DIVERSITY OF BACTERIA IN BREEDING WATER AND MIDGUT OF *Aedes albopictus*, AND DETERMINATION OF ITS PARATRANSGENIC CANDIDATES AND OVIPOSITIONAL ATTRACTANT

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UNIVERSITI SAINS MALAYSIA

2023

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by

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

June 2023

ACKNOWLEDGEMENT

I would like to take this opportunity to extend my greatest gratitude and appreciation to everyone involved in this journey of a lifetime of mine. First and foremost, I would like to thank my supervisor, Dr. Intan Haslina Ishak, Prof. Madya Dr. Hamdan Ahmad and Prof. Madya Dr. Abd. Hafiz for their invaluable input, detailed comments, support and encouragement for me to complete a PhD under their supervision. I would like to express my gratification to my beloved father, Mohd. Fadzlah, my late mother, Allahyarhamah Che Rosnani, husband, Mohamad Shafiq, daughter, Marissa Suraya and my parents in-law, Rugayah and Jamaluddin for their endless guidance, support and encouragement for me to complete a PhD. The conduct of this research would not have been possible without the generous help from the staff of School of Biological Sciences and Vector Control Research Unit (VCRU) especially NoorHazwani. I am forever thankful for their cooperation and kindness for assisting me with my data collection. Most important of all, I would like to thank my dearest family Rusbi, Farah and Fatin, and friends especially Nurul Akmar, Najihah and Syafika for their kindness and friendship who made this journey bearable at times when I was at my lowest and who were always there to celebrate my accomplishments. Last but not least, the funders of this studentship, Fellowship and Research Grant of Universiti Sains Malaysia are gratefully acknowledged for the financial support and opportunities. Thank you.

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LIST OF SYMBOLS

%	Percentage
°C	Degree celcius
bp	Base pair
cm	Centimeter
gm	Gram
g/L	Gram per liter
h	Hour
kb	Kilobase pair
L	Liter
m	Meter
mg	Miligram
min	Minute
mL	Mililiter
mm	Milimeter
N	Normality
sec	Second
μg/ml	Microgram per mililiter
μL	Microliter

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment search tool
cDNA	Complementary DNA
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
NA	Nutrient Agar
NB	Nutrient broth
BHI	Brain Heart Infusion
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
OD	Optical density
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
pH	Potential hydrogen
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
USM	Universiti Sains Malaysia

KEPELBAGAIAN BAKTERIA DALAM AIR PEMBIAKAN DAN USUS TENGAH *Aedes albopictus* DAN PENENTUAN CALON PARATRANSGENIK DAN TARIKAN OVIPOSISI

ABSTRAK

Para penyelidik telah mula memberikan tumpuan kepada pengurusan vektor dan pelaksanaan kawalan biologi untuk menggantikan kesan berbahaya daripada penggunaan racun serangga yang berlebihan. Kajian ini dijalankan untuk memberikan pengetahuan kukuh mengenai kepelbagaian bakteria dalam air pembiakan dan usus tengah *Aedes albopictus*. Kajian mengenai bakteria ini penting untuk penentuan paratransgenik dan tarikan oviposisi. Kaedah kultur-bergantungan dan kaedah kultur-tanpa bergantungan digunakan untuk penilaian perbandingan bakteria dari bekas tiruan dengan kehadiran dan ketiadaan larva. Menariknya, kaedah kultur-bergantungan menunjukkan kepelbagaian bakteria yang lebih tinggi dalam air pembiakan dengan kehadiran larva tetapi Unit Taksonomi Operasi (OTU) yang lebih tinggi jika tiada larva melalui kaedah kultur tanpa bergantungan. Keputusan ini menyerlahkan kepentingan sejenis bakteria yang bertanggungjawab sebagai tarikan atau penghalang terhadap nyamuk. Kepelbagaian mikrobiota yang lebih tinggi terdapat dalam usus tengah nyamuk peringkat dewasa berbanding peringkat larva menyimpulkan bahawa kedua-dua persekitaran dan perwarisan bertanggungjawab sebagai sumber mikrobiota dalam usus tengah. Kehadiran secara konsisten *Bacillus cereus* dan *Proteus mirabilis* dalam air pembiakan dan kedua-dua peringkat usus tengah *Ae.albopictus* berfungsi sebagai calon awal yang berpotensi untuk paratransgenik. Calon paratransgenik yang diuji adalah *B. cereus* dan *P. mirabilis* telah menunjukkan bahawa *B. cereus* berfaedah dan lebih sesuai sebagai calon paratransgenik. Larva dapat mengambil bakteria *B. cereus* daripada air pembiakan dan bakteria ini telah tersedia di

dalam usus tengah nyamuk bagi kedua-dua peringkat berdasarkan kepada kumpulan kawalan. Larva yang dirawat dengan *B. cereus* (114.09c) adalah lebih besar daripada larva yang dirawat dengan *P. mirabilis* (90.91b) dan kumpulan kawalan (26.00a), $x^2(2)=108.534$, p=0.000. Penentuan daya tarikan oviposisi bagi satu spesies bakteria dengan kepekatan sel bakteria yang berbeza adalah terdiri daripada *B. cereus*, *P. mirabilis* dan *Enterobacter cloacae*. Hasil kajian menunjukkan bahawa *E. cloacae* dengan kepekatan 10^{-6} memberikan daya tarikan oviposisi yang lebih tinggi jika dibandingkan dengan bakteria dan kepekatan lain, $F[_{3,20}] = 4.744$, p = 0.012, $P<0.05$. Kesimpulannya, kajian mikrobiota ini seterusnya akan membawa ke arah penemuan kawalan vektor yang lebih baik termasuk calon paratransgenik dan ovitrap yang cekap untuk pengawasan nyamuk.

DIVERSITY OF BACTERIA IN BREEDING WATER AND MIDGUT OF *Aedes albopictus,* AND DETERMINATION OF ITS PARATRANSGENIC CANDIDATES AND OVIPOSITIONAL ATTRACTANT

