



# Culturable microbial composition in the midgut of *Aedes aegypti* strains with different susceptibility to dengue-2 virus infection

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## Abstract

Dengue is the widest spread vector-borne viral disease around the world and is transmitted mainly by the urban mosquito, *Aedes aegypti*. At present, vector control is the most widely used strategy to decrease disease incidence. However, it has demonstrated limited success. A new control strategy, associated with the manipulation of vector competence (VC) using endosymbiotic microorganisms, may be more sustainable because these microorganisms can influence mosquito development, the vector immune response, and vectorial capacity for infection with dengue virus (DENV). Hence, we explored the diversity of culturable midgut microbiota from two field-derived *Aedes aegypti* strains that are either susceptible or refractory to DENV infection and evaluated how strain-level dissection of the gut microbiome modulates VC. Microbial identification was carried out by mass spectrometry using MALDI-TOF, Vitek-2, BD Phoenix, and 16 s rRNA sequencing. There were differences in the composition and density of midgut microbiota in both mosquito strains. The refractory strain showed the highest microbial diversity and density with the highest prevalence of Gram-negative bacteria including *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Escherichia* genera. In the susceptible strain, only Gram-positive bacteria of the *Bacillus* genus and *Candida* yeast were observed in the midgut. To evaluate the effect of midgut microbiota on DENV-2 infectivity in both *Aedes aegypti* strains, mosquitoes were treated with sugar and an antibiotic/antimycotic cocktail or sugar alone (the control) and were subsequently challenged with a mixture of blood and DENV-2. DENV-2 infection in the mosquitoes' heads (salivary glands) and midguts was evaluated after an extrinsic period of fourteen days with indirect immunofluorescence. A significant increase in DENV-2 susceptibility was observed in the treated refractory strain from 51.22% to 86.64% (Chi-square = 9.747,  $p < 0.05$ ), while no changes were observed in the susceptible strain. These results confirm that susceptible and refractory mosquito strains may influence or be influenced by the presence of different gut microorganisms that affect virus infection susceptibility.

**Keywords** Antibiotics · Aseptic mosquitoes · Immune system · Mass spectrometry · Microbiome · Pupae

## 1 Introduction

Dengue is the most widely-distributed viral vector-borne disease in the world (Nene et al. 2007) with around 128 countries in tropical and subtropical regions, containing 3.97 billion people (Brady et al. 2012), reported to be endemic. There are an estimated 390 million cases of dengue annually of

which 96 million manifests as clinical disease (dengue fever and severe dengue) (Bhatt et al. 2013). The transmission is primarily by *Aedes aegypti*, a highly anthropophilic mosquito (Nene et al. 2007), from which the females feed on vertebrate blood for their nutrition, development of eggs, and survival (Andrade et al. 2005). Dengue disease control focuses principally on decreasing mosquito populations. However, the rapid development of insecticide resistance has limited the effectiveness of this vector control method (Hill et al. 2014), resulting in an ineffective (Simmons et al. 2012) and expensive control strategy that does not reduce incidence of disease (Packierisamy et al. 2015; Shepard et al. 2013). In consequence, studies of the biology, behavior, and life cycle of *Aedes aegypti* are necessary for the development of new control strategies (Gusmão et al. 2010).

Manipulation of vector competence VC (the intrinsic ability of a vector to transmit a pathogen) can be used as a strategy

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for vector control. VC is related to anatomical barriers. Pathogens such as DENV must attach and penetrate barriers (specifically, the midgut infection barrier (MIB) and midgut escape barriers (MEB)) to replicate within salivary glands and transmit the virus to the next host (Serrato et al. 2017). Mosquito VC manipulation has been used to reduce its capacity to transmit pathogens to humans (Jupatanakul et al. 2014). Currently, there is growing interest in understanding how gut bacteria interfere with VC, which has led several studies of the microbiota associated with different species of mosquitoes in the last decades (Ricci et al. 2012). It has been reported that endosymbionts microorganisms affect mosquito development, immune response, and the capacity to be infected by a virus (Jupatanakul et al. 2014).

