Original Article

Interactions of chikungunya virus with Asian tiger mosquitoes (*Aedes albopictus*): vector competence and diversity of midgut bacteria

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Abstract

Chikungunya virus (CHIKV) is an important mosquito-borne virus. Previous studies have revealed the influence of midgut bacteria on infection with this pathogen. In this study, we examined the interactions of CHIKV with *Aedes albopictus* (Skuse) in terms of vector competence and the diversity of midgut bacteria. The diversity of midgut bacteria in CHIKV-infected and non-infected *Ae. albopictus* was observed after feeding with blood with different titers of CHIKV. CHIKV transmission rates on day 14 post-blood feeding in *Ae. albopictus* were 42%, 70%, 100%, 90% and 83% after being fed on 10^2, 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL of CHIKV, respectively. *Micrococcus* was the dominant genus in CHIKV-infected mosquitoes fed at CHIKV titers of 10^2, 10^3, 10^5 and 10^6 $TCID_{50}$/mL, but *Bacillus* was the dominant genus at 10^4 $TCID_{50}$/mL. Non-infected mosquitoes differed from the CHIKV-infected mosquitoes fed at 10^3 and 10^5 $TCID_{50}$/mL in that *Staphylococcus* was dominant. At the species level, *Micrococcus luteus* was the dominant species in *Ae. albopictus* fed on a CHIKV-negative blood meal, CHIKV non-infected mosquitoes fed on CHIKV at 10^2 $TCID_{50}$/mL., and CHIKV-infected mosquitoes fed at CHIKV titers of 10^2, 10^3, 10^5 and 10^6 $TCID_{50}$/mL.

Keywords: asian tiger mosquito, bacteria, chikungunya virus, microbiome, vector competence

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Introduction

Chikungunya virus (CHIKV), an important mosquito-borne virus in humans, is an *Alphavirus* of the Togaviridae family. It was first isolated in Tanzania in 1952, and its emergence and re-emergence have been widely reported from various countries, including China, France, Malaysia, India and Thailand (Thaikruea *et al*., 1997; Powers *et al*., 2000; Charrel *et al*., 2007; Thavara *et al*., 2009). CHIKV causes an acute illness in humans with symptoms such as high fever, headache, nausea, vomiting, rash and severe joint pain. The unique clinical sign of CHIKV-associated diseases is generalized arthralgia, which may continue for months or years. *Aedes aegypti* and *Ae. albopictus* are the primary mosquito vectors in the transmission of CHIKV and other arboviruses, including the dengue virus (DENV) and Zika virus (ZIKV) (Thavara *et al*., 2009; Chompoosri *et al*., 2016; Tuanudom *et al*., 2017; Suwanmanee *et al*., 2018; Gloria-Soria *et al*., 2020; Gutierrez-Bugallo *et al*., 2020; Vega-Rua *et al*., 2020). Co-infection with CHIKV, DENV, and ZIKV has been reported in humans (Vongpunsawad *et al*., 2017; Suwanmanee *et al*., 2018). However, the disease caused by CHIKV infection is more severe and more generalized than that caused by DENV (Pialoux *et al*., 2007). *Culex quinquefasciatus* is another mosquito that plays an important role as a competent vector for CHIKV (Lutomiah *et al*., 2021).

Outbreaks of CHIKV in East Africa and Comoros during 2004–2005 revealed *Ae. aegypt*i as the potential vector (Powers & Logue, 2007). However, the possible vector for the transmission of CHIKV in recent outbreaks in the Indian Ocean islands, Asia, Africa and Europe was *Ae. albopictus* (Bonilauri *et al*., 2008; Thavara *et al*., 2009; Paupy *et al*., 2010; Tuanudom *et al*., 2017; Fortuna *et al*., 2018). A variant of CHIKV with the substitution of A226V in the E1 glycoprotein (E1-A226V) was responsible for recent outbreaks in these countries, including southern Thailand, where *Ae. albopictus* was the competent vector for this virus (Reiskind *et al*., 2010; Tuanudom *et al*., 2017). However, this study indicated that E1:A226V mutation is not exclusively responsible for the ability of CHIKV to replicate in this mosquito species well (Fortuna *et al*., 2018).

"Vector competence" is a term referring to a vector's ability to acquire, maintain and transmit a specific pathogen. CHIKV infects the mosquito midgut following ingestion of viremic blood, replicates within the midgut, disseminates to the salivary glands and emerges in the saliva, to be transmitted when the mosquito bites another host. Many factors may either obstruct or facilitate infection and hence affect the dissemination and transmission of CHIKV by vectors. The midgut and salivary glands act as barriers to virus infection, dissemination and transmission. Understanding these mechanisms is essential for creating innovative strategies to control CHIKV transmission.