ABSTRACT

Researchers have started to focus on vector management and implementation of biological control to replace the hazardous effect of excessive insecticide usage. This study was conducted to provide fundamental knowledge of bacteria diversity in breeding water and midgut of *Aedes albopictus*. The identification of these bacteria is important for its roles as paratransgenesis and oviposition attractant. The culture-dependent and culture-independent methods were used for the comparative assessment of bacteria from artificial containers with the presence and absence of larvae. Interestingly, the culture-dependant method showed a higher bacterial diversity in breeding water with the presence of larvae but higher operational taxonomic units (OTUs) in the absence of larvae by culture-independent method. These results highlighted the importance of a type of bacterium that is responsible for the attraction or deterrent effects towards mosquitoes. Higher microbiota diversity present in an adult mosquito's midgut compared to larval stage concluded that both environmental and vertical inheritance are the microbiota sources in the midgut. The consistent presence of *Bacillus cereus* and *Proteus mirabilis* in breeding water and both stages of *Ae. albopictus* mosquito's midgut served as potential early candidates for the paratransgenesis. The paratransgenic candidates tested for *B. cereus* and *P. mirabilis* showed that the *B. cereus* was beneficial and more suitable as paratransgenic candidate. The larvae were able to take the *B. cereus* bacteria from the breeding water and was readily established inside the mosquito's midgut in both stages based on the control group. The larvae that was treated with *B. cereus* (114.09c) was significantly larger than those treated with *P. mirabilis* (90.91b) and control group (26.00a), $x^2(2)$=108.534, p=0.000. The determination of oviposition attractant of a single species of bacterium with different bacterial cell concentration consisted of *B. cereus*, *P. mirabilis* and *Enterobacter cloacae*. The result showed that *E. cloacae* with a concentration of 10^{-6} gave a significantly higher oviposition attractant when compared with other bacteria and concentration, $F_{[3,20]}$ = 4.744, p = 0.012, P<0.05. As a conclusion, this microbes studies will subsequently lead towards the discovery of better vector control including paratransgenic candidate and efficient ovitraps for mosquito surveillance.

CHAPTER ONE

INTRODUCTION

1.1 Background study

Aedes albopictus is an invasive vector that is rapidly expanding its territory worldwide (Cui et al., 2021). These mosquitoes exhibit tolerance to a broad range of temperatures, making them well adapted to temperate and tropical regions (Chuchuy et al., 2018). They are associated with diverse places that are inclined towards the outdoors than indoors, including the peridomestic environment, and also target artificial containers as their breeding sites (Cui et al., 2021). In addition, they revolve around human environment and feed on humans and domestic animals. *Aedes albopictus* is one of the principal vectors responsible for transmitting dengue, including the life-threatening haemorrhage syndrome (Nazni et al., 2019), chikungunya and yellow fever viruses. Additionally, *Ae. albopictus* is also linked to the current resurgence of a new public health threat known as Zika virus (ZIKV) that is associated with neurological complications and birth abnormalities (Cui et al., 2021; Ritwik et al., 2015; Waggoner et al., 2016). Dengue is declared among the fastest-growing global infectious disease with a devastating 100-400 million cases annually including 380171 cases and 113 deaths for 2023 and in Malaysia, 46495 dengue cases had been reported (Brady & Hay, 2020; ECDC, 2023).

The dramatic increase in dengue cases have negatively impacted the country and the diseases' control largely depend on the control of the vectors, hence the extensive description of *Ae. albopictus* life traits including survivorship and fecundity are important for a better understanding of the vector's biology (Cui et al., 2021). The World Health Organization (WHO) has taken this matter seriously and they have suggested a fundamental control approach to eradicate the mosquito's population, including investigation about reducing *Aedes* sp.

breeding sites, larvicides, insecticides as well as biological control (WHO, 2021; Mohd Ngesom et al., 2021). There are considerable research on vaccine and vector control management, including genetically modified mosquitoes (Bargielowski et al., 2011), insecticide-spraying programmes (thermal fogging and ultra-low volume sprays) as the primary interventions combating dengue cases (Mohd Ngesom et al., 2021) and sterile insect technique (Becker et al., 2022; Nolan et al., 2011). Unfortunately, they have recorded little success. Concerns have arisen on the long-term adverse effects due to excessive chemical insecticide usage and resistance on humans and the environment. The increased resistance impact on mosquitoes could be seen in major pesticide classes including carbamate, pyrethroids and organophosphate being used (Ansari et al., 2014; Mohd Ngesom et al., 2021) All these problems have triggered the urge to explore new areas of research in vector biology. With these problems in mind, researchers started exploring alternative methods, using broad range of persistent chemicals to specific biological control agents (Arbaoui & Chua, 2014). These alternatives demonstrated an increased optimism in biorational approaches, including using natural enemies, especially parasites and larvivorous predators (Noreen et al., 2017). Another approach appertains to manipulate the 'symbiotic control' for the development of novel control strategies. Such interaction varies between pathogenic and obligate symbiosis, including the bacterial aid in mosquito's nutrient synthesis that interferes with the mosquito's development (Engel & Moran, 2013; Minard et al., 2013; Ricci et al., 2012). The bacteria abilities in such interaction have attracted researchers to understand them further.

1.2 Problem Statements

The breeding containers are an ecosystem mesocosm related to oviposition and mosquito's population (Ponnusamy et al., 2008). As the mosquitoes feed and breed in different environments, they may acquire some bacteria from the environment in their midgut. For example, the midgut bacterial isolation from field-collected *Ae. albopictus* adult in Madagascar presented three phyla, namely *Proteobacteria*, *Firmicutes* and *Actinobacteria*, especially the genus *Pantoea* in both sexes' midgut (Moro et al., 2013). On the other hand, (Yadav et al., 2016) identified more bacterial species in the midgut of *Ae. albopictus* in Tezpur, India. These differences shows that the bacteria diversity in the mosquito's midgut is mainly affected by different habitat environments. This research was conducted in Penang, Malaysia because it is suitable place for the development of *Ae. albopictus* due to the densely packed human population; there for more sources of blood suitable for adult reproduction and has produced a variety of containers suitable for larval habitats. In addition, the warm microclimatic conditions favours larval development and survival, thus easily promoting dengue fever outbreak (Muhammad et al., 2020).

This study presented the entire fundamental analysis of the microbiota in breeding water, which was important for determining paratransgenic candidates and oviposition attractant. Previously, other researchers had conducted similar studies on the association of microbiota with mosquitoes, including Chandler et al. (2015), but insufficient data collection compromised the depth of diversity analyses. Next, there was a study from neighbouring Thailand on mosquito-associated microbial communities as a whole, which included bacteria and eukaryotic symbionts, but again there were some limitations due to insufficient replication for testing by site and species (Thongsripong et al., 2018). There was also a study in Malaysia by Maimusa et al. (2019) that used culture-dependent method and focused on the determination of *Lactobacilli* bacteria in the midgut of wild *Ae. aegypti* suitable for paratransgenic control.

Midgut-associated bacteria have outnumbered the insect host's cells and is associated with the survival, digestion, reproduction, commensalism interactions and protection.

Furthermore, midgut microbiota also plays a role in the host-pathogen interaction, ultimately influencing the potential of disease transmission (Coatsworth et al., 2018). The pathogen that enters the mosquitoes will interact with the resident bacteria in the midgut and epithelial cells. This barrier governs the susceptibility of mosquitoes towards the arboviruses, where the reduction of certain microflora by antibiotics increases the infectivity of *Plasmodium falciparum* (Mourya et al., 2002). Previous findings by Pidiyar et al. (2004) and Dharne et al. (2006) showed a dynamic pattern of bacterial communities in the mosquito's midgut. Different stages and sexes of the mosquito's life cycle produce different microbiota due to the derivation of bacteria, either from the breeding water or blood meals (Dharne et al., 2006; Minard et al., 2013), as well as differentiation of microbiota in the host's organs including the midgut and salivary organs. Since only female mosquitoes have access to the blood meals and have a role in transmitting viruses, they were selected for midgut bacterial screening.