Studies of the ecological mosquito-microorganism relationship have suggested that these symbiotic associations have implications for the evolutionary success of the mosquitoes and their wide geographic distribution (Ricci et al. 2012). Previous work has proposed that in Diptera larvae stages, some bacteria produce necessary nutrients for larval development and cytochrome *bd* oxidase as an important product for larval molting (Coon et al. 2017), while in the adult phase, these same microorganisms can produce enzymes which play a role in blood digestion (Gaio A de et al. 2011) and plant sap metabolism (Minard et al. 2013). Likewise, the presence of some bacteria in the midgut of the mosquito vectors can impact not only digestion but also other physiological characteristics such fertility, immune response or pathogen clearance, and embryonic development (Fouda et al. 2001; Dong et al. 2009). In this context, we propose to determine if there are differences in the microbial composition in midguts of field-derived strains of Colombian *Aedes aegypti* that differ in their CV or susceptibility (refractory strain: Cali-MIB or susceptible strain: Cali-S) to dengue-2 virus infection (Caicedo et al. 2013) and if the absence of microorganisms in the midgut affects mosquito vector competence. This is probable due to the impact of the microbiome in the immune response of mosquitoes or the antagonist effect of microorganisms over dengue infection or both.

## 2 Materials and methods

### 2.1 Strains and mosquito breeding

*Aedes aegypti* with different vector competence were chosen through isofamily selection from field derived *Ae. aegypti*, collected from Cali-Colombia, that were exposed to DENV-2. Offspring were selected from mothers with midgut infection barriers (Cali-MIB), midgut escape barriers (Cali-MEB), or no barriers (susceptible) to DENV-2 (Cali-S) (Caicedo et al. 2013). Studies were carried out with the Cali-S (F<sub>31</sub>) and Cali-MIB (F<sub>30</sub>) strains, which were 94 and 42% susceptible

to DENV-2 infection, respectively. Additional studies were conducted using a third field-collected strain from Cali (Serrato et al. 2017), Paso del Comercio (F<sub>4</sub>), which has a susceptibility of 55%.

Mosquitoes were maintained under standard conditions (28 °C, 85% relative humidity and 12:12 h light: dark cycle). Approximately, 300 larvae were hatched in 2 L of dichloride water and were fed with a stock solution of dehydrated beef liver (DIFCO™ Liver 8 g/mL). Adults were fed with a 10% sugar solution.

### 2.2 Mosquito dissections for microorganism isolation

Midguts were dissected from pupae and adult mosquitos from the three *Ae. aegypti* strains (Cali-S, Cali-MIB and Paso del Comercio) to compare the diversity of the culturable midgut microbiota in each developmental stage. For midgut dissection, the work surface was sterilized with sodium hypochlorite (2%) followed by ethanol (70%), before irradiation with UV light for 15 min. We used sterile dissecting tools (needles, tweezers, and slides). The surface of the mosquito was sterilized by immersion in ethanol (70%) for one minute; the midgut was then extracted, pooled (five intestines per tube, replicated five times for each strain), and stored in 1.5 mL tubes with 100 µL of sterile PBS. The environmental control was a 1.5 mL tube containing only sterile PBS that was opened while we extracted the midguts to expose the contents to the same conditions as the pooled solutions. Using a laminar flow cabinet, each pooled tube was macerated and the homogenized and control were seeded on Luria-Bertani broth (LB) for 24 h at 35 ± 2 °C. Subsequently, 1:200 dilutions from LB broth were plated and incubated for 24 h at 35 °C in blood and chocolate agar for testing hemolytic activity and in MacConkey agar for isolating Gram-negative bacteria.

### 2.3 Microorganism identification

Isolated bacterial colonies were purified in Mueller-Hinton agar, while Sabouraud agar was used in yeast colonies, for later identification by Vitek-2, MALDI-TOF, and BD Phoenix Yeast ID.

During the MALDI-TOF process, we added 1 µL of 70% formic acid over a steel anchor plate to every sample well. One colony from every purified culture was allowed to dry over the plate; then they were covered with 2 µL of matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid) dissolved in 50% acetonitrile, 47.5% water, 2.5% trifluoroacetic acid, and left to dry again. A positive control (*Staphylococcus aureus* ATCC 25923) and negative control (matrix solution and formic acid) were used in every run. Bruker Biotyper 3.0 software and library version 3.3.1.0 (4613 entries) were used to analyze spectra. Log-scores of 1.9 or greater were considered reliable for species-level identification, while scores of  $\geq 1.7$  and  $< 1.9$

were considered possible species-level identification. Identifications with scores below 1.7 were considered unreliable (Kathuria et al. 2015).