The midgut of mosquito vectors contains not only harmful pathogens but also diverse microbiota. The diverse midgut microbiota significantly affects the development, digestion, metabolism and immunity of mosquitoes. This microbiota can influence the susceptibility of the host to pathogens and its ability to transmit these pathogens (Dennison *et al*., 2014). Over the last few years, many studies have focused on the role of midgut bacterial communities on the fitness of the host and the competence of various mosquito vectors with respect to pathogen transmission (Azambuja *et al*., 2005; Gusmao *et al*., 2007). New mosquito and disease control strategies are required to lessen the human and animal health concerns caused by mosquito-borne diseases. Recent findings have established the importance of the mosquito microbiota in disease transmission. However, few studies have been conducted into the midgut microbiota of *Ae. albopictus* and its interactions with CHIKV infection. This study was performed to examine the interactions of CHIKV with *Ae. albopictus* in terms of vector competence and the changes of diversity of the midgut bacteria. The diversity of the midgut bacteria in CHIKV-infected and non-infected *Ae. albopictus* was observed after the mosquitoes were fed a CHIKV-infected blood meal. This study provided important information for future basic and advanced research on the interactions between midgut bacteria and CHIKV, which may be extended to infections with other pathogens. An examination of the relationships between mosquitoes and their microbiota may lead to the development of novel tools that can complement current strategies for disease prevention and control.

Materials and Methods

Mosquitoes and mosquito blood feeding: Laboratory-raised *Aedes albopictus* were used in this study. The mosquitoes were initially collected from Nonthaburi Province and raised in the Department of Medical Sciences, Ministry of Public Health, Thailand. Subsequently, they were maintained in the Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, for more than ten generations. The mosquitoes were divided into five groups and fed on blood meals with different CHIKV titers (0, 10^2, 10^3, 10^4, 10^5, and10^6 median tissue culture infective dose ($TCID_{50}$)/mL), using an artificial membrane feeder covered with porcine intestinal membrane. The blood meals contained the viral stocks, 20% fetal bovine serum, 1% sucrose, 70% (v/v) packed sheep erythrocytes, and 3 mM ATP (Smith *et al.,* 2005; Tsetsarkin *et al.,* 2007). The engorged females were maintained at 28°C ± 5°C, at 80% ± 5% relative humidity, with a 12:12 h photoperiod. This study was conducted in 2014, at which time mosquitoes were not included in the experimental animals that needed approval from the Chulalongkorn University Animal Care and Use Committee**.**

Chikungunya virus: An Indian Ocean lineage of CHIKV, isolated from a patient infected during an outbreak in Thailand in 2008–2009, was used in this study**.** The virus was kindly provided by the Faculty of Medicine, Chulalongkorn University**.** CHIKV was propagated in the African Green Monkey Kidney (Vero) cells and stored under liquid nitrogen. The CHIKV and viral infected mosquito suspensions were

titrated in Vero cells and confirmed for CHIKV infection using immunocytochemistry (ICC) assay and reverse transcription-polymerase chain reaction (RT-PCR).

Mosquito dissection and saliva collection: On day 14 post-blood feeding, the mosquitoes were kept at 4°C for 10 mins and their legs and wings were removed. The proboscis was inserted into a 20 µL sterile pipet tip containing 5% (w/v) sucrose solution in Modified Eagle's Medium (MEM) and 20% (v/v) fetal bovine serum (FBS) for 20 mins for saliva collection (Tiawsirisup *et al.*, 2005). Each saliva sample was then transferred into a separate tube containing 200 µL of 10% (v/v) FBS in MEM, and CHIKV was detected using immunocytochemistry (ICC) assay and RT-PCR. The presence of CHIKV in the mosquito saliva was taken to indicate the mosquito's ability to transmit the virus: the vector competence of the mosquito. Then, the midgut was dissected and processed for bacterial isolation and identification.

CHIKV detection: A 100 µL of mosquito saliva sample was inoculated into 96-well plates containing Vero cell monolayers. Cell cultures were observed for cytopathic effects for up to seven days and confirmed for CHIKV infection using ICC assay and RT-PCR. The cells were then fixed with 4% (w/v) formalin, washed with 0.5% (v/v) Tween-20 in phosphate-buffered saline and incubated with mouse monoclonal antibody (McAb) against CHIKV (Abcam, Cambridge, United Kingdom) for 1 h. After washing, they were incubated with rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP; Dako Cytomation, Carpinteria, California) for 1 hour and the color was developed using a chromogen aminoethyl carbazole substrate (Sigma-Aldrich, St. Louis, MO, USA). The infected cells, showing a red color, were recorded to calculate the $TCID_{50}$ of the virus (Reed and Muench, 1938). Viral nucleic acid was also extracted from the cell culture medium using a viral nucleic acid extraction kit II (Geneaid Biotech, Taiwan) and examined for CHIKV using RT-PCR as previously reported, using the DVRChk-R (5′-GGGCGGGTAGTCCATGTTGTAGA-3′) and DVRChk-F (5′-ACCGGCGTCTACCCATTCAT GT-3′) primers (Naresh Kumar *et al.*, 2007; Theamboonlers *et al.*, 2009).

