malaria vectors.

GUT BACTERIAL ISOLATION

From the mosquitoes collected in El Bagre and Istmina localities, 120 specimens corresponded to *A. darlingi* and 98 to *A. nuneztovari*. Eleven specimens collected during the last sampling night were used for microbiota characterization, six corresponded to *A. darlingi* and five to *A. nuneztovari*; each mosquito analyzed represented a sample replicate. The guts were individually homogenized with a mechanical pestle and 2 µL of the homogenate was surface spread plated on Nutrient Agar (NA) and Blood Agar (BA) in duplicates and grown at 24 °C for 48h. Microscopic and macroscopic morphology characterization of the bacterial isolates was performed by Gram staining to determine positive or negative staining and shape (e.g. cocci, bacilli), and by describing the bacterial colony appearance in terms of its shape, margin, elevation, surface, color, brightness, size and hemolytic properties when grown on BA. A large number of colonies representing the bacteria present in each mosquito gut were obtained by culture methods, but following colony characterization, single bacterial isolates of each morphotype were chosen to continue the characterization process. The morphotypes obtained were grown individually in NA and BA agars, cryopreserved in 50 % glycerol (1:1 ratio), and kept at -80 °C to generate a reservoir of bacterial isolates. *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli,* and *Pseudomonas aeruginosa* were used as controls in staining and biochemical tests. Biochemical tests were performed for each isolate as follows: for Gram-positive bacilli, hemolysis, spore formation, and motility (Sulfide Indole Motility test-SIM). Test for Gram-negative bacilli included lactose fermentation in MacConkey/Endo agar; lactose, sucrose, and glucose fermentation and H_2SO_4 and gas production in a Triple Sugar Iron (TSI) agar; motility and indole production in SIM medium and an oxidase test. For Gram-positive cocci, catalase, coagulase and mannitol fermentation; and for Gram-negative cocci oxidase, catalase, and sugar fermentation. Finally, the bacterial isolates were grouped into morphotypes, according to their microscopic, macroscopic morphology characterization and biochemical properties. To consolidate the number of bacteria to be sequenced, representative members of each morphotype were selected for further processing.

BACTERIAL DNA EXTRACTION, 16S rRNA AMPLIFICATION, AND SEQUENCE ANALYSES

To obtain sequences, the previously selected representative bacterial morphotypes were grown in Luria Bertani (LB) liquid medium and incubated at 37 °C for 24 h. Each culture was centrifuged at 13,000 rpm for 2-5 min, and the pellet was resuspended in 180 µL of lysis buffer (20 mM Tris, 2 mM EDTA, Triton X-100 1.2 %, pH 8.0). For Gram-positive bacteria, lysozyme was added to increase the chances of disrupting the cell wall (20 mg lysozyme/mL of lysis buffer). Samples were incubated at 37 °C for 30 min. Twenty-five µL of proteinase K were added and the suspension was incubated at 56 °C for 30 min and a final DNA ethanol-precipitation step was performed according to an adapted protocol (Rosero *et al*., 2010). For each isolate, a PCR was performed using the 16S rRNA gene universal primers 27F and 1492R (Lane, 1991), under the following conditions: a master mix with 20 ng/ µL DNA, 0.05 µM primers 27F y 1492R, 1U/reaction *Taq* DNA polymerase (Bioline), 0.4 mM dNTPs, 1.5 mM $MgCl_2$, 1X de Buffer NH_4, and MQ water to a 25 µL final reaction; cycle reactions were, 94 °C for 2 min, 30 cycles of 94 °C, 30 s; 53 °C, 1 min and 72 °C, 30 s, and a final extension at 72 °C, 10 min. Amplicons were sent for bidirectional sequencing by the Sanger method, using the universal primers 800R. DNA sequences were edited in Geneious software v. 8.0.2 (Kearse *et al*., 2012) (GenBank accession numbers MH757423 - MH757445). The identity of the isolates was obtained by comparing them to available sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast). The BLAST search, MUSCLE alignment, and NJ tree were generated in the Geneiuos software v. 8.0.2.