In the case of Vitek-2, the bacterial suspensions were prepared from pure colonies for all strains. The colonies were suspended in salt solution, and the turbidity was adjusted to 0.5 McFarland. The prepared bacterial cultures were analyzed using the GP ID card and the VITEK® 2 system (bioMérieux, Paris, France) according to the manufacturer. Results were expressed as defined by the manufacturer (96%–100%, excellent identification; 93%–95%, very good identification; 89%–92%, good identification; 85%–88%, acceptable identification; below 85%, no identification) (Pincus 2006).

For BD Phoenix Yeast ID, the panels were inoculated with a pure yeast suspension adjusted to 2.0 McFarland, as determined using the BD PhoenixSpec nephelometer. Then the panels were loaded into the BD Phoenix instrument, incubated at 35 °C, and interpreted automatically by the device. As the Phoenix does not report low confidence values, lower than 90%, results with scores of >90% were considered to represent positive identifications (Marucco et al. 2018).

In those identifications with a low level of confidence for these methodologies, we used 16S rRNA sequencing to corroborate the results. DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit. Total DNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE). Primers used for PCR reactions were: forward 5'-AGA GTT TGA TCH YTY AGA TGG-3' and reverse 5'-TTG TTA ACC ACG GYT CGA CTT-3' to a fragment of 1504 pb. PCR reactions were run at: 94 °C for one minute, and 35 cycles of 94 °C: 30 s, specific primer Tm 60 °C: 30 s, and 72 °C: 5 min, followed by 72 °C: 10 min. The PCR products were visualized on an agarose gel (1%), before being sequenced, and the sequences were analyzed by BLAST (NCBI) and Ribosomal Data Project (RDP) database.

## 2.4 Standardization of antibiotic and antimycotic concentrations

Two standard antibiotics reported in the literature, penicillin (100 ppm)/streptomycin (100 ppm) were initially tested; however, it was necessary to add gentamicin (75 ppm) and metronidazole (40 ppm) for the complete elimination of bacteria. Fluconazole (100 ppm) was used as an antifungal for yeast elimination. Efficiency tests were performed on solid LB culture media and thioglycollate broth for anaerobic bacteria.

## 2.5 Aseptic mosquitoes

Pupae from Cali-S and Cali-MIB strains were separated in two cages. In the first cage, adults were fed with 1 mL of sterile sugar solution (10%) containing antibiotics and antimycotic

cocktail previously tested and standardized. In the second cage, control adults were fed with 1 mL of a sterile sugar solution (10%) without antibiotics or antimycotics. Both treatments were given ad libitum using cotton balls replaced every day. Treatment was carried out for ten days to ensure removal of the microbiota in the midgut by antibiotic/antimycotic treatment. To validate the efficiency of antibiotic and antimycotic treatment, five midguts from each group (control untreated and antibiotic/antimycotic treated mosquitoes) were evaluated. The samples were dissected and macerated in sterile PBS, seeded in LB broth and after 24 h at 35 °C, and plated in blood agar, chocolate agar and MacConkey agar (35 °C for 24 h) to examine the growth of microorganisms.

## 2.6 Vector competence

Sterile and control female mosquitoes were fed with defibrinated rabbit blood mixed with an equal volume of a cell suspension infected with dengue virus serotype-2 (DENV-2, New Guinea C,  $10^{-8.5}$  TCID<sub>50</sub> (Tissue culture Infective Dose/mL)). The virus was grown in *Aedes albopictus* C6/36HT cells in Leivovitz 15 medium (L15) with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine at  $31 \pm 1$  °C, as described previously (Higgs et al. 1996; Caicedo et al. 2013). For 14 days the infected cells were incubated at 31 °C in L15 medium supplemented with 2% heat-inactivated FBS, 1% penicillin/streptomycin, and 1% L-glutamine. Females were allowed to feed for about two hours and subsequently introduced in their respective cages within safety cabinets. They continued with antibiotic/antimycotic treatment and control mosquitoes were fed with 1 mL of a sterile sugar solution (10%) without antibiotics and antimycotic during the post-infection period (14 days).

