Journal of Environmental Biology
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

JEB®

Original Research Paper
Volume 45 Issue 3 May 2024 pp. 277-287
DOI: http://doi.org/10.22438/jeb/45/3/MRN-5208

J. Environ. Biol., 2024
© **Triveni Enterprises**, Lucknow (India)
Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Molecular characterization of *Aedes aegypti* (L.), its bacterial microbiomes and their control in Saudi Arabia

S.E. Sharawi[1]*, D.H. Kaneetah[1], J.A. Mahyoub[1], H.M. Al-Solami[1], A.G. Alghamdi[1], Wafa Mohammed AL-Otaibi[2], T.S. Alghamdi[3], Naser A. Alkenani[1], M.A. Al-Shaeri[1], Manal E. Shafi[1] and H.S. Alyahya[1]

[1] *Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, 21589, Saudi Arabia*
[2] *Department of Biology, College of Sciences, Taif University, Taif, 21944, Saudi Arabia*
[3] *Biology Department, Faculty of Sciences, Al-Baha University, Al-Baha, 1988, Saudi Arabia*

Received: 13 December 2023 **Revised:** 10 February 2024 **Accepted:** 20 March 2024

***Corresponding Author Email:** sesharawi@kau.edu.sa ***ORCiD:** https://orcid.org/0000-0001-5765-2251

Abstract

Aim: This study aims to identify laboratory strains of *Aedes aegypti* at a molecular level and isolate bacteria from various developmental stages, while also assessing the effectiveness of environmentally friendly *Bacillus subtilis* compared to a commercial insecticide (Bacilod 5000 IU) containing *Bacillus thuringiensis* against the 4th larval stage of *Ae. aegypti.*

Methodology: The molecular analysis revealed a new strain of *Ae. aegypti* and identified 24 bacterial strains from 9 distinct families. These novel strains were documented and deposited in the GenBank for future reference. Notably, in this study *B. subtilis* at different concentrations (1000, 3000, 5000, 7000 and 9000 ppm) was compared with Bacilod 5000 IU (0.1, 0.3, 0.5, 0.8 and 1 ppm).

Results: The mortality rate of 4th instar larvae ranged from 12 to 93% for strains treated with the isolated bacteria *B. subtilis* and 14 to 92% for strains treated with the commercial insecticide *B. thuringiensis* IU. In comparison to *B. subtilis*, the commercial insecticide *B. thuringiensis* showed an approximate 8939.91 times higher relative resistance index.

Interpretation: The results obtained in this study highlights the need for further research into isolated bacteria as potential biological controls for *Ae. aegypti*.

Key words: *Aedes aegypti, Bacillus subtilis*, Biological control, Insecticides, Microbiomes

Triveni Enterprises
(Educational Services Set-up)

How to cite: Sharawi, S.E., D.H. Kaneetah, J.A. Mahyoub, H.M. Al-Solami, A.G. Alghamdi, W.M. Al-Otaibi, T.S. Alghamdi, N.A. Alkenani, M.A. Al-Shaeri, M.E. Shafi and H.S. Alyahya: Molecular characterization of the *Aedes aegypti* (L.), its bacterial microbiomes and their control in Saudi Arabia. *J. Environ. Biol.,* **45,** 277-287 (2024).

Introduction

In the global context, *Aedes aegypti* emerges as a widely distributed insect with a notable inclination towards human habitats (Alonso-Palomares *et al.,* 2018). Its substantial role in the transmission of diseases, such as Dengue, Yellow fever, Zika, and Chikungunya is well-documented (Alonso-Palomares *et al.,* 2018). Female mosquitoes, serving as vectors for arboviruses, pose a considerable health threat to human communities, emphasizing the critical need to comprehend their dynamics (Matthews, 2019; Ferreira-de-Lima *et al.,* 2018). In Saudi Arabia, meticulous cataloging has identified a total of 49 mosquito species, comprising 18 anophelines and 31 culicines, underscoring the intricate nature of disease transmission (Alahmed *et al.,* 2019). Notably, among them, *Ae. aegypti* harbors a higher bacterial diversity (Bennett *et al.,* 2019). Researches on the adult stageof *Ae. aegypti* has unraveled diverse microbiota dwelling in various organs, particularly the midgut. Specific bacteria, from the *Serratia* sp. and *Enterobacter* sp. genera, have been recognized for influencing crucial physiological processes, including altering the host's pH during blood ingestion and impeding arbovirus transmission (Gusmão *et al.,* 2010).