STATISTICAL ANALYSIS

Fisher's exact test and Chi-square statistic were calculated to determine differences in bacterial composition between *A. nuneztovari* and *A. darlingi,* and between locations and feeding status (0.05 significance level), using PAST software v. 3.14.

RESULTS

ANOPHELES GUT BACTERIAL COMMUNITY COMPOSITION

After the bacterial isolates were grouped into morphotypes according to microscopic, macroscopic characteristics and biochemical properties, 44 unique, representative morphotypes were selected; twenty-three were from Istmina locality and 21 from El Bagre. The obtained 16S rRNA sequences were edited and analyzed and the consensus sequences were grouped, as they were assumed to represent identical bacterial isolates. The 16S rRNA sequences (GenBank accession numbers MH757423 - MH757445), allowed taxonomic identification of the morphotypes. Most of the recovered bacterial isolates of *Anopheles* from Istmina were Gram-negative rods (70 %), the remaining were Gram-positive rods (15 %) and Gram-negative cocci (15 %). In El Bagre, most isolates were also Gram-negative bacilli (50 %), followed by 30 % Gram-positive bacilli, 10 % Gram-negative cocci, and 10 % Gram-positive cocci. Notably, the Gram-negative bacilli collected at Istmina were neither motile nor lactose fermenting, and most were positive in the oxidase test; Gram-positive bacilli were also non-motile. In El Bagre, most of the Gram-negative bacilli were non-fermenters of lactose and positive in the oxidase test.

The 16S rRNA sequence analyses allowed the grouping of the bacteria into 11 families, four classes, and three phyla (Table 1). Three bacterial phyla were detected in *A. darlingi* and *A. nuneztovari*, and included, Proteobacteria and Firmicutes in both mosquito species; Bacteroidetes were only detected in *A. darlingi* from Istmina, Pacific coast. The bacterial families present in both vector species and from both locations were, Bacillaceae, Flavobacteriaceae, Erwiniaceae, Moraxellaceae, Aeromonadaceae, and Enterobacteriaceae (Fig. 2a-2b). Of notice, Enterobacteriaceae was the most abundant and representative bacterial family in *A. nuneztovari* from the Istmina locality. The families Yersiniaceae, Staphylococcaceae, Neisseriaceae, and Paenibacillaceae were exclusive to *A. darlingi*, while Pseudomonadaceae was limited to *A. nuneztovari* from El Bagre, Bajo Cauca region (Table 1) (Fig. 2a-2b). Of the bacterial genera detected for both *Anopheles* species, four were exclusive to *A. darlingi*: *Brevibacillus*, *Staphylococcus*, *Chromobacterium,* and *Serratia*; which belonged to specimens collected in El Bajo Cauca. *Pseudomonas* sp. was only detected in *A. nuneztovari*; the remaining six, *Chryseobacterium*, *Aeromonas*, *Enterobacter*, *Acinetobacter*, *Pantoea,* and *Bacillus* were shared between both mosquito species (Table 1) (Fig. 2a-2b).

In the phylogenetic tree based on Maximum Likelihood, the 16S rRNA partial gene sequences were grouped into three main clades corresponding to the bacterial phyla detected (Proteobacteria, Firmicutes, and Bacteroidetes); bootstrap values were close to 1.0 (Fig. 3). Sequencing and phylogenetic analysis allowed the identification of a set of bacterial morphotypes at the species level (100 % identity, Table 1), and their corresponding genera were allocated into unique clades, e.g. *Pseudomonas*, *Serratia*, *Pantoea*, *Staphylococcus*, *Brevibacillus,* and *Chromobacterium* (Fig. 3). However, for a group of morphotypes, it was not possible to precisely determine the species and in the phylogenetic tree, they were grouped as separated branches of the same clade; they included morphotypes of the genera *Enterobacter*, *Chryseobacterium*, *Aeromonas*, *Acinetobacter* and *Bacillus*. As an example, seven sequences assigned to the *Acinetobacter* clade had a high percentage similarity with species of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb)

Table 1. Bacterial morphotypes identity. Taxonomical identification at the genus or species level of the bacterial morphotypes (M) obtained from the gut of *Anopheles nuneztovari* and *Anopheles darlingi* mosquitoes collected in Istmina, Chocó, W Colombia and El Bagre, Antioquia, NW Colombia.