Vector competence was assessed by determining the anatomical barriers to virus infection. After the post-infection period, indirect immunofluorescence (IFI) (Monoclonal antibody against DENV-2: sterile PBS (1:200), provided by the CDC, and fluorescein isothiocyanate fluorochrome: sterile PBS (1:200)) was measured in mosquito salivary glands. Mosquitoes with positive head IFI results were categorized as having a susceptible phenotype. In mosquitoes with negative head IFI results, their midguts were assessed for the presence of DENV using IFI. Mosquitoes that were negative for DENV infection in both the head and midgut were classified as having refractory phenotype via a midgut infection barrier (MIB), while those with a negative head IFI but positive midgut IFI were classified as having a refractory phenotype via a midgut escape barrier (MEB).

## 2.7 Statistical analysis

Chi-square was used to compare if there were differences in vector competence after treatment with antibiotic/antimycotic

cocktail in Cali-S, Cali-MIB and Paso del Comercio strains, using PAST v3.16 software (Hammer et al. 2001).

### 3 Results

#### 3.1 Microorganism identification

Microorganisms were successfully cultured in the three different mediums used. The plated dilutions (1:200) of LB broth containing each group of midguts allowed isolation of pure colonies, without overlapping, for identification. The chocolate and blood agar culture mediums allowed for the growth of an equal number of gut microbiota species, whereas MacConkey mainly favored the growth of Gram-negative bacilli (Fig. 1). There was no microbial growth in the negative control.

The different identification methodologies allowed identifying a greater quantity of species in the adult stage of the refractory Cali-MIB strain, compared with the susceptible Cali-S and Paso del Comercio strains. Adults of Cali-MIB strain presented mainly Gram-negative species from *Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* families, and yeast of Saccharomycetales order (*Candida* sp.), while the susceptible strains, Cali-S and Paso del Comercio strains, presented only yeast (Saccharomycetales) and Gram-positive *Bacillus megaterium* in (Table 1).

The culturable microbial community comparison (Fig. 2) between adult and pupa stages showed only one species of gut microbiota, *Pseudomonas aeruginosa*, was present in both Cali-MIB and Paso del Comercio strains. However, a comparison of the adult stage between the two strains showed the presence of the *Candida* genus in them; while in the pupa stage, *Pseudomonas aeruginosa* was the species in common. Adult Cali-MIB mosquitoes had the highest number of culturable gut microbiota species ( $n = 5$ ).

#### 3.2 Aseptic mosquitoes

Different experiments were carried out to standardize gut microbiota sterilization in mosquitoes. Initially, mosquitoes were challenged for 5 days with penicillin (100 ppm), streptomycin (100 ppm) and gentamycin (75 ppm) (Dong et al. 2009). However, following treatment, bacterial and yeast growth was detected in Muller Hinton Agar and anaerobic bacteria was detected in thioglycollate broth (data not shown). Subsequently, we increased the concentrations of penicillin and streptomycin to 150 ppm and 200 ppm, respectively, keeping the concentration of gentamicin constant. Bacteria were eliminated at 150 ppm, but mosquito mortality was observed at 200 ppm. However, we still saw evidence of anaerobic bacteria growth at 200 ppm. Considering these results, a cocktail of 150 ppm of penicillin, 150 ppm streptomycin, 75 ppm of gentamicin and 40 ppm of metronidazole for