Bacterial isolation and identification: After dissection, the midgut content from each mosquito was suspended in 300 µL of 60% (v/v) glycerol, and a 100 µL aliquot of the suspension was spread on Tryptose Soya Agar supplemented with 5% (v/v) sheep blood and incubated at 37°C for 24 hours. Individual bacterial isolates from the mosquito midguts were subcultured in 2 mL of Tryptose Soya Broth at 37°C for 24 hours and harvested by centrifugation at 20,000 rcf for 15 mins and the pellet was washed in distilled water. A total of 40 µL of distilled water was added to the pellet, which was resuspended by vortexing and then kept at 100°C for 10 mins before being cooled on ice and clarified by centrifugation at 20,000 rcf for 10 mins. The 16S ribosomal RNA gene was then amplified using two pairs of eubacteria-specific primers. The first primer pair was the 16SF (5′–AGTTTGATCCTGGCTCAG–3′) and 16SR (5′–GCTACCTTGTTACGACTT C–3′) (Dinparast Djadid *et al.*, 2011) and the second pair was 63F (5′–CAGGCCTAACACATGCAAGTC–3′) and 1387R (5′–GGGCGGWGTGTACAAGGC–3′) (Marchesi *et al.*, 1998). The amplified fragments were purified using Gel/PCR DNA Fragments Extraction Kits (Geneaid Biotech) and submitted for sequencing. The partial 16S rRNA gene sequences were assembled and analyzed using the Lasergene package version 5.03 (DNASTAR, Inc., Madison, Wisconsin, USA). The sequences were compared with those in the GenBank database using the BLAST algorithm (NCBI, 2016). Homologous sequences were retrieved from GenBank using BLASTn search and aligned using the ClustalW program (Thompson *et al.*, 1994). Phylogenetic relationships were determined by tree reconstruction using the neighbor-joining (NJ) method with the Kimura-2 parameter for distance calculation incorporated in the MEGA 7.0.26 package (Kumar *et al.*, 2016). The robustness of the phylogenetic tree was examined using 1000 bootstrap replicates and the consensus tree was used for analysis.

Results

Chikungunya virus transmission rates in Aedes albopictus: There were 30 blood-fed mosquitoes in each group, except for the group fed on 10^2 $TCID_{50}$/mL of CHIKV, which contained only 24 blood-fed mosquitoes, due to a shortage of living mosquitoes on day 14 post-blood feeding (PBF). The CHIKV transmission rates in mosquitoes fed on 10^2, 10^3, 10^4, 10^5, and 10^6 $TCID_{50}$/mL of CHIKV on day 14 PBF were 42%, 70%, 100%, 90% and 83%, respectively (Table 1).

Midgut bacterial composition of Ae. albopictus fed different levels of CHIKV-infected blood: The midgut bacterial genera were identified from *Ae. albopictus* fed on blood containing different titers of CHIKV. The genera varied between the mosquito groups but there was no significant difference among all CHIKV-infected groups (Table 1). The bacterial genera were dominated by *Micrococcus* in the CHIKV-infected mosquitoes that were fed on 10^2 $TCID_{50}$/mL of CHIKV and were significantly different in the infected and non-infected mosquito groups ($P < 0.05$). In mosquitoes fed on 10^3 $TCID_{50}$/mL of CHIKV, the dominant bacterial genera were *Micrococcus*, followed by *Staphylococcus*, while the main bacterial genera in the non-infected mosquitoes were *Staphylococcus* followed by *Micrococcus*. The dominant bacteria in CHIKV-infected mosquitoes fed on 10^4 $TCID_{50}$/mL of CHIKV were *Bacillus* followed by *Staphylococcus*. *Micrococcus* was the most prevalent bacterial genus found in CHIKV-infected mosquitoes fed on 10^5 $TCID_{50}$/mL of CHIKV while *Staphylococcus* was the only bacterial genus found in the midgut of non-infected mosquitoes. The midgut bacteria in the CHIKV-infected mosquito group fed on 10^6 $TCID_{50}$/mL of CHIKV were also dominated by *Micrococcus*, followed by *Bacillus*, but no midgut bacteria were identified in non-infected mosquitoes. Lastly, the dominant midgut bacterial genera in *Ae. albopictus* after being fed on a CHIKV-negative blood meal were *Micrococcus* (Table 2). A total of 37 phylotypes were

observed when using a cutoff of 99% DNA sequence similarity. The 16S rRNA gene sequences from a variety of phylogenetic groups are shown in Fig. 1.

Midgut bacterial species of Ae. albopictus fed on different levels of CHIKV-infected blood: Eleven bacterial species were identified from the midgut of *Ae. albopictus* after being fed a CHIKV-negative blood meal, with the dominant species being *Micrococcus luteus* (Table 2). However, when fed on CHIKV at 10^2 $TCID_{50}$/mL, 13 bacterial species were identified, with the dominant bacterial species in both the CHIKV-infected and non-infected mosquitoes being Micrococcus luteus (Table 3).

When fed on CHIKV at 10^3 $TCID_{50}$/mL, the dominant midgut bacterial species in CHIKV-infected mosquitoes was *Micrococcus luteus*, while the dominant species in the non-infected mosquitoes was *Micrococcus yunnanensis*. However, there was no significant difference between the CHIKV-infected and non-infected mosquitoes (Table 4). After being fed on CHIKV at 10^4 $TCID_{50}$/mL, 11 bacterial species were identified from the CHIKV-infected mosquitoes, with an equal bacterial infection rate (Table 5). When mosquitoes were fed on CHIKV at 10^5 $TCID_{50}$/mL, the dominant midgut bacterial species was *Micrococcus luteus*, while in non-infected mosquitoes, the dominant species were *Staphylococcus haemolyticus* and *Staphylococcus warneri*. However, there was no significant difference between the CHIKV-infected and non-infected mosquitoes (Table 6). Finally, after being fed on CHIKV at 10^6 $TCID_{50}$/mL, 13 bacterial species were identified in the CHIKV-infected mosquitoes, with the dominant species being *Micrococcus luteus* (Table 7).