Dengue virus has been reported to be isolated from the larvae around the locality of dengue hot spots (Rohani et al., 1997). The identification of the viruses that was maintained in the larvae stage indicated a transovarial transmission by its parents. Hence, for a successful mosquito control programme, studies have suggested a paratransgenesis strategy by manipulating the midgut microbiota of mosquitoes, involving the insertion of an effector molecule in the bacteria that can inhibit the pathogenic viruses, especially in the larval stages (Rohani et al., 1997; Moll et al., 2001). However, to achieve the paratransgenesis strategy, the priority is that the candidate bacteria can be taken by the larvae during larval stages. Additional advantage is that these bacteria are able to maintain and survive during the metamorphosis process so that the transformed expressing effector molecules could remain in both stages. Hence, it is crucial to identify whether the larvae can take the bacteria from its environment and withstand the metamorphosis process, or the bacteria have previously established themselves by means of transovarial transmission from their parents.

Since offspring develop without the protection of an adult, oviposition behaviour is a very important skill for mosquito survival. Therefore, it is essential for the mosquito to select suitable breeding sites, taking into consideration the factors of predators, chemical and physical aspects (Kaw et al., 2004). However, there is a lack of investigation into the bacteria's role in relation to the mosquito's oviposition and only few researchers had shown interest in this area. As demonstrated by (Arbaoui & Chua, 2014), unfiltered bamboo leaf infusion (BLI) attracted significantly more egg-laying mosquitoes than the filtered BLI in which the microorganisms were physically removed by 0.45µm membranes. This result highlights the essential role of microbes in the production of attractant for oviposition. Similarly, Ritwik et al. (2015) found that four species of *Bacillus* species isolated by the conventional culture method attracted *Aedes aegypti* to oviposition. The research conducted by Ponnusamy et al. (2015) also successfully tested different types of individual bacterial species and a combination of bacteria that attracted both species of dengue mosquitoes, *Ae. albopictus* and *Ae. aegypti,* to oviposit. However, data on oviposition attractant are still limited, especially in the local context. Therefore, the current study highlighted the importance of isolating a single bacterium for mosquito oviposition attractant.

Overall, the research involving microbiota is very important to help discover the common and diverging effects of this microbiota on the mosquito's host physiology. The fundamental knowledge of *Ae. albopictus* and its association with bacteria is limited, especially in this local area. Thus, the research of *Ae. albopictus* and its relationship with the microbiota in breeding water and midgut is very important in the search for a paratransgenic candidate as well as oviposition attractant. Consequently, it serves as the platform for successful vector management strategies in the future.

1.3 Significance of the study

The research was conducted to (1) determine the association of *Ae. albopictus* with bacteria in breeding water and midgut, and (2) search for paratransgenic candidates, development association and oviposition attractant. The comparative assessment of bacterial communities in the breeding water artificial containers associated with the presence and absence of *Ae. albopictus* larvae, serves as a fundamental evaluation. The objective was achieved by combining conventional culture-dependent and sophisticated culture-independent methods to better understand this research. Additionally, the next objective is focused on the midgut bacteria that harbours the immature and adult stages of *Ae. albopictus*. The breeding water and life stages of *Ae. albopictus* midgut showed the persistence of two similar bacteria, known as *Bacillus cereus* and *Proteus mirabilis*. Therefore, there is an uncertainty whether the bacteria were acquired from its environment or transovarially from its parents that enabled them to survive. To answer this problem, further tests of these bacteria were undertaken to provide an insight into successful paratransgenesis candidates as a control management of the mosquitoes. In addition, its association with the larval sizes was also investigated. Finally, to complete the research, the function of a single bacterium as an oviposition attractant that entice the gravid female mosquitoes to oviposit and breed inside the breeding substrates was evaluated. All three interested bacteria of *B. cereus*, *P. mirabilis* and *E. cloacae*, previously isolated from the breeding water were used as the single bacterium attractant, with the lowest bacterial cell dilution to test if the lowest bacterial cell can elicit oviposition responses. The future plan is to examine the formulation of bacteria using an alginate encapsulation method as an attractant bait in the field, which could be used as an enhance ovitrap surveillance.

1.4 Hypothesis

The diversity of bacteria differs depending on the breeding water with the presence or absence of larvae. There are also differences in the midgut microbiota of immature and adult stages. The bacteria are present in both stages of the mosquito, indicating that the selected bacteria can be transmitted transstadially from larva to adult stage as well as transavorially from parents. Determination of selected beneficial microbiota as paratransgenic candidates and as attractant for oviposition may benefit the vector control of *Ae. albopictus*.

1.5 Objectives

To provide information about the bacteria association in breeding water and midgut microbiota of *Ae. albopictus*, as well as subsequently using the beneficial larvae for the development of bacterial attractant candidates, the following objectives have been outlined:

Objective 1: To determine the diversity and composition of bacterial faunas in breeding water with the presence and absence of larvae using the culture-dependent and culture-independent methods.

Objective 2: To determine bacterial diversity and abundance in different stages of the field-collected *Ae. albopictus* midgut from artificial breeding sites.

Objective 3: To determine the best paratransgenic candidates via previously isolated breeding water and midgut bacteria from both stages of *Ae. albopictus*.

Objective 4: To obtain the best bacterium that were previously isolated from the breeding water of *Ae. albopictus* as oviposition attractant.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Aedes albopictus*

Knowing the biology of mosquitoes is vital before it can be exploited for effective control measure. *Aedes albopictus*, the most invasive mosquitoes species has spread worldwide and is associated with public health threat. The mosquito's larval habitat in tropics and subtopics areas are well diversified depending on urban areas and forested areas. The main breeding water habitat during immature stages can be divided into indoor environments (e.g flower pots and water containers), semi-shaded area (e.g tires), nearly fully shaded area (e.g forest) and nearly fully open areas (e.g open containers). *Aedes albopictus* prefers outdoor and forested areas than indoor, whereas breeding water differs according to the environment (Cui et al. 2021). FIGURE 2.1