Moreover, bacteria like *Wolbachia* and *Asaia* may possess the capacity to modulate *Ae. aegypti* vector competence, potentially reducing its ability to transmit pathogens to humans. *Wolbachia* has been employed in large-scale biological control strategies due to its ability to invade and persist within arthropod species by manipulating host reproduction (Raoult, 2008; Sohail *et al.,* 2024). Despite the information present on the midgut microbiota, there exists a paucity of studies focusing on the microbiota associated with other vital organs, such as salivary glands and ovaries. The ovaries, in particular, are of interest, as they serve as sites for virus replication, and specific bacteria may influence the establishment and displacement of other symbionts with demonstrated biotechnological and biological control applications for *Ae. aegypti* (Ricci *et al.,* 2012). Existing studies indicate that the bacterial load and diversity in the salivary glands and ovaries are comparatively lower than in the midgut (Alvarado *et al.,* 2021; Gaio *et al.,* 2011). For the larval and pupal stages of *Ae. aegypti,* a comprehensive Indian study utilizing 16S rRNA gene sequences identified isolated bacterial communities, revealing 24 distinct species spanning four major phyla (Yadav *et al.,* 2015; Lan *et al.,* 2024).

A meticulous examination of larvae and adult *Ae. aegypti* from diverse Sri Lankan regions have highlighted the intricate bacterial diversity, emphasizing the role of species, developmental stage, and strain in shaping bacterial diversity (Ranasinghe *et al.,* 2021). Molecular identification techniques, such as PCR amplification and partial 16S rRNA gene fragment sequencing, have identified 25 bacterial species, including *Bacillus endophyticus* and *Pantoea dispersa* (Kai *et al.,* 2019; Bouzid *et al.,* 2024). Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing provide profound insights into mosquito-associated bacteria, with *Proteobacteria* and *Firmicutes* emerging as major phyla and *Bacillus* dominating the isolated bacterial genera (Kai *et al.,* 2019; Ding *et al.,* 2024). Intriguingly, double infections by maternally inherited *Wolbachia pipientis* are prevalent, indicating the complex nature of bacterial associations (Kulkarni *et al.,* 2019; Raharimalala *et al.,* 2012). Combining culture-dependent and culture-independent methods, along with rDNA sequences, facilitate the detection of bacteria from the egg stage of *Ae. aegypti* (Gusmão *et al.,* 2010). Furthermore, laboratory strains of *Ae. aegypti* are associated with bacteria belonging to genera like *Bacillus, Elizabethkingia, Enterococcus, Klebsiella, Pantoea, Serratia,* and *Sphingomonas*. Particularly, *Pantoea stewartii* isolates from *Ae. aegypti* have displayed higher survival rates within *Ae. aegypti,* indicating potential avenues for controlling mosquito-borne diseases (Terenius *et al.,* 2012).

Wolbachia, another isolated bacterium, has been found to possess the potential to reduce *Ae. aegypti's* vector competence by altering its reproductive capabilities (Hoerauf and Rao, 2007; Ye *et al.,* 2013). *Bacillus thuringiensis* (Bt), widely used as a biological larvicide, produces toxins harmful to mosquito larvae, providing an environmentally safe method for controlling *Ae. aegypti* populations (World Health Organization, 2005). Larval source management (LSM) with bacterial larvicides *B. thuringiensis* israelensis (Bti), disrupts the mosquito life cycle by treating breeding sites and killing larvae (World Health Organization, 2011). *B. subtilis,* emerging as a promising biological control agent, showcases versatility in combatting *Ae. aegypti* populations at different life stages, and many studies suggest its impact extends to adult mosquitoes, presenting a holistic approach for integrated vector management programs (Paulraj *et al.,* 2018; Lacey *et al.,* 2015, Elsayed *et al.,* 2020). Building on this comprehensive overview, this study aims to identify isolated bacteria from various stages of *Ae. aegypti,* both within and outside lab strains. Additionally, it seeks to compare environmentally friendly isolated bacteria with commercial insecticide, assessing their efficacy as larvicides against the 4th larval stage of *Ae. aegypti*.