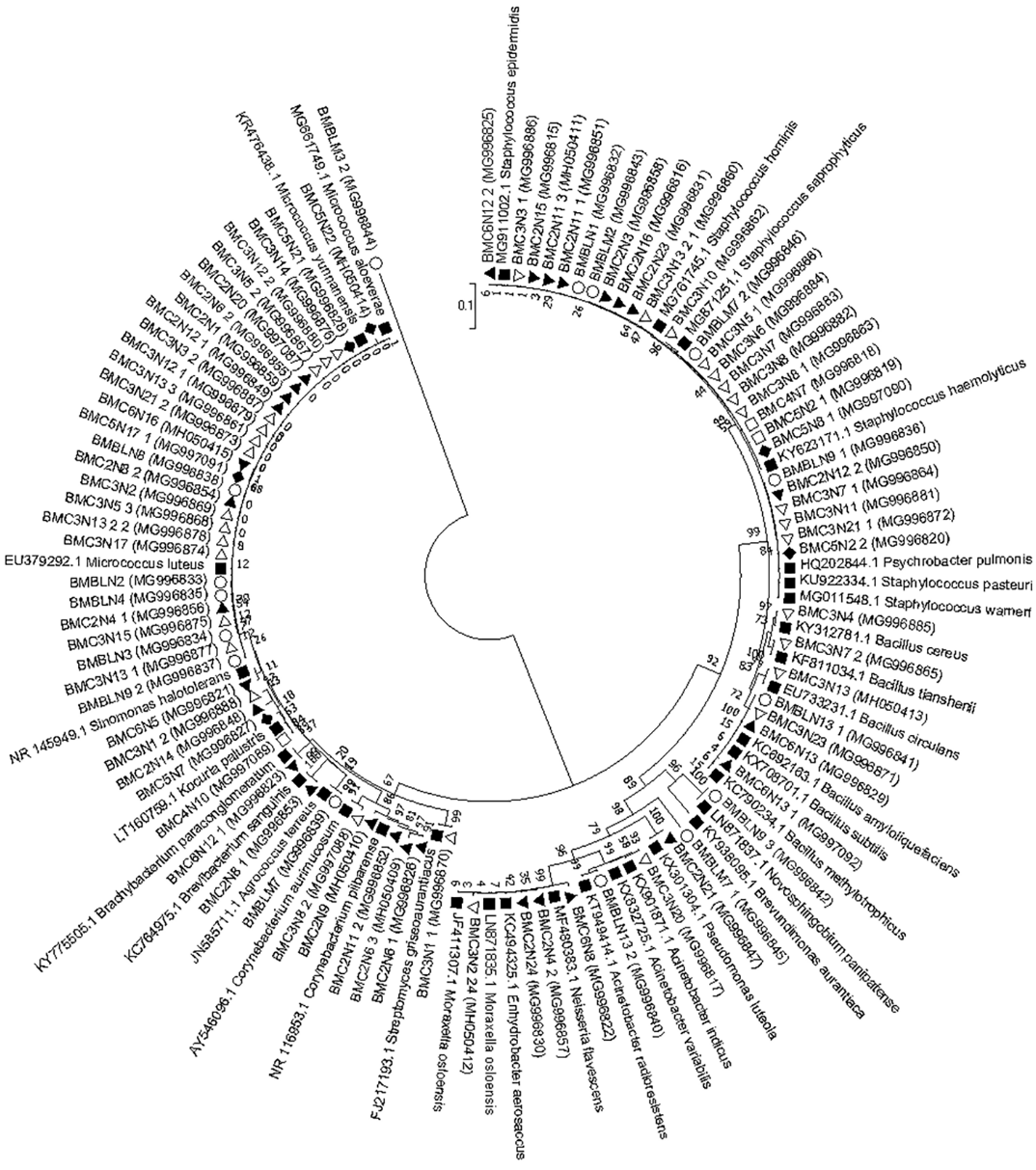

Figure 1 Phylogenetic tree (NJ) constructed from the partial 16S rRNA gene sequences (1,500 bp) from isolates cultured from CHIKV-infected and non-infected *Aedes albopictus*, with BS values given at the nodes. Entries with a black square represent reference names and accession numbers (in parentheses). Entries from this study are represented as strain number, accession number (in parentheses). (■reference names, ○ non-infected blood meal group, and blood meal infected with CHIKV at ▲ 10^2 $TCID_{50}$/mL, △ 10^3 $TCID_{50}$/mL, □ 10^4 $TCID_{50}$/mL, ◆ 10^5 $TCID_{50}$/mL, and ▼ 10^6 $TCID_{50}$/mL)

Table 1 Midgut bacterial infection rates in *Aedes albopictus* fed on different levels of CHIKV-infected blood