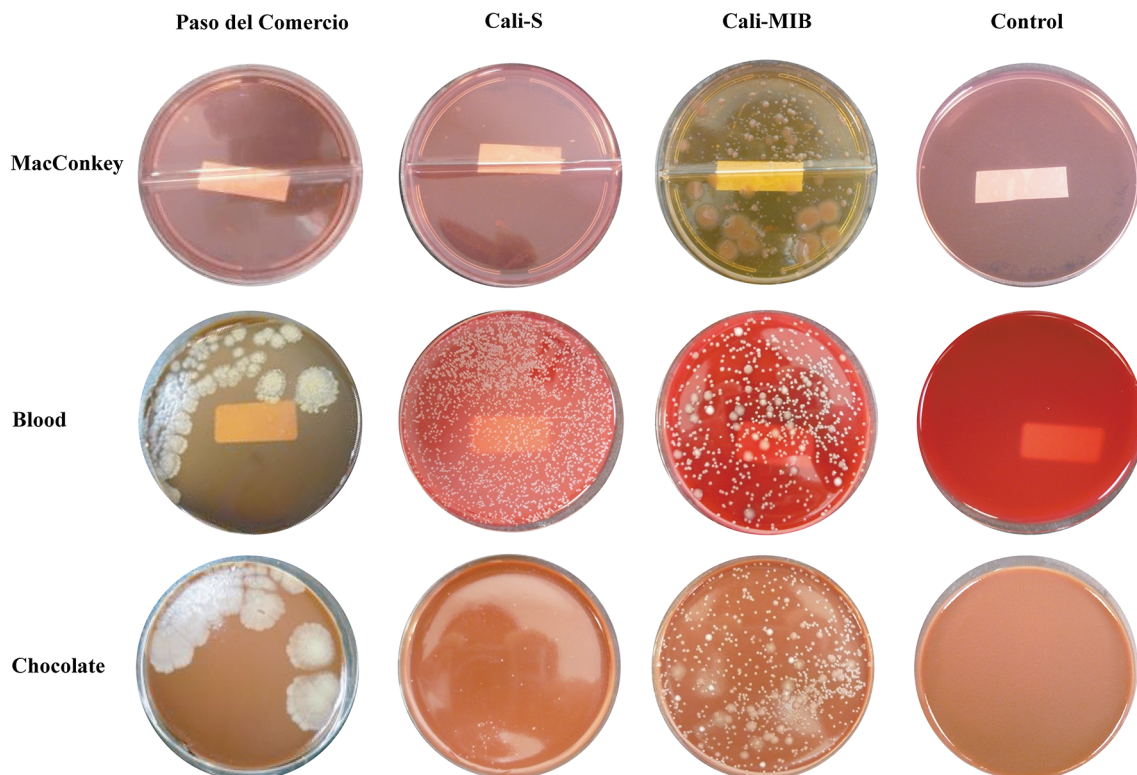


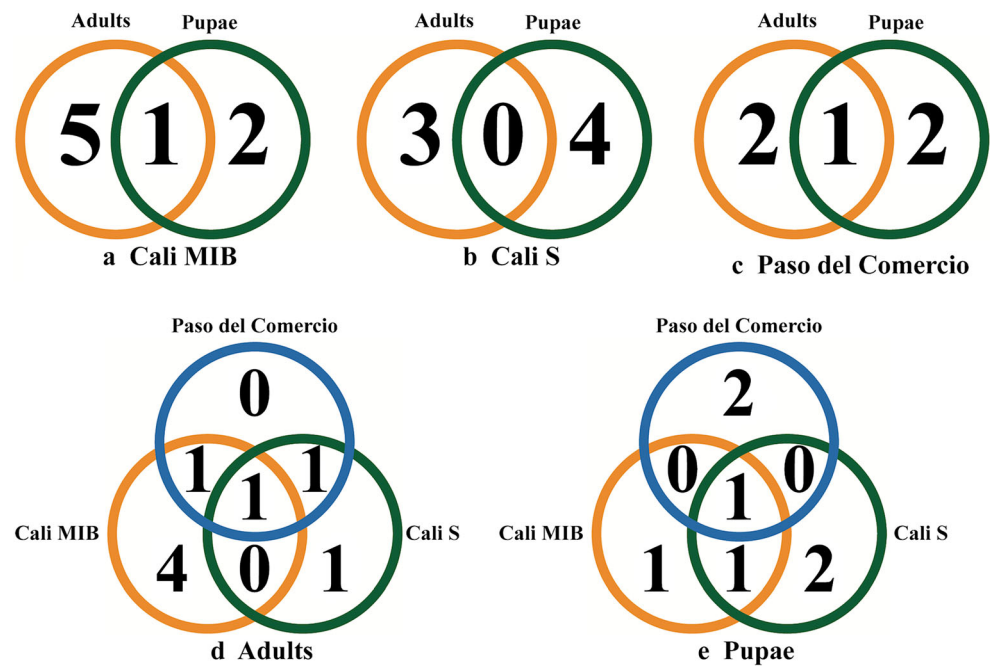
Fig. 1 Microorganism colonies isolated from midguts of Paso del Comercio, Cali-S, and Cali-MIB strings in MacConkey, blood and chocolate agars