Locality	Species	Phylum	Class	Family	Genus or Species of Morphotypes	Identity (%)
Istmina	*A. darlingi*	Bacteroidetes	Flavobacteria	Flavobacteriaceae	**M1** *Chryseobacterium gleum*	100
					M2 *Chryseobacterium hominis*	98.9
		Proteobacteria	γ-Proteobacteria	Moraxellaceae	**M7** *Acinetobacter* sp.	99.9
				Enterobacteriaceae	**M3** *Enterobacter cloacae/ E. asburiae*	100
				Erwiniaceae	**M5** *Pantoea dispersa*	100
		Firmicutes	Bacilli	Bacillaceae	**M6** *Bacillus aryabhattai/ B. megaterium/B. flexus*	100
	A. nuneztovari	Bacteroidetes	Flavobacteria	Flavobacteriaceae	**M1** *Chryseobacterium gleum*	100
					M2 *Chryseobacterium hominis*	100
		Proteobacteria	γ-Proteobacteria	Moraxellaceae	**M7** *Acinetobacter* sp.	99.9
				Enterobacteriaceae	**M3** *Enterobacter cloacae/ E. asburiae*	100
					M4 *Enterobacter asburiae*	100
				Erwiniaceae	**M5** *Pantoea dispersa*	100
El Bagre	*A. darlingi*	Proteobacteria	β-Proteobacteria	Neisseriaceae	**M8** *Chromobacterium violaceum*	100
			γ-Proteobacteria	Aeromonadaceae	**M10** *Aeromonas caviae/A. hydrophila*	100
				Moraxellaceae	**M9** *Acinetobacter soli/A. calcoaceticus*	100
				Yersiniaceae	**M13** *Serratia marcescens*	100
		Firmicutes	Bacilli	Bacillaceae	**M14** *Bacillus* sp.	100
				Staphylococcaceae	**M15** *Staphylococcus saprophyticus*	100
				Paenibacillaceae	**M16** *Brevibacillus* sp.	99.9
	A. nuneztovari	Proteobacteria	γ-Proteobacteria	Aeromonadaceae	**M10** *Aeromonas caviae/A. hydrophila*	100
				Pseudomonadaceae	**M11** *Pseudomonas* sp.	100
				Moraxellaceae	**M9** *Acinetobacter soli/A. calcoaceticus*	100
				Enterobacteriaceae	**M12** *Enterobacter* sp.	100
		Firmicutes	Bacilli	Bacillaceae	**M14** *Bacillus thuringiensis/B. cereus*	100

complex and were separated into two different branches of the clade (M7 and M9 morphotypes) (Fig. 3) (Table 1).

BACTERIAL RICHNESS DECREASES IN BLOOD-FED FEMALES

There were differences in the composition of the bacterial gut microbiota depending on the presence or absence of blood. For both vector species, a decrease in bacterial richness at the genus level was detected in specimens that had blood in their guts, as compared to non-blood-fed mosquitoes. In Itsmina, the genera *Enterobacter, Bacillus, Acinetobacter,* and *Chryseobacterium* were detected in blood-fed and non-blood-fed specimens, while *Pantoea* was not detected in blood-fed mosquitoes. In El Bagre, *Bacillus, Acinetobacter,* and *Aeromonas* were detected in both types of fed mosquitoes, while *Brevibacillus*, *Staphylococcus*, *Chromobacterium,* and *Pseudomonas* were only found in non-blood-fed mosquitoes and *Enterobacter* and *Serratia* were present in the blood-fed mosquitoes (Fig. 4). The statistical analysis on bacterial composition, at the genus level, showed no significant difference in the number of bacterial genera between mosquito species (Chi^2= 0.92; p> 0.05) or between localities (Chi^2= 0.68; p> 0.05); however, when comparing morphotypes, a significant difference in gut bacterial composition was found between mosquitoes according to locality (Chi^2= 0.039; p< 0.05). Furthermore, a significant difference in composition was found at the bacterial genus level in blood-fed versus non-blood-fed mosquitoes in El Bagre (Chi^2= 0.054; p< 0.05).