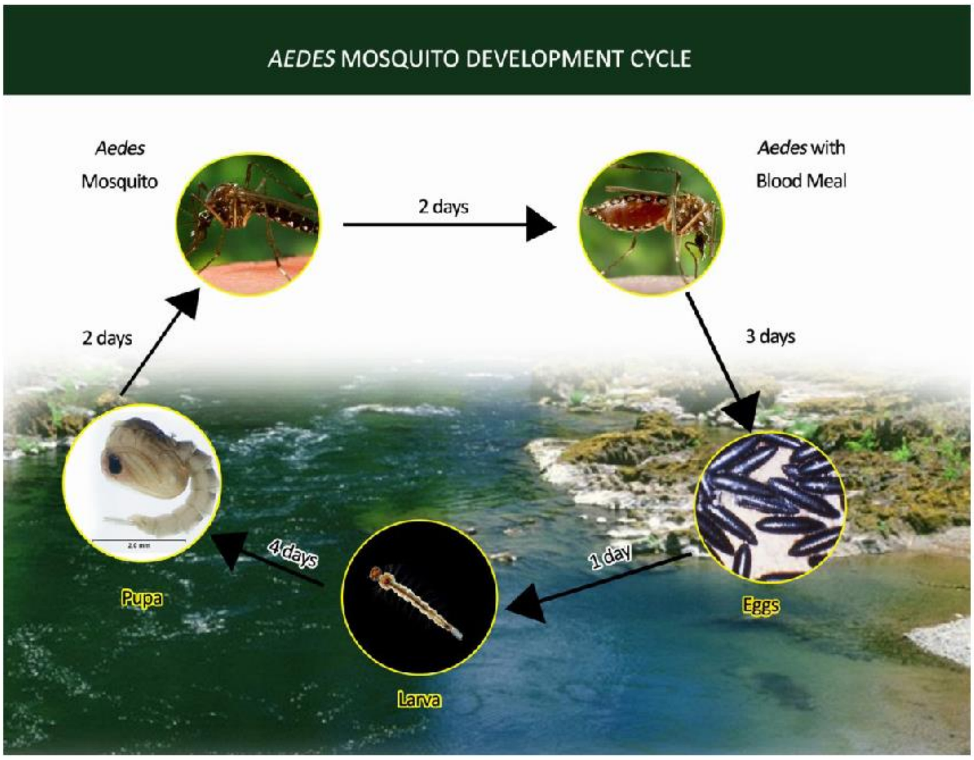

Figure 2.1 *Aedes albopictus* development cycle from eggs, larva, pupa and adult. Retrieved from Anoopkumar et al. (2017)

2.1.1 Life stages of *Aedes albopictus*

2.1.1(a) Eggs

The characteristic of *Ae. albopictus* eggs (Figure 2.2) can be described as a smooth black structure laid on a moist surface near the waterline, elongated/or ovoid in shape and are typically 0.5 mm in length. However, the eggs do not encompass floats and micropylar collars (Anoopkumar et al., 2017). During the oviposition, the eggs are soft and pale before they get darker and harder. The formation of serosal cuticle is important for preventing the desiccation (Noh et al., 2021). The eggs deposited adhere on the walls of the breeding habitat by chorionic pad that contains hyaluronic acid (hygroscopic compound) that also prevent them from desiccation, enabling them to withstand any adverse climatic change (Martínez-García et al., 2022). The duration of gonotrophic cycle before the production of eggs, is defined as the time elapsed between the blood-feeding of gravid females that will usually occur at a minimum of three days. Usually, gravid females can lay between 60-70 eggs in the waterline surface of stagnant water in various artificial containers, such as gardening utensils, tires, discarded tins and other plastic containers. The eggs take up to 72 hours to hatch, with the successful percentage of eggs hatching between 85.9% to 86.32% (Anoopkumar et al., 2017; Saifur et al., 2010). Embryonic development is the most crucial part that determines the embryo's survival success rate. Besides optimum moisture requirement, the temperature can also play a role in embryogenesis, ranging from 12°C to a maximum temperature of 35°C, with relative humidity above 80%. However, under certain dry weather circumstances, the eggs can remain dormant and survive for months.

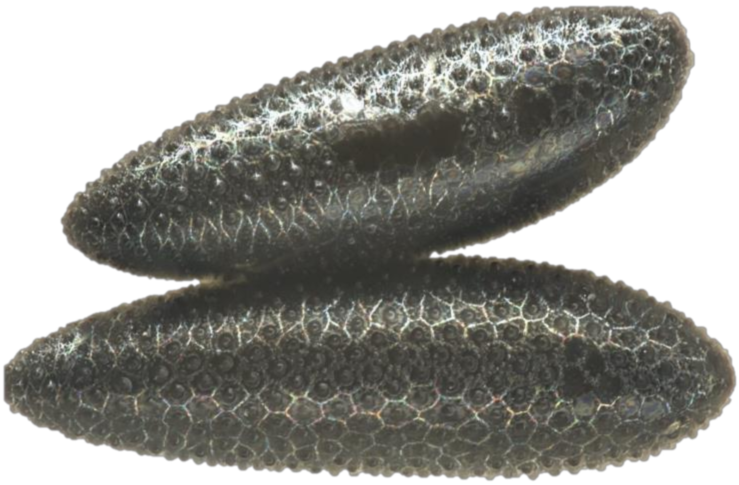

Figure 2.2 Image of egg taken by using specialized equipment (Macropod Micro Kit 40X). Retrieved from Shragai et al. (2019).

2.1.1(b) Larvae

The larvae stage comes next after the eggs have hatched. At this stage, the larvae's characteristics include (1) having a comb hair without the lateral denticles located at the 8th abdominal segment, with the double lateral saddle; (2) having short siphons or a snorkel-like air tube located at their posterior body for air-breathing and (3) containing a single pair of hair tuff attached to the breathing tube to let them grow in clear water but not necessarily in clean water (Dieng et al., 2010) as seen in Figure 2.3. The soft texture of siphon gets darker and harder until the later stage of larvae, together with the increases of siphon's length and width. Usually, the length is double the size of the width (Hossain et al., 2022).

The body sizes of the larvae are proportionate to the food available, in addition to other factors such as temperature, sex and the number of larvae in that habitat. They move by wriggling in selected breeding water habitat and are vigorous feeders that feed on the organic matter present (Zettel & Kaufman, 2013). The larval filter out organic matter including heterotrophic microorganism such as detritus bacteria and protozoans from the habitat with modified mouth parts or known as “mouth brushes” (Chouin & Santos, 2017) . The breeding container for the immature are man-made container and natural containers. Natural container habitat that contributes space for mosquitoes breeding sites include drains, puddle, pits, and freshwater swamps while the man-made container habitats include jars, discarded tin cans and plastic containers. The larvae will keep feeding until the fourth instar larval moults into pupae (Anoopkumar et al., 2017).

Next, the moulting process increases the body length, which including shedding their skins three times to be able to grow from the first to the fourth instar (L1, L2, L3, L4). This entire process has been reported to occur between several days to few weeks, depending on the climate and food availability (Chouin & Santos, 2017). Usually, larvae develops into four phases into a pupa in three to five days that could be 1/8 inch long at their first instar and grows to 1/2 inch at their fourth instar (Hossain et al., 2022). After the fourth instar and the larvae has adequate with appropriate size and energy, the metamorphosis trigger the larvae to change into pupae. Usually, males develop faster than females, that eventually will pupate earlier and emerge first, faster than females. (Chouin & Santos, 2017).