Materials and Methods

Bacterial isolation from the external surface of *Ae. aegypti*: The study encompassed the systematic collection of various developmental stages of *Ae. aegypti* (eggs, larvae, pupae and adults), from the Dengue Mosquito Experimental Station (DMES), at King Abdulaziz University, Jeddah province, Kingdom of Saudi Arabia. The protocol for isolating bacteria from the exterior surface of *Ae. aegypti* mosquitoes adhered to the established methodology of Herms (1950). Each developmental stage was represented by a sample comprising twenty individuals of eggs, pupae, larvae and adult mosquitoes, meticulously collected into sterile test tubes. Following this, 10 ml of distilled water was introduced into each tube and mixed thorough by for 2 minutes. Subsequent to the preparation of various dilutions of the wash, 100 µl of the resultant solution was evenly dispersed onto nutrient agar plates. These plates were then subjected to incubation at a controlled temperature (27°C) for a period of 72 hrs, facilitating the growth and observation of bacterial colonies.

Bacterial isolation from the internal surface of *Ae. aegypti*: Bacterial isolation from *Ae. aegypti* specimens was conducted following the methodology outlined by Herms (1950). Ten individual samples from each developmental stage were subjected to sterilization using 99% ethanol for a duration of 2 min in Eppendorf tubes. Subsequently, nutrient agar plates were streaked with these samples, and the incubation period was established at 72 hr to facilitate bacterial growth. To attain homogeneous bacterial populations from the external and internal surface of *Ae. aegypti,* the process of subculturing was executed. Following isolation, the bacterial entities were cultured in a solution comprising 70% glycerol at the King Fahd Medical Research Center situated in Jeddah Province, Saudi Arabia, facilitating the establishment of a stock culture.

DNA extraction from *Ae. aegypti* and isolated bacteria: DNA extraction procedures were performed on both *Ae. aegypti* specimens and isolated bacteria (Gram-positive and negative), utilizing the QIA amp DNA Mini Kit protocol.

Polymerase Chain Reaction: The PCR technique, universal primers were utilized for both *Ae. aegypti* and isolated bacteria. The Cytochrome Oxidase subunit 1 (CO1) gene of *Ae. aegypti* was amplified using the universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). In the case of bacteria, the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3') were employed. The amplification process took place in a thermocycler (VeritiTM 96-well Thermal Cycler, Applied Biosystems, US). Each amplification reaction consisted 25 µl of 1x GoTaq_Green Master Mix (Promega, USA), incorporating 2 µl of DNA template and 1 µl each of the forward and reverse primers (10 pmol). The amplification proceeded through an initial heating step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, and an extension at 72 °C for 45 seconds (for both mosquito and bacterial). The amplification was concluded with a final extension step at 72 °C for 10 minutes. Subsequently, the temperature was lowered to 4 °C and held indefinitely as the final step in the process according to Somia *et al.* (2023).

Gel Electrophoresis: This part was implemented according to Somia *et al.* (2023). To assess the size of the molecules, 10 µl of a molecular weight marker (Direct Load™ PCR 100 bp Low Ladder D3687-1VL) was loaded into the first well. The gel was electrophoresed at 127 V in the electrophoresis power supply for one hour. To prepare the gel, 1% agarose powder was dissolved in 1X TAE buffer by heating the suspension in a microwave oven Somia *et al.,* 2023. Ethidium bromide (EtBr), at a concentration of 0.1 g ml^{-1}, was added to the agarose solution. Subsequently, the gel tray was filled with the prepared agarose, and a comb was inserted at one end to create wells for sample loading. After one hour, the gel was carefully removed from the comb and placed in an electrophoresis tank filled with 1X TAE buffer as a running buffer. For sample loading, a 5X loading dye was added to the DNA samples at a volume ratio of 5:1 (dye to sample). Horizontal gel apparatuses were used to run the gels for 60–90 minutes at 127 volts. The DNA fragments were visualized and photographed using the Viber Lourmat Gel Imaging System (AC input 115 V, 60 Hz). To determine the molecular weight of the DNA fragments, DNA ladders were utilized as markers. In TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), 2 µl of DNA amplicon was electrophoresed at 100 V for 25 minutes for DNA fragment amplification. Specifically, 100 bp DNA ladders from Promega, USA, were employed as reference markers.