DISCUSSIÓN

This study characterized the culturable bacteria residing in the gut of two of the three Colombian main malaria vectors, *A. nuneztovari,* and *A. darlingi;* in addition, the bacterial composition according to geography, mosquito species, and feeding status was explored. In general, the results showed significant differences in mosquito gut bacterial composition at the morphotype level, between localities; while there was not significant difference in the number of bacteria at the genus level, between mosquito species or

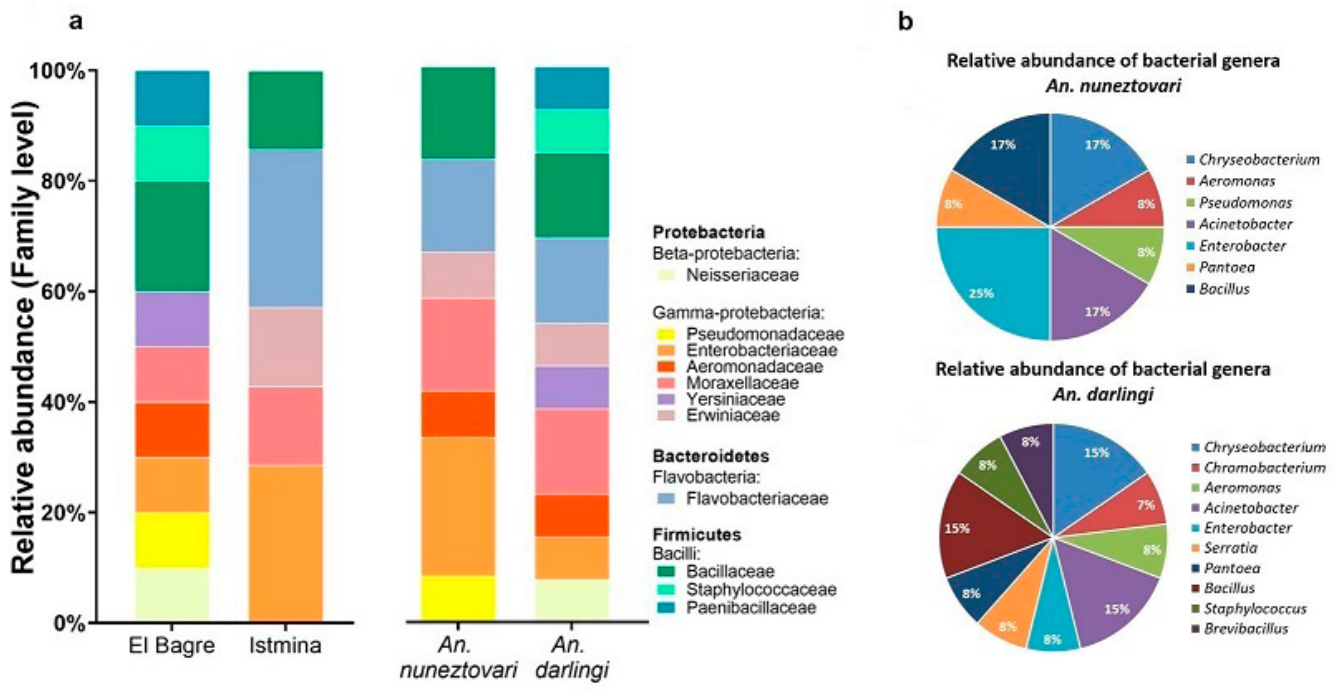

Figure 2. a. Comparisons of bacterial families per locality and mosquito species, b. Bacterial genera per mosquito species. a. Relative abundance of the bacterial taxa at the family level in samples from the gut of *Anopheles nuneztovari* and *Anopheles darlingi* mosquitoes collected in two localities: Istmina, W Colombia and El Bagre, W Colombia, according to locality (left panel) or mosquito species (right panel). **b.** Pie charts showing the relative abundance of bacterial genera in the gut of the vector species *A. nuneztovari* and *A. darlingi* from both localities.