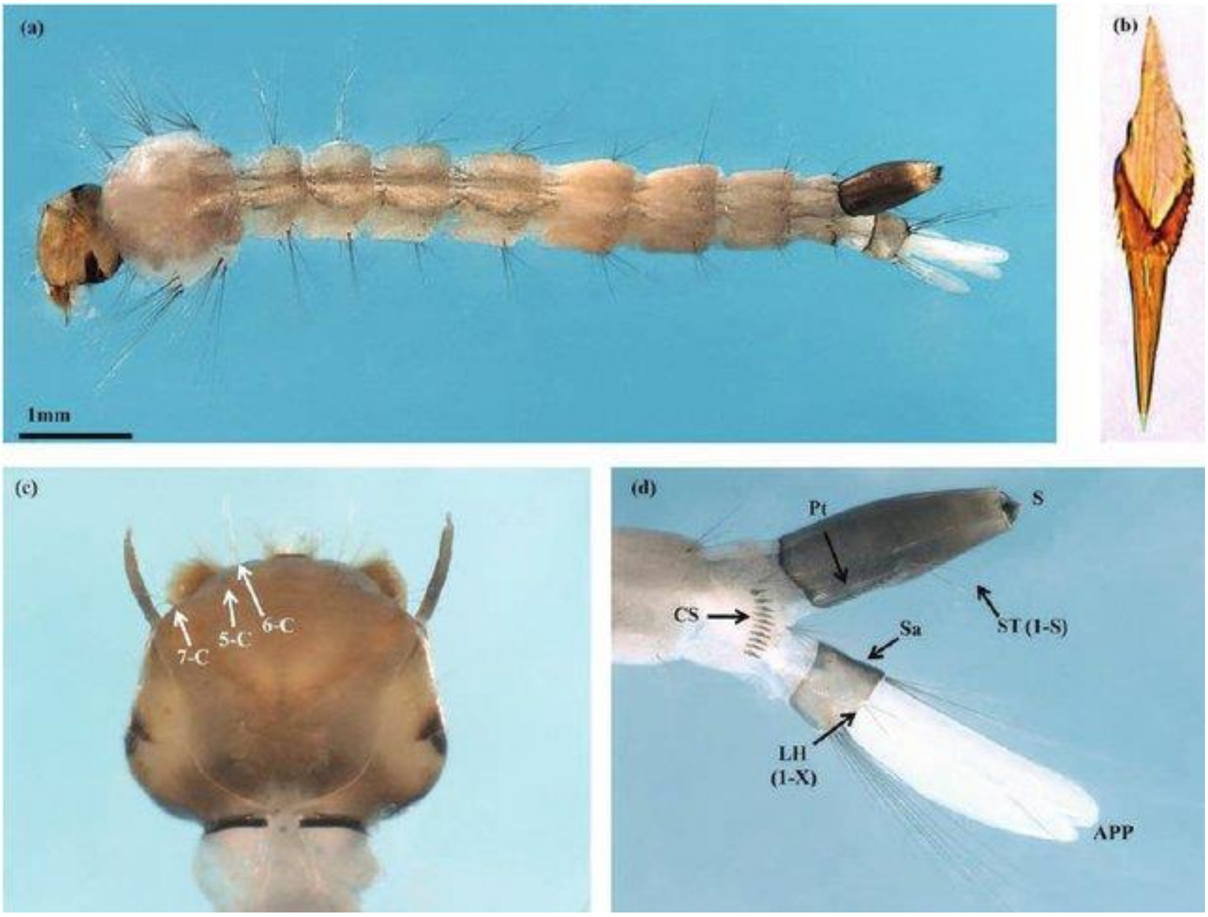

Figure 2.3: Full description of *Ae. albopictus* larvae made by Farajollahi & Price (2013). (a) Entire *Ae. albopictus* dorsal view. (b) Comb scale. (c) head of 4th instar larvae. (d) Terminal segment compose of anal papilla (APP), comb scale (CS), lateral hair (seta 1-S), siphon (S), saddle (Sa), pectin teeth (Pt), siphonal tuff (ST).

2.1.1(c) Pupae

The subsequent life stage of mosquitoes is known as the pupae stage (Figure 2.4). The morphology of pupae can be described as comma shaped (Ha et al., 2017). In this stage, there is no requirement for food due to the lack of mechanism for food uptake, but they still move actively in a jerky motion. The pupae have the shortest life span, usually only one to three days, depending on the environment before they form into an adult (Anoopkumar et al., 2017). They rely on the oxygen obtained aerially because they cannot use the dissolved oxygen, due to their thick and nonpermeable cuticles. A study by Focks and Chadee (1997) reported that the male pupae stage is approximately 32 to 36 hours, while for the female pupae, it takes between 49 to 52 hours. That explains why the emergence rate of males is faster than females. Under an ideal environment, which is between 27 to 37 °C, these pupae can complete their development in one to three days. The difference between a larvae and pupae is that the pupae can remain alive when exposed to a completely free water environment, while the larvae are not able to survive those conditions.

There are natural occurring phenomena during the pupae stage that is known as protandry which is useful for sex separation, where the emergence of males are faster than females, due to their earlier sexual maturation. This phenomenon is naturally present in *Ae. albopictus* (Bellini et al., 2018).

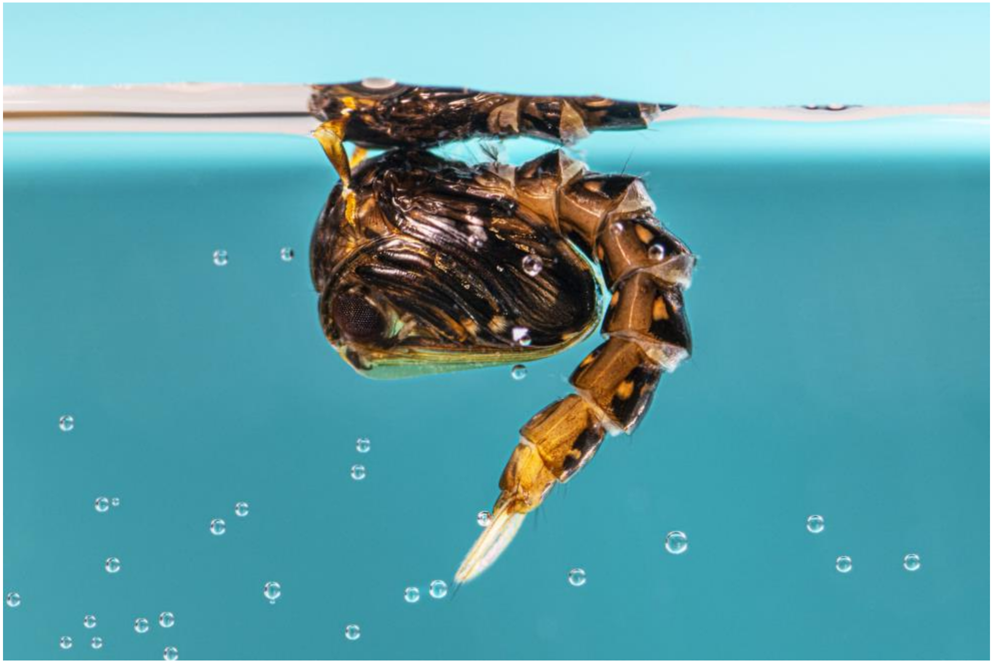

Figure 2.4: Morphology of pupae stage of *Ae. albopictus* (CDC, 2022).