Sequencing Reaction: The DNA Sanger Sequencing method was performed by the Macrogen Company in Korea. Subsequently, the sequence obtained were compared with the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (10.1093/nar/gkh435).

Biological control of *Ae. aegypti* larvae using free cells of isolated bacteria and comparison with a commercial insecticide: The bacterial strain employed in this investigation, *Bacillus subtilis,* exhibits non-pathogenicity towards both human and animal hosts, thus rendering it a safe choice for experimentation. Its environmentally friendly attributes are notable, although its effectiveness against various strains of *Ae. aegypti* in the Jeddah region remains largely unexplored. Colonies of *B. subtilis* were meticulously selected, introduced into a nutrient-rich broth, and maintained at 28 °C within a shaking incubator for a duration of 48 min. The concentration of bacterial cells within the broth suspension was estimated based on the number of cells utilized in the experiments. Various dilutions were prepared, and subsequent to filtration through a bacterial filter with a pore size of 0.2 µm, these suspensions were employed at different concentrations over a 72-hour period to yield bacteria-free cell solutions. *Ae. aegypti* larvae were treated with the bacteria-free cells to generate varied concentrations, following the method of Syed *et al.* (2013).

Distilled water served as a solvent in plastic containers to produce a gradient of concentrations. Each concentration had three replicates, with each plastic cup containing a standardized volume of the final test solution along with 20 larvae. These cups were maintained under controlled temperature (25 °C), following a recommended 12:12 hour light/dark photoperiod. The commercial insecticide Bacilod 5000 IU, comprising *B. thuringiensis* var. israelensis, Serotype H-14, Strain 164, with a potency of 5000 ITU/Mg, was juxtaposed with a naturally isolated bactericide. In the experimental protocol, larvae were completely randomized after 72 hr, and subsequent mortality rates were assessed after an additional 72 hr interval.

Phylogenetic analysis: Phylogenetic analysis of *Ae. aegypti* and the isolated bacteria was conducted utilizing sequences acquired from reference sequences available in the National Center for Biotechnology Information (NCBI) database through BLAST. Evolutionary distances were computed employing the maximum composite likelihood method, and bootstrap support values (1000 replicates) were employed to construct the phylogenetic tree.

Statistical Analyses: The Least Significant Difference (LSD) test was employed for comparing the means utilizing SAS software (version 9.4). Laboratory toxicity results and Resistance Ratio (R.R) of the bacteria against the mosquito larvae were evaluated using LDP Line Software (version 1.0), following the guidelines delineated by Bakr (2005). Sequence comparison and phylogeny reconstruction of *Ae. aegypti* and the isolated bacterial strains, owing to their elevated computational complexity, were analyzed using MEGA software (version 11.0.13) to illustrate the evolutionary relationships among them (DOI: 10.1093/ molbev/ msab120).

Results and Discussion

The mosquitoe genus *Aedes* plays a crucial role in urban infection cycles, characterized by high arbovirus rates perpetuating continuous infection cycles (Beaty *et al.,* 2016). Morphological identification of *Aedes* species poses challenges, especially when specimens are damaged or their external characteristics gets altered. Consequently, accurate identification of species relies on DNA barcoding. In this study, molecular identification led to the discovery of a new strain of *Ae. aegypti* (SHARAWI_3), submitted to the GenBank database (NCBI) under accession number OR287717. The emergence of this new strain may be attributed to environmental and geographical changes. Our findings are consistent with Gupta *et al.* (2016), who highlighted the pivotal role of geographical factors in influencing genetic variations within *Ae. aegypti* populations. Low genetic variation in the COI gene of *Ae. aegypti* compared to various strains suggests specific genetic patterns in different regions (Gupta *et al.,* 2016; Urdaneta-Marquez *et al.,* 2008).

Local ecological factors also contribute to population dynamics, underscoring the importance of comprehensive studies comparing local mosquito species and understanding ecological influences on population variations (Ramirez *et al.,* 2012). Furthermore, Table 1 presents the GenBank accession numbers of sequences demonstrating the highest similarity to the partial sequence of the mitochondrial DNA gene (CO1) in Ae.