Bacteria phylum	Bacterial genus and Gram status	Midgut bacterial infection rates***									
		10^2 $TCID_{50}$/mL		10^3 $TCID_{50}$/mL		10^4 $TCID_{50}$/mL		10^5 $TCID_{50}$/mL		10^6 $TCID_{50}$/mL	
		CHIKV +*	CHIKV -**	CHIKV +	CHIKV -	CHIKV +	CHIKV -	CHIKV +	CHIKV -	CHIKV +	CHIKV -
		42% (n = 10)	(n = 14)	70% (n = 21)	(n = 9)	100% (n = 30)	(n = 0)	90% (n = 27)	(n = 3)	83% (n = 25)	(n = 5)
Actinobacteria	*Actinomyces* (+)	0	0	0	0	0	–	0	0	4.0 (1/25)	0
	Brachybacterium (–)	0	0	0	0	0	–	0	0	8.0 (2/25)	0
	Brevibacteriu (+)	10.0 (1/10)	0	0	0	3.3 (1/30)	–	0	0	0	0
	Corynebacterium (+)	20.0 (2/10)	0	0	11.1 (1/9)	0	–	3.7 (1/27)	0	0	0
	Kocuria (–)	0	7.1 (1/14)	4.8 (1/21)	0	3.3 (1/30)	–	3.7 (1/27)	0	0	0
	Micrococcus (+)	70.0 (7/10)	28.6 (4/14)****	52.4 (11/21)	22.2 (2/9)	3.3 (1/30)	–	33.3 (9/27)	0	16.0 (4/25)	0
	Sinomonas (+)	0	0	0	0	0	–	0	0	4.0 (1/25)	0
	Streptomyces (+)	0	0	0	11.1 (1/9)	0	–	0	0	4.0 (1/25)	0
Firmicutes	*Bacillus* (+)	0	0	14.3 (3/21)	11.1 (1/9)	10.0 (3/30)	–	0	0	12.0 (3/25)	0
	Paenibacillus (–)	0	0	0	0	3.3 (1/30)	–	0	0	4.0 (1/25)	0
	Staphylococcus (+)	50.0 (5/10)	21.4 (3/14)	38.1(8/21)	33.3 (3/9)	6.7 (2/30)	–	3.7 (1/27)	33.3 (1/3)	8.0 (2/25)	0
	Streptococcus (+)	0	0	0	0	3.3 (1/30)	–	3.7 (1/27)	0 (0/3)	0	0
Proteobacteria	*Acinetobacter* (–)	0	0	4.8 (1/21)	0	0	–	0	0	0	0
	Brevundimonas (–)	0	0	0	0	3.3 (1/30)	–	0	0	0	0
	Enhydrobacter (–)	10.0 (1/10)	0	0	0	0	–	0	0	0	0
	Moraxella (–)	0	7.1 (1/14)	0	11.1 (1/9)	0	–	7.4 (2/27)	0 (0/3)	4.0 (1/25)	0
	Pseudomonas (–)	0	7.1 (1/14)	0	0	0	–	0	0	0	0
	Psychrobacter (–)	0	7.1 (1/14)	0	0	0	–	0	0	0	0

*Indicated by CHIKV detection in mosquito saliva

**Indicated by no CHIKV detection in mosquito saliva

***Bacterial infected mosquitoes/tested mosquitoes

****Significant difference of midgut bacteria between CHIKV-infected and non-infected *Ae. albopictus*, determined by a Chi-square test ($P < 0.05$)

Table 2 Midgut bacterial infection rates in *Aedes albopictus* fed on CHIKV-negative blood

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **
Actinobacteria	*Agrococcus terreus*#	4.2 (1/24)
	Janibacter indicus	4.2 (1/24)
	Micrococcus luteus	12.5 (3/24)
	Micrococcus yunnanensis	8.3 (2/24)
Firmicutes	*Bacillus amyloliquefaciens*#	4.2 (1/24)
	Staphylococcus hominis	4.2 (1/24)
	Staphylococcus cohnii	4.2 (1/24)
	Staphylococcus pasteuri	4.2 (1/24)
Proteobacteria	*Acinetobacter radioresistens*	8.3 (2/24)
	Neisseria perflava	4.2 (1/24)
	Novosphingobium panipatens	4.2 (1/24)

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes

Table 3 Midgut bacterial infection rates in *Aedes albopictus* fed on 10^2 $TCID_{50}$/mL of CHIKV

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **	
		CHIKV +	CHIKV -***
Actinobacteria	*Brevibacterium sanguinis*#	10.0 (1/10)	0 (0/14)
	Corynebacterium jeikeium#	10.0 (1/10)	0 (0/14)
	Corynebacterium pilbarense#	20.0 (2/10)	0 (0/14)
	Kocuria marina#	0 (0/10)	7.1 (1/14)
	Micrococcus luteus	60.0 (6/10)	21.4 (3/14)
	Micrococcus yunnanensis	10.0 (1/10)	7.1 (1/14)
Firmicutes	*Staphylococcus epidermidis*	20.0 (2/10)	14.3 (2/14)
	Staphylococcus haemolyticus	10.0 (1/10)	0 (0/14)
	Staphylococcus hominis	30.0 (3/10)	7.1 (1/14)
Proteobacteria	*Moraxella osloensis*	0 (0/10)	7.1 (1/14)
	Pseudomonas luteola	0 (0/10)	7.1 (1/14)
	Psychrobacter pulmonis#	0 (0/10)	7.1 (1/14)
	Enhydrobacter aerosaccus#	10.0 (1/10)	0 (0/14)

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes
***CHIKV + indicated by CHIKV detection in mosquito saliva and CHIKV - indicated by no CHIKV detection in mosquito saliva on day 14 post-blood feeding

Table 4 Midgut bacterial infection rates in *Aedes albopictus* fed on 10^3 $TCID_{50}$/mL of CHIKV