2.1.1(d) Adults

The fundamental knowledge of the adult stage is highly required because this is where the transmission of disease will occur (Figure 2.5 and 2.6). It is known as a zoophilic species attacking both wild and domestic animals but preference for human blood (Anoopkumar et al., 2017 ; Benelli et al., 2020). Only female mosquitoes feed on the host for the eggs production, where the blood will be degraded into amino acids by digestive enzyme secreted from the midgut epithelial cells (Malassigné et al., 2020). Both mosquito sexes feeds on glucose solution from the flower nectar as their basic food, while the blood provides important protein for the egg development (Cui et al., 2021). Generally, the mosquito's body parts consist of three main segments: head, thorax and abdomen. The distinct morphologic characteristic of *Ae. albopictus* can be easily recognised by their narrow and pointed abdomen with cerci protruding through and a single broad line, white marking on legs and longitudinal silvery-white dorsal stripe on thorax, black and white scales on the tarsal segment of its legs and black body with silver-white levels on the palpus and tarsi. In addition, it is entirely black on its unscaled clypeus and midfemur (Martin & Mustapha., 2020). The time needed to complete their life cycles starting from eggs to adults is 10 days and their lifespan is between 20 to 30 days (Clemons et al., 2010 ; Anoopkumar et al., 2017). The length of an adult mosquitoes is between 2 to 10 mm, where male mosquitoes are usually 20% smaller than females (Anoopkumar et al., 2017). Both female and male mosquitoes can be easily differentiated by looking at their antennae and size. Male antennae are plumous and contain auditory receptors, while female antennae are less dense. Male mosquitoes only required nectar as their meals, while there are additional blood meals required by female mosquitoes. The adults will take some time on the water's surface until they are strong enough to fly freely. In addition, females start to draw blood meals from humans or other animals after 48 hours of their emergence and can take multiple blood meals during the different gonotrophic cycle. This cycle is a crucial part where disease can easily transmit when the females draw the infected blood meals from the infected persons or animals and transmit them to an uninfected person (Delatte et al., 2010). In *Ae. albopictus*, the male has a degree of multiple mating, proving the occurrence of male territoriality than females that has a small rate of mating (Bellini et al. 2018).

Another factor that influences the mosquito’s development is the extreme microclimatic condition, such as extreme heat and humidity that decreases the reproductive capacity of female *Ae. albopictus* (Cui et al., 2021). The researchers also found that the adult cycle can achieve up to 70 days indoors with the reproduction cycle to continue for more than 50 days in both indoors and outdoors.

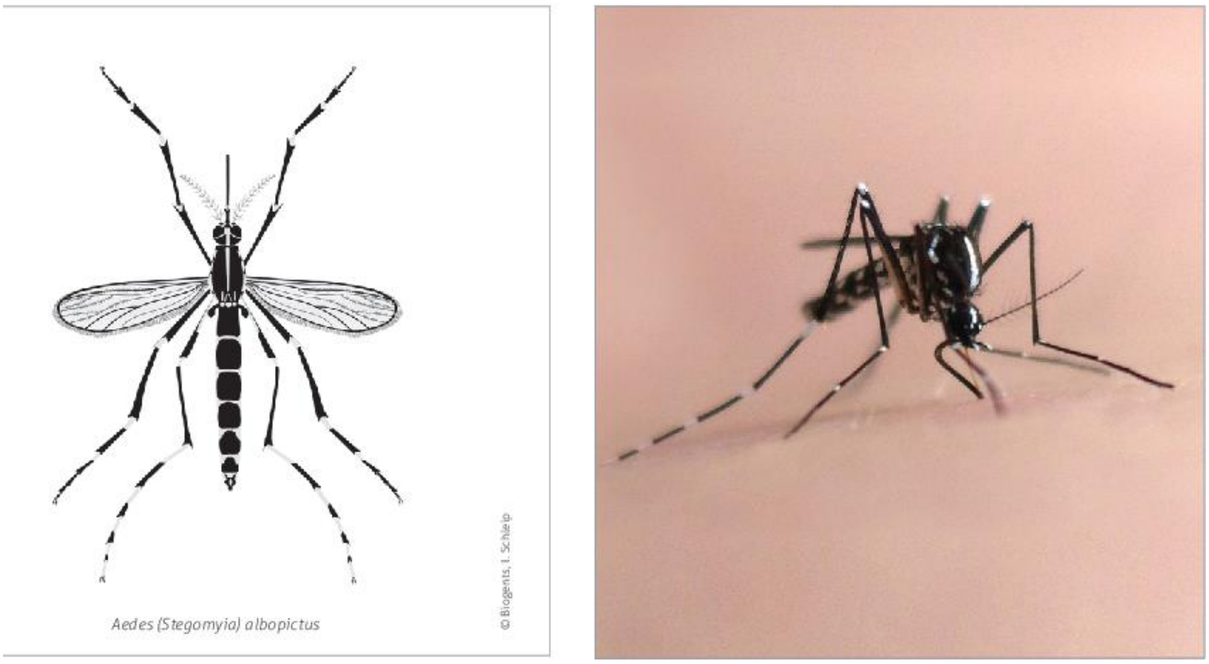

Figure 2.5: The morphology of *Ae. albopictus*. Retrieved from Benelli et al. (2020)

Figure 2.6: Adult female of *Ae. albopictus* while taking a blood meal (ECDC,2022)

2.1.2 *Aedes albopictus* behaviour

Aedes albopictus is a holometabolous insect that is also considered as exophagic and exophilic (Egid et al. 2022; Reinhold et al. 2018). The complete cycle consists of eggs, larvae, pupa and adults that live in two different environments without resemblance to each other. The first stages are the aquatic breeding stages, including eggs, larvae and pupae, followed by the adult stage that lives freely in a subaerial environment. These mosquitoes are known as a zoophilic feeder that feeds on mammals, reptiles, birds, amphibians and plant nectar (Clements, 2000; Egid et al., 2022). Shepard et al. (2013) have detected that the dengue disease is often associated with an impoverished population because of the abundance of discarded human-made containers, such as garden utensils, tires, food containers, tins and plastic containers. Others habitat preference widely includes near the forested areas, urban areas, agricultural fields and rubber plantations that varies on indoor environments and outdoors environment that are in conditions of semi-shaded, nearly fully shaded and open areas (Cui et al., 2021). Although the mosquitoes can breed in an urban and peridomestic environment, they generally prefer to breed in rural areas in peninsular Malaysia (Yap, 1975) ; Rozilawati et al., 2007), including forest fringes and secondary forest habitats. These mosquitoes dynamically revolve around the outdoor area targeting artificial containers. The selected containers provide a safe environment for the larvae, including food availability, without competition and predator (Hawley,1988; Sunahara et al., 2002).