localities; this last result did not allow us to draw further conclusions on differences in bacterial genera composition driven by geography or mosquito species. A predominance of Gram-negative bacilli in both vector species was observed, in particular of the Proteobacteria phylum. This is interesting since a study on field-collected *A. gambiae* mosquitoes showed that Gram-negative isolates of Proteobacteria, specifically, the genera *Acinetobacter*, *Pseudomonas,* and *Enterobacter* reduced *P. falciparum* prevalence, whereas no effect was observed when using a Gram-positive isolate of the species *Bacillus pumilus* on the Firmicutes phylum (Cirimotich *et al*., 2011). Furthermore, Proteobacteria are reported to be the most abundant and common class in *Anopheles* mosquito guts from various continents (Villegas and Pimenta, 2014). In a previous study on *A. albimanus*, the Proteobacteria phylum was found to be the most abundant in the aquatic habitat (~75 %) (Galeano-Castañeda *et al*., 2020). In our work, this phylum was still present in the adult stage, but it is known that although a large part of the microbiota is acquired from the aquatic environment of the larvae, there is also a large microbiota richness lost during metamorphosis, in the pass from the pupa to adult; in addition, the feeding of the circulating mosquito affects microbiota composition (Galeano-Castañeda *et al*., 2020). Bacterial genera found in this study such as *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Pantoea*, *Chryseobacterium*, *Enterobacter* and *Serratia*, have been frequently described in the gut microbiota of the African malaria vector *A. gambiae* (Boissière *et al*., 2012), the Asian mosquito *A. stephensi* (Rani *et al*., 2009; Dinparast *et al*., 2011) and the Latin-American malaria vectors *A. albimanus* and *A. darlingi* (González-Ceron *et al*., 2003; Galeano *et al*., 2019). These studies suggest that mosquitoes acquire most of their gut microbiota from the environment (Rani *et al*., 2009; Dada *et al*., 2014; Galeano-Castañeda *et al*., 2020), which significantly contributes to the great heterogeneity of the intestinal symbionts. Also, another study showed that the microbiota differs among larvae from diverse aquatic habitats, but it is similar if larvae are collected in the same site (Coon *et al*., 2016).

In this study, three isolate types were common to the two mosquito vectors and geographic sites, which belonged to the genera *Enterobacter*, *Acinetobacter,* and *Bacillus.* Interestingly, a study performed in *A. stephensi* showed that isolates of wild and genetically modified *Enterobacter cloacae* expressed effector proteins that interrupt the development of *Plasmodium berghei* in the vector (Dehghan *et al*., 2022). Indeed, a later study showed that an isolate of *E. cloacae* strongly induces the immune response of *A. stephensi* mosquitoes, reducing the malaria parasite (Eappen et al., 2013).

Furthermore, *Enterobacter Esp_Z*, an isolate originally obtained from a Zambian population of *A. arabiensis*, likewise reduces *Plasmodium* development at the oocyst and ookinete stages in *A. gambiae* (Cirimotich *et al*., 2011; Dennison *et al*., 2016). Also, in this vector species, a positive effect on mosquito survival was observed by the reintroduction of *E. cloacae* (Ezemuoka *et al*., 2020). This is important because native bacteria such as *Enterobacter*, whether genetically modified or naïve, could improve the effectiveness of a strategy directed to protect against the parasite without affecting the mosquito population. In particular, the *Enterobacter* isolate found in *A. darlingi* and *A. nuneztovari* species, genetically similar to the species *E. cloacae*, represents an appropriate candidate to study its anti-*Plasmodium* activity or potential as a suitable symbiont for a paratransgenesis strategy.