Table 1: Percentage of similarity to the closest neighbor(s) of the isolate

Nearest neighbor(s)	Accession number	Similarities (%)
Ae. aegypti	MN299016.1 MN299014.1 MN299008.1 MK542380.1 MK542379.1	99.35

Table 2: Bacterial species and families isolated from both laboratory and wild strains of *Ae. aegypti*.

Body surface	*Ae. aegypti* stages	Species	Genera	Families
Internal	Egg	*Bacillus sp.*	*Bacillus*	*Bacillaceae*
	Egg	*Bacillus cereus*	*Bacillus*	*Bacillaceae*
	Larva	*Delftia sp.*	*Delftia*	*Comamonadaceae*
	Larva	*Delftia lacustris*	*Delftia*	*Comamonadaceae*
	Larva	*Delftia lacustris*	*Delftia*	*Comamonadaceae*
	Pupa	*Aeromonas caviae*	*Aeromonas*	*Aeromonadaceae*
	Pupa	*Delftia sp.*	*Delftia*	*Comamonadaceae*
	Pupa	*Elizabethkingia anophelis*	*Elizabethkingia*	*Weeksellaceae*
	Adult	*Delftia sp.*	*Delftia*	*Comamonadaceae*
External	Egg	*Brevundimonas sp.*	*Brevundimonas*	*Caulobacteraceae*
	Larva	*Bacillus subtilis*	*Bacillus*	*Bacillaceae*
	Larva	*Serratia oryzae*	*Serratia*	*Yersiniaceae*
	Larva	*Pseudomonas sp.*	*Pseudomonas*	*Pseudomonadaceae*
	Larva	*Elizabethkingia anophelis*	*Elizabethkingia*	*Weeksellaceae*
	Pupa	*Comamonas sp.*	*Comamonas*	*Comamonadaceae*
	Pupa	*Delftia sp.*	*Delftia*	*Comamonadaceae*
	Pupa	*Elizabethkingia anophelis*	*Elizabethkingia*	*Weeksellaceae*
	Pupa	*Microbacterium sp.*	*Microbacterium*	*Microbacteriaceae*
	Pupa	*Bacillus halotolerans*	*Bacillus*	*Bacillaceae*
	Adult	*Bacillus tequilensis*	*Bacillus*	*Bacillaceae*
	Adult	*Bacillus subtilis*	*Bacillus*	*Bacillaceae*
	Adult	*Staphylococcus pasteuri*	*Staphylococcus*	*Staphylococcaceae*
	Adult	*Bacillus amyloliquefaciens*	*Bacillus*	*Bacillaceae*
	Adult	*Bacillus subtilis*	*Bacillus*	*Bacillaceae*

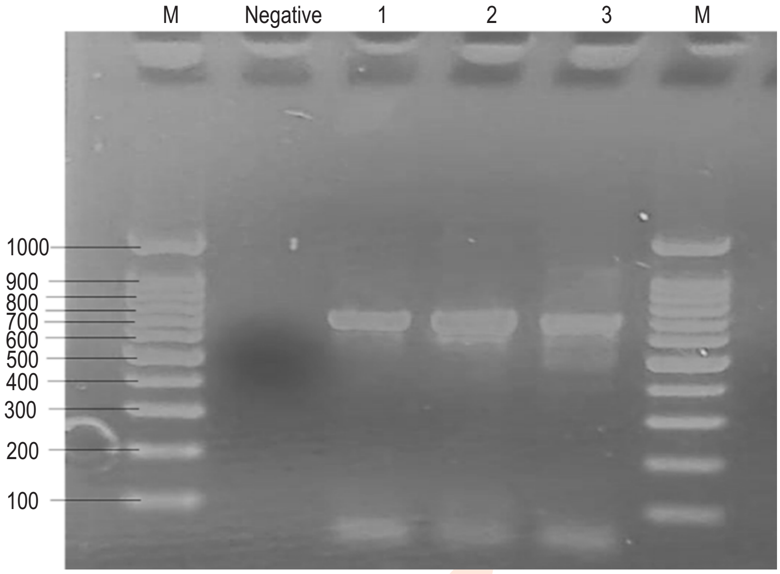

Fig. 1: Gel electrophoresis of CO1 gene. M: Marker; Negative: negative control;1-3: Repeated samples.