The insect reproduction biology consists of feeding, mating and oviposition. It is known as a diurnal biter that is actively biting during the day (Kraemer et al., 2015). Blood meals not only provide the necessary food to survive, but they are also advantageous for the female mosquitoes to transmit their pathogen efficiently. During the biting process, the pathogen enters together with the blood feeding into the mosquito's midgut. Then, it will infect the gut epithelial cells before accumulating and invading the salivary glands. Eventually, the mosquitoes would pick up the pathogen and transmit them to another host during the biting process (Malassigné et al., 2020). The mosquitoes are required to complete the cycle before taking another blood meal from a susceptible host. Therefore, the frequency of contact between the host and infected female mosquitoes can increase the chances of disease transmission. Moreover, blood proteins can trigger a reproductive output necessary for the production of eggs (Roitberg et al., 1993).

Food resources during larval development is essential in the determination of body sizes. Thus, bigger mosquitoes feed lesser than the smaller ones (Scott et al., 2000b), while smaller mosquitoes tend to feed more often due to the increased speed of blood meal digestion (Scott et al., 1997). The mosquito's sex also plays an essential role in microbiota composition.

Both sexes differ in terms of their ecological behaviour, including nutritional and dispersal capabilities. They have the same dietary factors, which are sucrose hydrolyses, but differ in the hematophagous characteristics shown by female mosquitoes. In addition, female salivary glands contain proteins for the haemostasis of the host blood for feeding adaptation and viral sustenance (Shettima et al., 2021).

2.1.3 Medical significance of *Aedes albopictus*

Aedes albopictus (Skuse) (Diptera: Culicidae) is a mosquitoes capable of enduring various climates, ranging from humid tropical climate to colder temperate regions. The adaptive capabilities of these mosquitoes gradually increase, making them a competent vector of public health. It was first recognised as one of the principal vectors for dengue transmission in Malaysia in 1984 (Yap, 1984), which can cause dengue fever (DF) and also dengue haemorrhagic fever (DHF). These types of fevers can affect people at any age. Besides dengue, it was well documented that, *Ae. albopictus* is also a vector for other diseases, including yellow fever, chikungunya and zika (Cui et al., 2021; Yadav et al., 2016). Zika viruses are associated with microcephaly, while chikungunya causes debilitating arthralgia and additional endemic viral infections such as West Nile fever and Mayaro (Weerantunga et al., 2017). These mosquitoes have a global impact which affected various sectors, mainly health and the economy.

The main vector of dengue is *Ae. aegypti* due to being dominant in DENV transmission. However, *Ae. albopictus* is highly susceptible to DENV midgut infection due to the higher accumulation of DENV RNA in its abdominal tissues, despite having less infectious saliva than *Ae. aegypti* (Whitehorn et al., 2015). *Aedes albopictus* usually completes their life cycle within residential areas due to its limited dispersing distance. Hence, the dengue also spreads by the infected persons' movement rather than the infectious mosquitoes themselves (Edman et al., 1998). This mosquito was responsible for the first transmission of dengue outbreaks in Japan in 1942. In addition, chikungunya outbreaks in Europe, Hawaii, Madagascar and Gabon was also initiated by *Ae. albopictus*, due to its vector competency (Chuchuy et al., 2018).

As reported by Martin & Mustapha (2020), this Asian tiger mosquitoes is native to tropical and subtropical Southeast Asia. Various Asian countries besides Malaysia, such as India, Indonesia and Thailand, have been affected with the arbovirus carried by this mosquito (Gratz, 2004). The researcher also linked the possibility of *Ae. albopictus* serving as a bridge vector for Eastern equine encephalitis (EEE) and La Crosse viruses (Gubler et al., 2001).

2.2 Dengue vector surveillance and control strategies

For the past 50 years, arbovirus transmitted by *Aedes* that causes dengue, chikungunya and Zika has repeatedly appeared worldwide. The expansion is related to various factors, including global trade and the lack of vector control that affects the economy, causing billions of dollars in annual losses (Roiz et al., 2018). Previously, different control strategies were carried out, including reduction of breeding sources, insecticides and larvicides but only little success were recorded (Mohd Ngesom et al., 2021). The control strategies include insecticide-spraying programmes (residual spraying and insecticide-treated bed net) as the primary interventions and using natural insecticide such as eco-friendly pesticide, pyriproxyfen that degrades and provides carbon source for the microorganism (Amusan et al., 2005; Mohd Ngesom et al., 2021; Weeratunga et al., 2017), biological agents (Scholte et al., 2007), genetically modified mosquitoes (Bargielowski et al., 2011), sterile insect technique (Morán-Aceves et al., 2021) and also vaccine for human (Coudeville et al., 2016). The implementation of dengue vaccine Dengvaxia is limited to the public health program in Brazil and the Philippines but is associated with limited efficacy and can increase dengue severity (Da Silveira et al., 2019; Thomas & Yoon, 2019). To eradicate the mosquito's population or diseases is impossible, therefore, the disease transmission prevention can only depend on controlling and reducing the vector populations of *Aedes* sp. (Wilson et al., 2020). For a more strategic control, those endemic countries should aggressively implement intensive efforts and systematic paradigm, including surveillance, strategies and product development that are more attractive and effective.

Vector surveillance is an essential requirement for the quantitative and qualitative assessments before any treatment can be conducted. The information can be used to forecast and assemble prevention strategies to manage disease outbreaks (Beech et al., 2009; Pley et al., 2021). The surveillance of the *Aedes* sp. population dynamics can be employed using ovitrap, a cost-effective and convenient tool. The prediction of dengue outbreaks using these surveillance system is a sensitive tool for *Aedes* sp. population monitoring predicting dengue outbreaks that also determines the distribution, spatiao-temporal risk factors and larval habitats (Sasmita et al., 2021).

Larvicide is the chemical used to control the vector by targeting their breeding sites to kill the immature mosquitoes, specifically larvae. An example of a larvicide is methoprene, a residual insect growth regulator used in discarded human-made containers, roofs and large containers to reduce the overall population (Sharon, 2017). However, this larvicide method is known to be associated with several disadvantages which are the activation of insecticide resistance and the high cost associated with this method. There are four mechanisms underlying the mosquito's insecticide resistance including cuticle modification, insecticide detoxification, behavioural avoidance of insecticides and the insensitivity of the insecticide's sites of target (Fazal et al., 2023). The wide-scale disease outbreaks in Cambodia in 2007 was related to the presence of abundant, discarded containers and cryptic breeding sites that had complicated the control programmes since the use of larvicide for mitigation and prevention were not compatible. Since the larvicide only targeted larvae, the adult mosquitoes remain unaffected, and disease transmission still occurs among those infected by the adult mosquitoes (Chang et al., 2011).