Regarding the *Acinetobacter* isolates, they presented high sequence homology with *A. nosocomialis*, *A. calcoaceticus,* and *A. baumannii*. All the mentioned species belong to the *Acinetobacter baumannii-calcoaceticus*-ABC complex composed of four phenotypically indistinguishable species (Maslunka *et al*., 2014). Although not much is known about the antiparasitic activity of these bacterial species, it is believed that *A. baumanii* contributes to the blood digestion process of *Aedes albopictus* Skuse, 1894, mosquitoes (Minard *et al*., 2013), which is essential for mosquito egg production. Interestingly, in the present study, isolates of this genus were present in blood-fed and non-blood-fed mosquitoes of both species and localities; therefore, they are suitable candidates for further investigation. Additionally, *Bacillus* isolates were present in all specimens analyzed. In particular, *Bacillus thuringiensis* var. *israelensis* (Bti) has been largely studied for its efficacy in reducing mosquito larval populations by toxin production which is contained in a parasporal crystal (Nartey *et al*., 2013); further analysis of the *Bacillus* isolates should reveal their potential for their biocontrol properties. The anti-*Plasmodium* activity of an isolate of *B. pumilus* was assessed in *A. gambiae* mosquitoes, but no effect on the

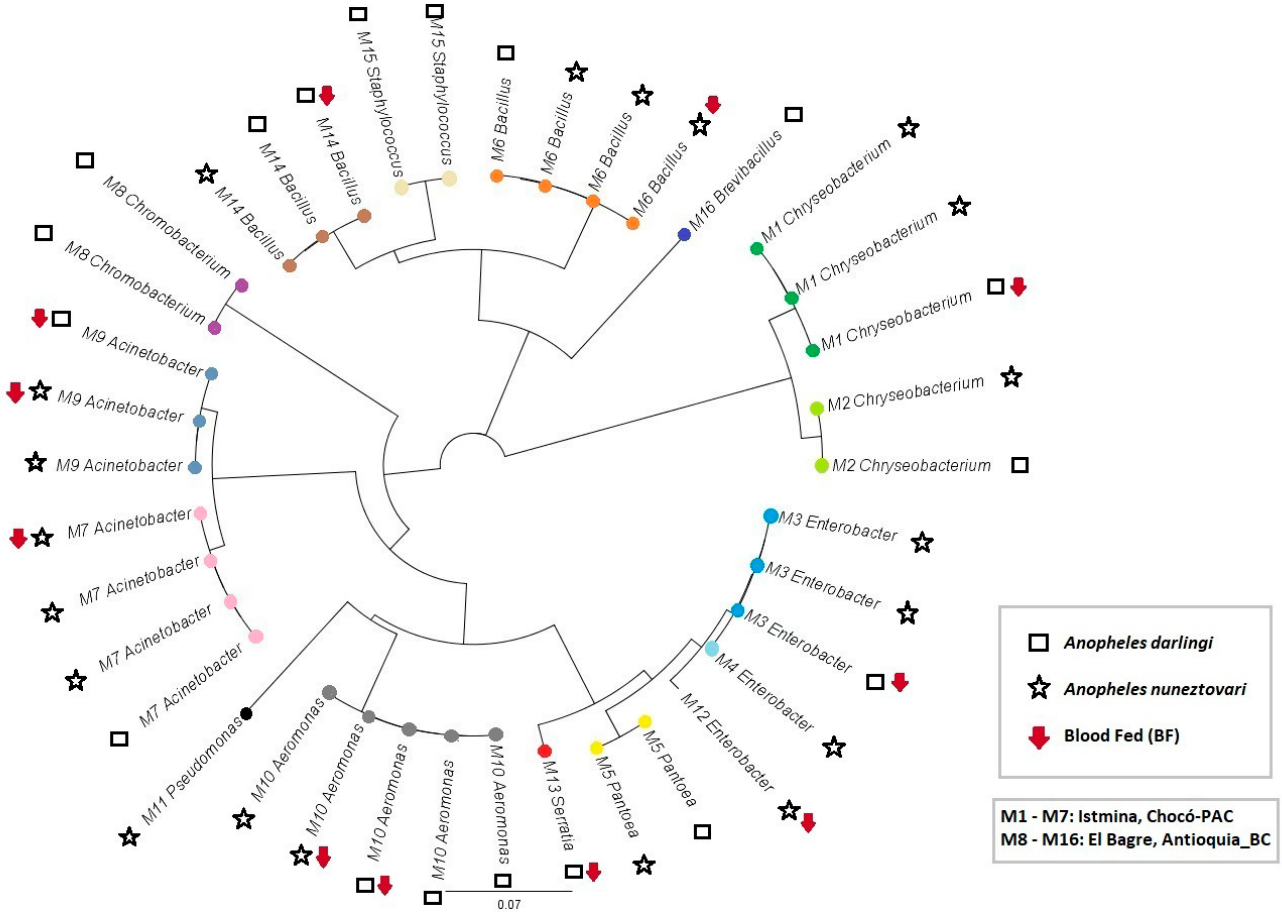

Figure 3. NJ tree representing bacterial genera identified per locality, mosquito species, and feeding status. Bacterial taxa at the genera level in samples from the gut of *Anopheles nuneztovari* and *Anopheles darlingi* mosquitoes collected in two localities of two malaria-endemic regions of Colombia: Istmina, Chocó in the Pacific region, W Colombia and El Bagre, Antioquia in the Urabá-Bajo Cauca-Alto Sinú region, NW Colombia. Grouped according to morphotypes and 16S rRNA sequences. Feeding status: Blood-fed (BF) or non-blood-fed (NBF).

oocyst or ookinete parasite stages was found (Cirimotich *et al*., 2011). Similarly, no toxic activity was observed when exposing *A. gambiae* adult mosquitoes to *B. thuringiensis israelensis* (Terbot *et al*., 2015). All of this reinforces that the parasporal crystal toxins are only effective in the mosquito's immature stages.