**Table 1** Comparison of culturable microorganisms isolated from Cali-S, Cali-MIB and Paso del Comercio strains. I.M.: Identification method; VIT: Vitek-2; MAL: MALDI-TOF; BDP: BD Phoenix, 16S: Sequencing of 16S rRNA (NCBI and RDP databases analysis)

Family	Strain	Cali-S			Cali-MIB		
		Adults	I.M (Identity %)	Pupae	I.M (Identity %)	Adults	I.M (Identity %)
Pseudomonadaceae	<i>Pseudomonas aeruginosa</i>		VIT (99)	<i>P. aeruginosa</i>		VIT (99)	
	<i>Pseudomonas putida</i>		MAL (96.5); VIT (97)				
Enterobacteriaceae	<i>Serratia marcescens</i>		MAL (99.9); VIT (99)				
	<i>Escherichia hermannii</i>		VIT (99)				
Xanthomonadaceae	<i>Stenotrophomonas maltophilia</i>		MAL (99.9)				
Aeromonadaceae							
	<i>Aeromonas hydrophila</i>			<i>Aeromonas hydrophila</i>		VIT (98); MAL (99)	
Microbacteriaceae				<i>Microbacterium paraoxydans</i>		MAL (99)	
Moraxallaceae							
Staphylococcaceae							
Bacillaceae							<i>Bacillus megaterium</i> BDP (99)
Debaryomycetaceae							<i>Candida guilliermondii</i> MAL (99.9); VIT (91)
	<i>Candida</i> sp.		VIT (99)				<i>Candida parapsilosis</i> MAL (99)
Strain							
Cali-S							
Pupae							
		I.M (Identity %)		I.M (Identity %)			I.M (Identity %)
<i>P. aeruginosa</i>			VIT (99)	<i>P. aeruginosa</i>		VIT (99)	VIT (99)
<i>Pseudoxanthomonas mexicana</i> .			16S (99 NCBI; 0.842 RDP)				
	<i>A. hydrophila</i>		VIT (98); MAL (99)				
<i>Acinetobacter bereziniae</i> .			16S (97 NCBI; 0.901 RDP)	<i>Leucobacter komagatae</i> .			16S (92 NCBI; 0.519 RDP)
				<i>Staphylococcus haemolyticus</i>			16S (98 NCBI; 0.324 RDP)
				<i>B. megaterium</i>		BDP (99)	
				<i>C. guilliermondii</i>		MAL (99.3)	

**Fig. 2** Venn diagram with the number of culturable species shared between adult and pupa stages from the same strain **a.** Cali-MIB; **b.** Cali-S; **c.** Paso del Comercio; and among the different strains for a stage **d.** adults and **e.** pupae



anaerobic bacteria was empirically selected due to its ability to eliminate growth of all microorganisms without an impact on mosquito fitness.

Finally, for yeast elimination, an antifungal agent, fluconazole (100 ppm) was added. Combined, this tested cocktail of five drugs was used to generate aseptic mosquitoes for the VC tests.

### 3.3 Vector competence

The refractory strain showed significant changes to VC (Cali-MIB, Chi-square = 9.747,  $p < 0.05$ ): the susceptible phenotype increased from 51.22% to 86.64%, and the refractory phenotypes (MIB and MEB) decreased after antibiotic/antimycotic treatment (Table 2). In contrast, the susceptible strains, Cali-S and Paso del Comercio, did not have significant changes in VC phenotypes (Cali-S; Chi-square = 3.0265,  $p = 0.220198$  and Paso del Comercio; Chi-square = 0.4277,  $p = 0.807461$ ) (Table 2).