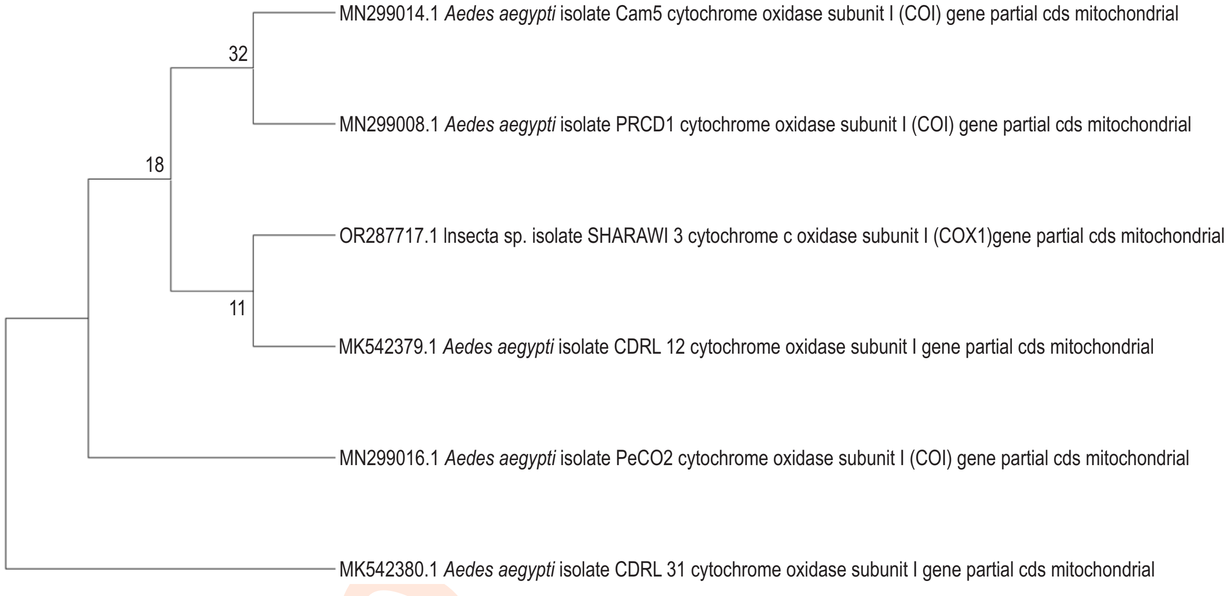

Fig. 2: The phylogenetic among the DNA isolates of *Ae. aegypti* and its closest species depicted using MEGA 11 software.

aegypti, along with their nearest neighbours. The length of *Ae. aegypti* DNA is reported to be 600 base pairs (bp), indicating the size of the genetic sequence. This information is crucial for genetic studies and molecular analysis of the mosquito's traits and characteristics, which is in agreement with previous study (Pitt *et al.,* 2024; Choudhary *et al.,* 2024; Somia *et al.*, 2023). Subsequently, Figure 2 depicts a phylogenetic tree show casing the evolutionary connection between *Ae. aegypti* and its closely related species. The tree is generated through DNA sequence alignments conducted with the MEGA software, facilitating a comparative analysis of genetic similarities and differences among these mosquito species. This method aids in understanding the evolutionary history and genetic diversity within the *Aedes* genus. Furthermore, this study unveiled a varied spectrum of bacterial families and species residing within *Ae. aegypti* laboratory strains. We effectively isolated bacteria from

Table 3: Identification of bacterial isolates (external and internal isolation)