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **	
		CHIKV +	CHIKV -***
Actinobacteria	*Corynebacterium aurimucosum*#	0 (0/21)	11.1 (1/9)
	Kocuria palustris	4.8 (1/21)	0 (0/9)
	Micrococcus luteus	42.9 (9/21)	0 (0/9)
	Micrococcus yunnanensis	28.6 (6/21)	22.2 (2/9)
	Streptomyces griseoaurantiacus#	0 (0/21)	11.1 (1/9)
Firmicutes	*Staphylococcus epidermidis*	4.8 (1/21)	0 (0/9)
	Staphylococcus haemolyticus	19.1 (4/21)	0 (0/9)
	Bacillus cereus	4.8 (1/21)	0 (0/9)
	Bacillus circulans#	4.8 (1/21)	0 (0/9)
	Bacillus methylotrophicus#	4.8 (1/21)	0 (0/9)
	*Bacillus tiansheni*i#	0 (0/21)	11.1 (1/9)
	Staphylococcus hominis	4.8 (1/21)	11.1 (1/9)
	Staphylococcus pasteuri	9.5 (2/21)	11.1 (1/9)
	Staphylococcus saprophyticus#	0 (0/21)	11.1 (1/9)
Proteobacteria	*Moraxella osloensis*	0 (0/21)	11.1 (1/9)
	Acinetobacter indicus#	4.8 (1/21)	0 (0/9)

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes
***CHIKV + indicated by CHIKV detection in mosquito saliva and CHIKV - indicated by no CHIKV detection in mosquito saliva on day 14 post-blood feeding

Table 5 Midgut bacterial infection rates in *Aedes albopictus* fed on 10^4 $TCID_{50}$/mL of CHIKV

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **	
		CHIKV +	CHIKV -***
Actinobacteria	*Brevibacterium casei*#	3.3 (1/30)	-
	Kocuria palustris	3.3 (1/30)	-
	Micrococcus luteus	3.3 (1/30)	-
Firmicutes	*Bacillus aquimaris*#	3.3 (1/30)	-
	Bacillus cereus	3.3 (1/30)	-
	Bacillus clausii#	3.3 (1/30)	-
	Paenibacillus lautus#	3.3 (1/30)	-
	Staphylococcus epidermidis	3.3 (1/30)	-
	Staphylococcus haemolyticus	3.3 (1/30)	-
	Streptococcus mitis	3.3 (1/30)	-
Proteobacteria	*Brevundimonas diminuta*#	3.3 (1/30)	-

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes
***CHIKV + indicated by CHIKV detection in mosquito saliva and CHIKV - indicated by no CHIKV detection in mosquito saliva on day 14 post-blood feeding

Table 6 The midgut bacterial infection rates in *Aedes albopictus* fed on 10^5 $TCID_{50}$/mL of CHIKV

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **	
		CHIKV +	CHIKV -***
Actinobacteria	*Corynebacterium ihumii*#	3.7 (1/27)	0 (0/3)
	Kocuria palustris	3.7 (1/27)	0 (0/3)
	Micrococcus luteus	29.6 (8/27)	0 (0/3)
	Micrococcus yunnanensis	11.1 (3/27)	0 (0/3)
Firmicutes	*Staphylococcus cohnii*	3.7 (1/27)	0 (0/3)
	Staphylococcus haemolyticus	0 (0/27)	33.3 (1/3)
	Staphylococcus warneri	0 (0/27)	33.3 (1/3)
	Streptococcus mitis	3.7 (1/27)	0 (0/3)
Proteobacteria	*Moraxella osloensis*	7.4 (2/27)	0 (0/3)

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes
***CHIKV + indicated by CHIKV detection in mosquito saliva and CHIKV - indicated by no CHIKV detection in mosquito saliva on day 14 post-blood feeding

Table 7 Midgut bacterial infection rates in *Aedes albopictus* after being fed on 10^6 $TCID_{50}$/mL of CHIKV

Bacterial phylum	Closest related bacterial species*	Midgut bacterial infection rates **	
		CHIKV +	CHIKV -***
Actinobacteria	*Actinomyces naeslundii*#	4.0 (1/25)	0 (0/5)
	Brachybacterium nesterenkovii	4.0 (1/25)	0 (0/5)
	Brachybacterium paraconglomeratum#	4.0 (1/25)	0 (0/5)
	Micrococcus aloeverae#	4.0 (1/25)	0 (0/5)
	Micrococcus luteus	12.0 (3/25)	0 (0/5)
	Sinomonas halotolerans	4.0 (1/25)	0 (0/5)
	Streptomyces pseudogriseolus#	4.0 (1/25)	0 (0/5)
Firmicutes	*Bacillus megaterium*#	8.0 (2/25)	0 (0/5)
	Bacillus subtilis	4.0 (1/25)	0 (0/5)
	Paenibacillus timonensis#	4.0 (1/25)	0 (0/5)
	Staphylococcus epidermidis	4.0 (1/25)	0 (0/5)
	Staphylococcus hominis	4.0 (1/25)	0 (0/5)
Proteobacteria	*Moraxella osloensis*	4.0 (1/25)	0 (0/5)

#Represents the bacterial species that were only found in this group
*All bacterial species were identified on the basis of a 16S DNA sequence similarity of higher than 99%
**Bacterial infected mosquitoes/tested mosquitoes
***CHIKV + indicated by CHIKV detection in mosquito saliva and CHIKV - indicated by no CHIKV detection in mosquito saliva on day 14 post-blood feeding