Other bacterial genera detected in the specimens of relevance to further evaluate for their properties to counteract *Plasmodium* include, *Chromobacterium*, *Serratia,* and *Pseudomonas*. *Chromobacterium* isolates were recovered from *A. darlingi* of Istmina and had a 100 % identity with *C. violaceum*. Notably, *C. violaceum* exerts an inhibitory activity against *Plasmodium* at micromolar concentrations, by the action of violacein pigment production (Gnambani *et al*., 2020). *Chromobacterium* strain Csp_P isolated from the gut of *Ae. aegypti*, affected the susceptibility of the mosquito to get infected by *Plasmodium* or dengue virus, becoming a candidate to control malaria and dengue; in addition, it reduced the larvae and adult survival, directly affecting vectorial capacity (Ramirez *et al*., 2014).

Furthermore, it is suggested that *Chromobacterium* could be useful in the control of other diseases due to its antileishmanial, trypanocidal, bactericidal, and cytotoxic activity (de Carvalho *et al*., 2006; Ferreira *et al*., 2004). Furthermore, some bacterial isolates recovered from blood-fed *A. darlingi* guts were biochemically and genetically identified as *Serratia marcescens*. This bacterial species has shown potential for malaria control through modulation of the immune system against *Plasmodium* or by larvicidal activity (Bai *et al*., 2019; Jupatanakul *et al*., 2020). Transcriptomic studies demonstrated that *Serratia* strain *Y1* is capable of activating the immune system of *A. stephensi* mosquitoes in response to infection by *Plasmodium bergei*, in addition to silencing parasite genes, and has a stronger response after blood ingestion (Bai *et al*., 2019). In addition, an *S. marcescens* has been identified which is capable of causing mortality in *Anopheles* (Diptera: Culicidae) *dirus* Peyton and Harrison, 1979, larvae by the secretion of protease and chitinase-like macromolecules (Jupatanakul *et al*., 2020). Therefore, it is important to explore the anti-*Plasmodium* activity or larvicidal capacity of the *S. marcescens* isolate found in the *A. darlingi* blood-fed specimens, to evaluate its potential for malaria control strategies in the endemic regions of Colombia. Furthermore, gut bacteria of the *Pseudomonas* genus were detected in *A. nuneztovari*. In a culture-dependent study with *Anopheles* mosquitoes collected in Iran, it is proposed that some *Pseudomonas* species are suitable paratransgenesis candidates since they were found in specimens from different localities, both, in larvae and adult samples (Chavshin *et al*., 2014). In addition, a metagenomics study showed that members of the Pseudomonadaceae family proliferate and persist as part of the mosquito gut microbiome upon blood ingestion (Das De *et al*., 2022). Therefore, it will be relevant to evaluate if isolates of the *Pseudomonas* genus detected in *A. nuneztovari* could establish and persist among the mosquito-target populations, a requirement for a paratransgenesis candidate.