Besides targeting the larvae, the adult mosquito must also be controlled. Insecticides targeting the adult phase are used in control programmes through spraying or also known as insecticide spraying. Insecticide spraying is a massive reduction targeting adult vector populations during the outbreaks by decreasing their lifespan. Insecticide specifically temephos has been used as a method of mosquito control for over 40 years in the Asian region (Gubler, 2011; Setha et al., 2016). This method requires the persistence and expertise to conduct specific application methods such as spraying operations that consider the functionality of sprays, sufficient coverage and correct dosage. There are several disadvantages on using this spraying method such as there are chances of these mosquitoes being unaffected during the wide-scale spraying (Perich et al., 2000). Moreover, the gap in the timing between the reported cases and spraying management will cause severe outbreaks (Chang et al., 2011), especially in rural areas, where the time interval between when the cases are reported and the commencement of spraying operations will be long. The excessive usage of insecticide in these spraying programmes have also affected environmental health and consequently, developing resistance among mosquitoes (Flores et al., 2006).

Maintaining the conventional control method was the most crucial part. The complexity of the control method, such as Region Plan by the Italian region, involved millions in terms of costs (Canali et al., 2017). Additionally, insecticide resistance that emerged in *Ae. albopictus* population was also reported (Moyes et al., 2017). All these problems triggered the urge to explore new novel research avenues in vector biology that are eco-friendlier and complement the conventional vector control system. In the meantime, the controlling management of mosquitoes relied more upon vector population control or restricted contact between the human and vector (Roiz et al., 2018). The first principle in dengue vector control is to ensure its efficiency, whereby a profound understanding of the vector's biology and ecology is needed.

Additionally, in December 2016, a phase III trial of vaccine efficacy developed by Sanofi-Pasteur known as Dengvaxia was conducted (Coudeville et al., 2016). However, it was associated with safety concerns for mass administration. Also, the worry arises for the potential transmission of a new virus by *Aedes* sp. vector (Aguiar et al., 2016)

The strategies employed all depend on the cases, resource availability, application techniques and cultural context. Even though the strategies differ according to areas, they apply the same dengue control pattern, by moving from relying solely on insecticide towards safer strategies such as removing the vector's reservoir, biological control and environmental management. However, the dependency on the insecticide still serves as the primary method of control (Chang et al., 2011).

2.3 Biological approaches

Biological approaches take advantage of the predator-prey concept to reduce the vector's density. The chosen biological agents must be safe, inexpensive, can be produced in large-scale and acceptable to the targeted population. The intervention of non-chemical larvicide includes larvivorous fish, larvae mass trapping and oil coating that interrupts the vector's life cycle (Weeratunga et al., 2017). Other examples are the copepod *Mesocyclops* for controlling *Aedes* mosquitoes in Vietnam as community-based biological control agents (Nam et al., 2000). The method has successfully reduced the cases on houses community with positive dengue vectors by 1.5%. Other examples include using certain species of fish (e.g. guppy) over these arthropods, especially in household water containers (Seng et al., 2008).

Only two to three guppies were needed to control those mosquitoes, plankton and algal growth in 200-400 litres of water per container. Similarly, in the biological process of insect reproduction, there is an interaction between mosquitoes and microbes called the 'symbiotic control,' and the interaction between them varies from pathogenic to obligate symbiosis (Dillon & Dillon., 2004).

Bacteria help mosquitoes by supporting nutrient synthesis from the mosquito meal or their development (Minard et al., 2013;Ricci et al., 2012). Different bacterial communities play important roles depending on the species, sex, developmental stage and habitat (Minard et al., 2013). This microbiota can be isolated from the mosquito's organs, midgut and reproductive organ to haemolymphs and salivary glands (Dillon & Dillon., 2004). The midgut barrier determines mosquito susceptibility to arboviruses, and reducing certain microflora with antibiotics increases *Plasmodium falciparum* infectivity (Mourya et al., 2002). Based on previous findings by Pidiyar et al. (2004) and Dharne et al. (2006), there is a dynamic pattern of bacterial communities. The immature stage of mosquitoes obtains its bacteria from breeding water, and mosquitoes that consume blood meals could also affect the microbiota as well. The uncertainty is whether the bacteria are transtadial or acquired by feeding, which allows them to survive, or whether the bacteria are transient during digestion or the developmental stage (Minard et al., 2013).

2.4 Microorganism as the mosquitoes control method

Microbiota provide the nutritious food for the mosquitoes and can also affect the physiological impact including mosquitocidal and antipathogen effects. These interaction of mosquito (host), viral pathogen and microbiota leads to the manipulation and altering of the microbiota for vector control, that is also known as paratransgenesis (Scolari et al., 2019). Paratransgenesis aim to genetically modify the microbiota, expressing the antipathogen effector molecule inside the mosquito (Wang & Jacobs-Lorena, 2017). In addition, some undigestible larvae during feeding, also form a symbiosis mutualism that raises an eco-friendly approach in controlling mosquitoes (Ranasinghe & Amarasinghe, 2020). Recently, there has been interest in the novel approach of *Wolbachia* sp., that maternally transmit and create an endosymbiont that blocks the dengue transmission, especially in the primary vector mosquito, *Aedes aegypti* (Nazni et al., 2019). It is known to impact the reproductive level affecting the cytoplasmic incompatibility and the *Wolbachia* sp. infected females can successfully breed with infected and uninfected males (Ross et al., 2019). The reproductive manipulation quickly spread to the high population of mosquito causing a crossing sterility and demonstration of dengue transmission that eventually supress and reduced the human dengue infection (Nazni et al., 2019). Additionally, the *Wolbachia* sp. protects against the arboviruses and reduces the mosquito's lifespan. These findings show the complexity of manipulations among the interaction of host, viral pathogen and microbiota. However, it has been proposed that DENV inhibition with certain strains of *Wolbachia* may be temperature sensitive (Yixin et al., 2016). Moreover, the density and cytoplasmic incompatibility also reduced when *Ae. aegypti* larvae reared at high temperature, especially when the wild larvae breeding water is usually exposed to sunlight during the daylight (Nazni et al., 2019). This has suggested that further explorations are needed in searching for other novel microbiota candidates that fits the paratransgenic requirements and can be utilise as mosquito control. There is also the biological larvicide, *Bacillus thuringiensis israelensis* (Bti) targeted to control dipteran insects especially mosquitoes. *Bti* produces multiple toxins that interfere with the organisms' digestive wall making it alkaline and disrupts the gut. However, the disadvantages of using *Bti* is the interference with the ecosystem's function and food webs (Land et al., 2019).

Besides *Wolbachia* sp., and *Bti* other microbiota including entomopathogenic fungi were also associated with the antipathogen activities that interfere with the blood feeding