Discussion

Aedes albopictus mosquitoes are important vectors that can be found worldwide and are responsible for the transmission of various arboviruses. The current knowledge about the bacterial communities associated with *Ae. albopictus* mosquitoes is very limited and, consequently, their contribution to CHIKV infection is unclear. Therefore, the diversity and differences in midgut bacteria in CHIKV-infected and non-infected *Ae. albopictus* mosquitoes fed on infected blood meal were investigated. To the best of our knowledge, this is the first study in which an attempt has been made toward a comprehensive study and understanding of the correlation of varying CHIKV titers in blood meals and CHIKV infection in *Ae. albopictus* and the differences in the bacterial communities in the midguts of CHIKV-infected and non-infected *Ae. albopictus*. Due

to the limitations of bacterial isolation and identification, this investigation was limited to the characterization of culture-dependent aerobic bacteria from mosquito midguts. We were able to isolate and identify several midgut bacterial isolates using a combination of culture and 16S rRNA sequence-based approaches. These bacteria belonged to three major phyla of bacteria: Actinobacteria, Firmicutes, and Proteobacteria (Fig. 2).

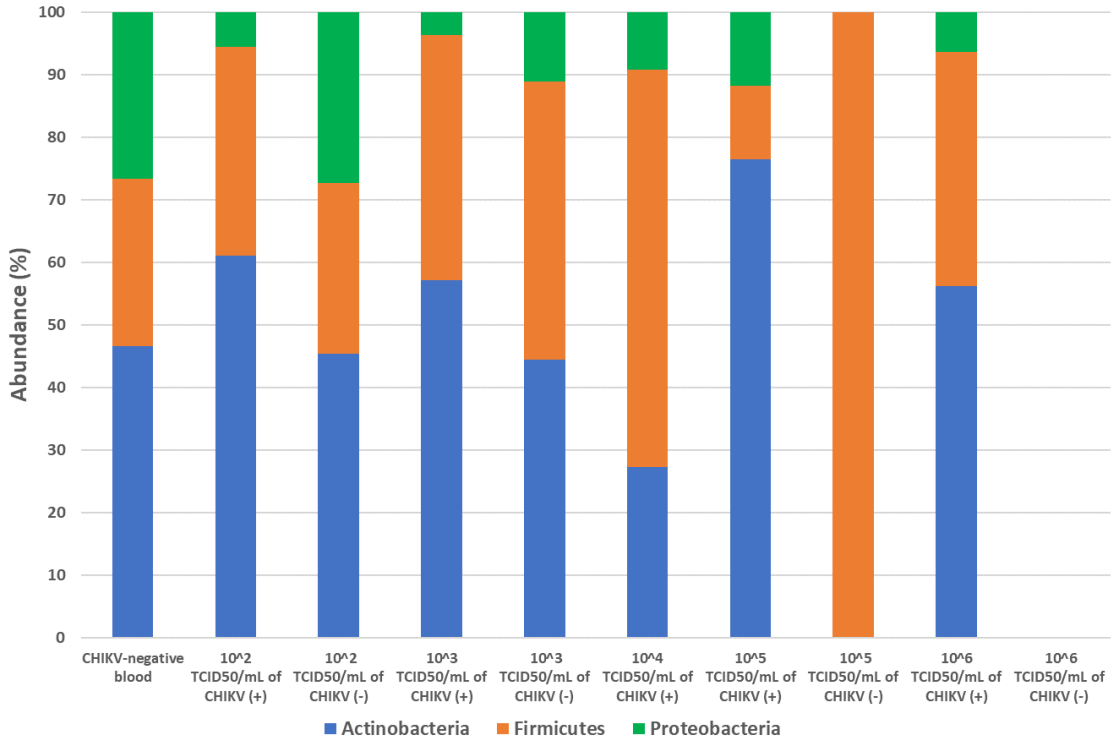

Figure 2 Abundance of identified bacterial species by phylum, isolated from the midgut of *Aedes albopictus* fed on CHIKV-negative blood and 10^2 - 10^6 $TCID_{50}$/mL of CHIKV

Ae. albopictus saliva samples were tested for CHIKV on day 14 PBF, to determine the mosquito's vector competence for CHIKV transmission. Transmission rates in *Ae. albopictus* were 42, 70, 100, 90 and 83% after fed on 10^2, 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL of CHIKV, respectively. However, CHIKV infection and dissemination rates in *Ae. Albopictus* are not shown in this study since they were previously published (Tuanudom *et al.*, 2017). According to this study, *Ae. albopictus* are efficient vectors for CHIKV transmission and the CHIKV titers in blood meals affect viral transmission in *Ae. albopictus*. The amount of CHIKV in blood meals also had an impact on virus infection and dissemination (Tuanudom *et al.*, 2017). CHIKV transmission rates increased following the increase of the virus titers in the blood meal. However, these rates are not exactly increased when the virus titer is rising. Many factors are involved in mosquito infection, dissemination and transmission, i.e., internal physiological barrier and amount of blood meal up taking. These variations are also found in West Nile virus infection, dissemination and transmission in mosquitoes (Tiawsirisup *et al.*, 2005).