**Table 2** Vector competence tests in antibiotic/antimycotic treated and control samples, n: refers to individuals. Phenotypes, Susceptible: Head infected by DENV-2, MIB: Midgut infection barrier, and MEB: Midgut escape barrier

Phenotype	Strains											
	Cali-MIB				Cali-S				Paso del Comercio			
	Control		Antibiotics		Control		Antibiotics		Control		Antibiotics	
	n	%	n	%	n	%	n	%	n	%	n	%
Susceptible	21	51.22	26	86.67	34	91.89	25	78.13	20	64.52	21	56.76
MIB	11	26.83	2	6.67	2	5.41	3	9.38	7	22.58	10	27.03
MEB	9	21.95	2	6.67	1	2.7	4	12.5	4	12.9	6	16.22
Total	41	100	30	100	37	100	32	100	31	100	37	100

## 4 Discussion

A larger number of microorganisms were isolated from the Cali-MIB strain, compared with the Cali-S and Paso del Comercio strains, suggesting that the susceptible strains have a reduced diversity of gut microbiota, but it needs to be emphasized that microbial diversity was assessed through culturable techniques and the majority of microorganisms are not culturable using the current methods. Additionally, when these strains are exposed to DENV and their gut microbiota diversity is controlled with antibiotics/antimycotics, the susceptible strains maintain VC, while the refractory strain increases susceptibility. Previous studies by our group have demonstrated differences in the innate immune response between these strains when they are exposed to DENV-2 (Caicedo et al. 2018; Ocampo et al. 2013; Serrato et al. 2017). The refractory strain Cali-MIB has shown an increase in the innate immune response, especially of apoptosis-related genes when these mosquitoes are exposed to DENV, while the

susceptible strains Cali-S and Paso del Comercio did not demonstrate this type of reaction (Ocampo et al. 2013; Serrato et al. 2017). Recently, we reported the potential function of a *Gram-negative bacteria binding protein* (GNBP) gene in the Cali-MIB strain during viral clearance (Caicedo et al. 2018). This gene could be related to the following hypothesis: the presence of bacteria and/or yeasts could induce the immune system by producing specific molecules that interact directly with DENV, such as antiviral or antiparasitic compounds, which influences VC. There exists the potential that these differences in susceptibility to DENV infection in response to differential midgut microbiota may be associated with genetic characteristics related to the innate immune response.

It has been described that microbiota associated with mosquitoes is mainly composed of bacteria (Pidiyar et al. 2002; Touré et al. 2000). Common bacterial species from *Escherichia*, *Serratia*, and *Pseudomonas* genera were also identified in this work. Similar with our results, previous studies have also noted the presence of yeasts from the *Candida* genus in *Aedes* (Gusmão et al. 2010). The few colonies isolated in the pupae stage supports the process of gut-sterilization, which consists of “cleaning” the microflora associated with larvae during metamorphosis and the acquisition of a new set of microbes during adulthood. Pupal midgut contents included microbes that are encased in meconial peritrophic matrices and egested (Moll et al. 2001).

Recently, Charan et al. (2013) conducted a metagenomic study in African strains of *Ae. aegypti* with differential susceptibility to DENV, finding a greater diversity of microorganisms in the midgut of refractory strains, compared with susceptible and field strains. Similarly, they found that only the refractory strain had bacteria of *Pedobacter* sp. and *Janthinobacterium* sp. genera. They suggest that these organisms may inhibit the establishment and incubation of DENV given their production of metabolites with antiparasitic and antiviral characteristics. Moreover, Wu et al. (2019) identified that *Serratia marcescens* facilitates arboviral infection in field-derived strains of *Ae. aegypti*. The abundant presence of *S. marcescens* increased ratios of DENV-positive whole mosquitoes, midguts, heads and salivary glands in two field strains, which indicates a potential association between the gut-inhabiting *S. marcescens* and the dengue prevalence in *Aedes* mosquitoes.