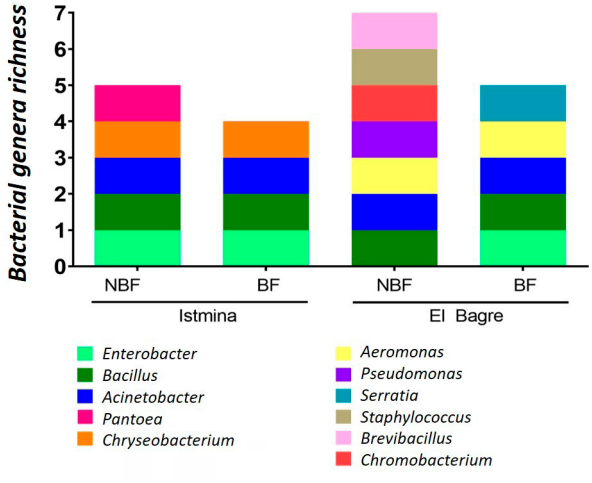

Figure 4. Bacterial community composition according to feeding status. Bacterial genera richness present in the main vectors *Anopheles nuneztovari* and *Anopheles darlingi* by locality and feeding status: blood-fed (BF) or non-blood-fed (NBF).

Of notice, a decrease in bacterial richness at the genus level was observed in female mosquitoes with blood in the intestine compared to those not blood-fed. A semi-field study on *A. gambiae* mosquitoes reported important changes in their intestinal microbial composition after blood meal digestion; a drastic reduction in community diversity occurred with a rapid increase in enteric bacteria capable of surviving under oxidative and nitrosative stress (Singh *et al*., 2022). Of the genera detected in blood-fed mosquitoes, *Enterobacter* is of particular interest considering that it is frequently found in the *Anopheles* gut and is capable of coping with the oxidative stress generated by blood ingestion (Dennison *et al*., 2016). As observed in the present study, *Enterobacter* significantly increased upon blood-feeding in *A. gambiae*; however, bacterial proliferation, and restructuring during and after blood-feeding should be aspects considered when choosing native candidates for *Plasmodium* biocontrol (Singh *et al*., 2022).

Among the limitations of our study are the sample size. However, we expect that this did not greatly influence the results, since the bacterial phyla and genera detected in the microbiota of the *Anopheles* species analyzed are considered part of the core microbiota (*Enterobacter*, *Bacillus,* and *Staphylococcus*), and this is important for the design of paratransgenic methods (Galeano-Castañeda *et al*., 2020). Also, the culture-based methodology used in this work could be considered a limitation, because of the impossibility of detecting nonculturable bacteria and compared to metagenomics, fewer bacteria will be detected; however, our group has characterized the microbiota of epidemiological

important *Anopheles* species by metagenomics (Bascuñán *et al.*, 2018; Galeano *et al.*, 2019; Galeano-Castañeda *et al.*, 2020) and this work constituted a complement to those studies. The advantage over metagenomic studies is the possibility of characterizing culturable bacteria which could be isolated for the creation of a bacterial bank and had the potential to be further evaluated for is antiparasitic or vector control potential.

CONCLUSIONS

This study expanded the knowledge of the culturable gut microbiota composition of two of the three main Colombian malaria vectors, *A. nuneztovari,* and *A. darlingi*. This constitutes an advancement in the field of mosquito-microbe interactions focused on the Colombian malaria vectors, of importance since the Latin American malaria vectors have largely been neglected in this regard. While most recent microbiome studies are based on genomic data generated from Next Generation Sequencing of non-viable microorganisms, the culture-based approach and characterization of bacterial communities using classical microbiology methods, although of a laborious nature, strengthens the study of the microbiota by generating data based on culturable, viable bacteria with potential for the development of disease mitigation strategies based on paratransgenesis.

AUTHOR'S PARTICIPATION

Conception/design of the research: PB, MMC; Data collection and experimental work SP, PUA, PB; Data analysis SP, PUA, PB, MMC; SP, PB, MMC wrote the manuscript. All authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available in the GenBank repository, under the accession numbers MH757423 - MH757445.

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