The bacteria in the midgut were simultaneously investigated. The authors were concerned that the midgut bacteria detected may change between day 0 and day 14 PBF, a limitation of the research design. On day 14 PBF, the bacteria found may not be the same bacteria that interact with CHIKV on day 0 PBF. The dynamics of the bacterial community are influenced by disease propagation in mosquitoes, which may alter the mosquito vector competence. Members of the phyla Alphaproteobacteria and Gammaproteobacteria, as well as Bacteroidetes, responded to CHIKV infection, according to taxonomy microarrays and quantitative PCR. The variety of symbiotic bacteria in mosquitoes was also influenced by CHIKV. Upon CHIKV infection, the quantity of *Enterobacteriaceae* bacteria increased but that of *Wolbachia* and *Blattabacterium* declined (Zouache *et al.*, 2012).

Wolbachia and cultivable bacteria of the genera *Acinetobacter*, *Comamonas*, *Delftia* and *Pseudomonas* were isolated from the bodies and guts of both males and females of *Ae. albopictus* initially collected in La Réunion and maintained as colonies in insectaries (Zouache *et al.*, 2009). Isolated bacterial genera from wild *Ae. albopictus* and *Ae. aegypti* collected in Madagascar were dominated by *Bacillus*, followed by *Acinetobacter*, *Agrobacterium*, and *Enterobacter* (Zouache *et al.*, 2011). In Thailand, *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Cellulomonas*, *Chryseomicrobium*, *Dietzia*, *Enterobacter*, *Klebsiella*, *Microbacterium*, *Pantoea*, *Pseudomonas* and *Staphylococcus* were identified from laboratory-reared *Ae. aegypti* originally collected from Bangkok, while *Bacillus*, *Cellulomonas*, *Microbacterium*, *Micrococcus*, *Moraxella*, *Neisseria*, *Staphylococcus* and *Streptococcus* were found in *Ae. aegypti* collected from

Bangkok (Tiawsirisup *et al.,* 2018). *Staphylococcus* and *Micrococcus* were the most common bacterial genera in laboratory-colonized *Ae. albopictus*, whereas *Rhizobium* and *Agrobacterium* were more common in field-collected *Ae. albopictus* from Thailand. Only *Staphylococcus* differed significantly between laboratory-colonized and field-collected *Ae. albopictus* (Tuanudom *et al.,* 2021).

Wolbachia is an endosymbiotic gut bacterium that can prevent the chikungunya and dengue viruses from replicating in *Aedes* mosquitoes (Jayakrishnan *et al.*, 2018). *Wolbachia* infection has been identified in abundance in wild-caught *Ae. albopictus* in Malaysia and Thailand (Ahantarig *et al.*, 2008; Ahmad *et al.*, 2017). Previous studies have shown that the presence of *Wolbachia* does not significantly impact CHIKV infection and replication in the midgut or the dissemination to salivary glands in *Ae. albopictus*. Nonetheless, *Wolbachia* has a minimal effect on the CHIKV titer in the salivary glands (Ahmad *et al.*, 2017). However, the bacterial isolation and culture methods used in this study could not examine the *Wolbachia* infection and the involvement with CHIKV infection in the mosquitoes.

The bacterial species isolated from mosquitoes fed on CHIKV-negative blood meals were dominated by *Micrococcus luteus*, followed by *Micrococcus yunnanensis* and *Acinetobacter radioresistens*. *Agrococcus terreus* and *Bacillus amyloliquefaciens* were only isolated from this group of mosquitoes. These bacteria have been isolated from various environments, including soil samples, potato plants, dried seaweed and the air (Groth *et al.*, 1996; Zhang *et al.*, 2010). They might be found in the food upon which the mosquito larvae fed or in the blood meal provided to the adult mosquitoes. However, these bacteria were not isolated from other groups of mosquitoes fed under the same conditions.

Micrococcus was found to be the dominant culturable midgut bacterial genus found in CHIKV-infected *Ae. albopictus.* A previous study showed that these bacteria produce proteins involved in antibiotic tolerance and biofilm formation (Mali *et al.*, 2017), which may induce CHIKV susceptibility in *Ae. albopictus. Micrococcus luteus* was the most common bacterial species identified from mosquitoes fed on CHIKV-negative blood meals and from CHIKV-infected mosquitoes fed on CHIKV at 10^2, 10^3, 10^5 and 10^6 $TCID_{50}$/mL. *Micrococcus luteus* was also isolated from CHIKV-infected mosquitoes fed on CHIKV at 10^4 $TCID_{50}$/mL but with the same infection rate as other bacteria isolated from this group of mosquitoes. However, *Micrococcus luteus* was also the most prevalent bacterial species isolated from non-infected mosquitoes fed on CHIKV at 10^2 $TCID_{50}$/mL. In contrast, the most prevalent bacterial species isolated from non-infected mosquitoes fed on CHIKV at 10^3 $TCID_{50}$/mL was *Micrococcus yunnanensis* and those from non-infected mosquitoes fed on CHIKV at 10^5 $TCID_{50}$/mL were *Staphylococcus haemolyticus* and *Staphylococcus warneri*.

In conclusion, differences in the community composition of the midgut bacteria in CHIKV-infected and non-infected *Ae. albopictus* were found in this study. The bacterial communities were diverse in each mosquito group but there was no correlation between the midgut bacteria and CHIKV infection status in *Ae. albopictus* nor any specific pattern of midgut bacteria between CHIKV-infected and non-infected *Ae. albopictus*. These inconsistencies may have resulted from differences in mosquito generation, blood meal source, diet, or environment, all of which may affect bacterial infection in mosquitoes. More studies need to be conducted to identify the role of each midgut bacterial species and its association with CHIKV infection in mosquitoes.

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