On the other hand, strain-specific mosquito gut microbiota might inhibit the development of vector-borne pathogens (Cirimotich et al. 2011) due to their strong influence on host immunity. Some bacteria can directly interfere with mosquito VC, such as the impact of *Serratia* and *Enterobacter* species on *Plasmodium* development in *Anopheles* mosquitoes (González-Cerón et al. 2003); however, regulation of viral resistance in *Ae. aegypti* can also occur through the expression of specific genes (Ocampo et al. 2013; Caicedo et al. 2018.). Increased microbiota

density triggers the host innate immune response to control the bacterial load; in this way, microbiota associated with the midgut of *Ae. Aegypti* indicates a complex interaction between mosquito, parasites, and flora (Ricci et al. 2012).

Meanwhile, we found that antibiotic treatment leads to increased susceptibility of the Cali-MIB strain to DENV. Other studies in *Anopheles gambiae* identified a subset of immune genes upregulated by mosquito microbiota using transcription profiles of septic and aseptic mosquitoes (Dong et al. 2009). These genes correspond to several anti-*Plasmodium* factors such as cecropins, defensins, and gambicins. Aseptic mosquitoes showed increased susceptibility to *Plasmodium falciparum*, while mosquitoes fed with bacteria and gametocytes of *P. falciparum* showed low levels of infection. That suggests that the anti-*P. falciparum* effect induced by endogenous bacteria is mediated by the antimicrobial immune response of mosquitoes that likely occurs through modulation of gene expression of the immune system by the microbiota (Dong et al. 2009). It has been shown that gut microflora is capable of activating the innate immune system of *An. gambiae* and indirectly protects and prevents possible reinfection of *P. falciparum* (Rodrigues et al. 2011). Conversely, a mechanism was identified that inhibits *Plasmodium* establishment in *An. gambiae* without apparent involvement of the innate immune response. Instead, the mechanism is due to factors produced by enterobacteria (Cirimotich et al. 2011). Equally, in 2014, Ye and colleagues conducted a study comparing mosquitoes with and without antimicrobial treatment, showing little effect on fitness after microbial infection or DENV infection. Also, there is evidence that some bacteria species facilitate the establishment of the pathogens in *Ae. aegypti*. For example, *Serratia odorifera* can significantly increase the viral susceptibility in aseptic *Ae. aegypti* females, while females fed only with another bacteria or DENV do not change (Apte-Deshpande et al. 2012). It was similarly found a decrease in the susceptibility to DENV when *Proteus* sp. or *Paenibacillus* sp. were incorporated in aseptic females (Ramirez et al. 2012).

In general, our results contribute to the understanding of vector-pathogen interactions and the identification of candidate targets for the future design and development of control strategies that include genetic manipulation of mosquito VC through transgenesis or paratransgenesis by altering gut symbionts. This method suggests that bacteria can be genetically modified to express anti-pathogenic molecules capable of interfering in the infection and replication of pathogens in mosquitoes (Coutinho-Abreu et al. 2010).

In conclusion, we used one field-collected strain of *Ae. aegypti* and two laboratory-selected strains (Cali-S and Cali-MIB) from the CIDEIM insectary based on their susceptibility to DENV-2. Aseptic mosquitoes were produced through an empirical standardization method which included an optimal mixture of antibiotics and antimycotic in sugar sterile feed. We use

methods different from the traditional ones used in this type of studies and noted differences in the culturable microbial communities present in Cali-S and Cali-MIB strains. The susceptible strain has the lowest quantity of identified species, but it is unclear how this process takes place. Elimination of gut microbiota in the refractory strain increased susceptibility to DENV, suggesting that the antipathogenic effect induced by endogenous bacteria or yeasts is mediated by the antimicrobial immune response of the mosquitoes. This likely occurs through modulation of immune system gene expression by the mosquito due to a microbial infection. However, further studies should identify how DENV-susceptible mosquitoes control the presence of midgut microorganisms and, therefore, do not activate their immune system when infected with DENV.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Experimental protocols and the use of hamsters and rabbit blood to feed and maintain insect colonies at CIDEIM were approved by the CIDEIM Institutional Review Committee for Research in Animals (#1021) (Comité de Ética para la Investigación en Animales Experimentales- CEIA). The CEIA is governed by law 84 of 1989 and resolution 8430 of 1993 of the National Ministry of Agriculture of Colombia in which scientific, technical and administrative standards are established for animal research.

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