Location	Species	Similarity	Accession number	Strains
	Bacillus sp.	99.57%	OR225844.1	Bacteria_JED_1
	Bacillus cereus	99.43%	OR225845.1	Bacteria_JED_4
	Delftia sp.	99.56%	OR225847.1	Bacteria_JED_7
Internal surface	*Delftia lacustris*	92.88%	OR225846.1	Bacteria_JED_6
	Delftia lacustris	98.53%	OR225848.1	Bacteria_JED_8
	Aeromonas caviae	96.19%	OR225849.1	Bacteria_JED_9
	Delftia sp.	99.27%	OR225864.1	Bacteria_JED_15
	Elizabethkingia anopheles	99.84%	OR225850.1	Bacteria_JED_10
	Delftia sp.	99.57%	OR225865.1	Bacteria_JED_16
	Brevundimonas sp.	99.25%	OR229082.1	Bacteria_JED_13
	Bacillus subtilis	99.71%	OR225866.1	Bacteria_JED_17
	Serratia oryzae	98.88%	OR225867.1	Bacteria_JED_18
	Pseudomonas sp.	99.84%	OR225868.1	Bacteria_JED_19
	Elizabethkingia anophelis	99.84%	OR225869.1	Bacteria_JED_20
	Comamonas sp.	99.85%	OR225870.1	Bacteria_JED_21
	Delftia sp.	98.55%	OR225871.1	Bacteria_JED_22
External surface	*Elizabethkingia anopheles*	99.84%	OR225872.1	Bacteria_JED_23
	Microbacterium sp.	99.84%	OR225873.1	Bacteria_JED_24
	Bacillus halotolerans	99.70%	OR225874.1	Bacteria_JED_25
	Bacillus tequilensis	99.43%	OR225875.1	Bacteria_JED_26
	Bacillus subtilis	99.43%	OR225876.1	Bacteria_JED_27
	Staphylococcus pasteuri	99.29%	OR225877.1	Bacteria_JED_28
	Bacillus amyloliquefaciens	99.52%	OR225878.1	Bacteria_JED_29
	Bacillus subtilis	99.71%	OR225879.1	Bacteria_JED_30

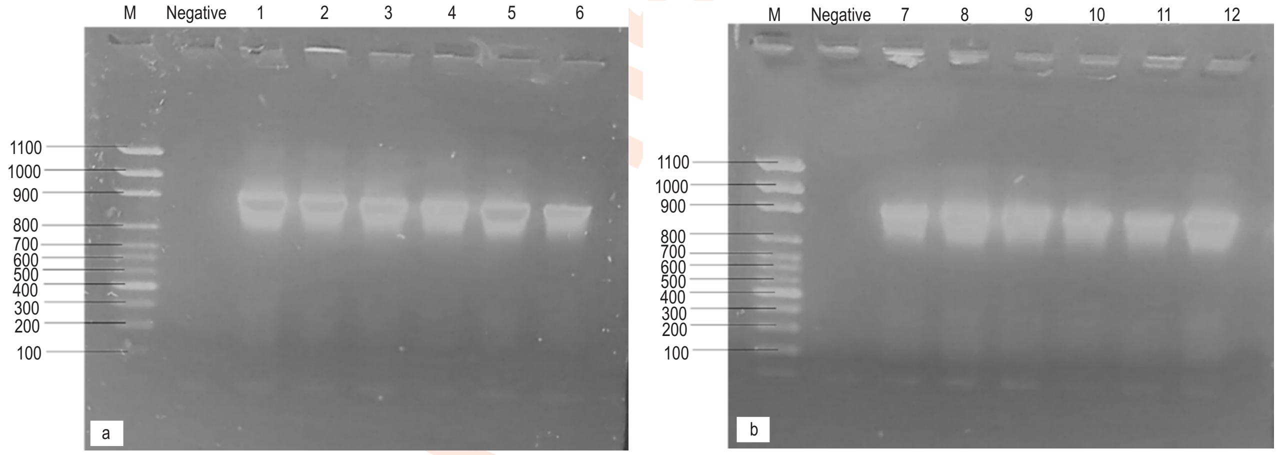

Fig. 3: PCR products of some isolated bacteria. M: Marker; negative: negative control. (A) Sample from 1:6, (B) sample from 7:12.

internally and externally within the body of *Ae. aegypti* spanning 9 families encompassing 10 genera and 16 distinct species, as elucidated in Table 2. The table lists the positions of various isolates found in different developmental stages of *Ae. aegypti* mosquitoes, along with their respective species, genera, and families. It indicates the presence of diverse bacterial species, such as *Bacillus, Delftia, Aeromonas*, and *Pseudomonas* across different life stages, both internally and externally within the body of *Ae. aegypti*. This information provides insights into the microbiota associated with *Ae. aegypti* at different stages of its life cycle. Table 3 furnishes comprehensive information regarding the newly isolated bacterial strains, all of which have been deposited in the GenBank database (NCBI). It was observed that isolated bacteria from external sources exhibited a greater diversity of families compared to those from internal sources, possibly due to direct contact with the external environment. The most commonly

Table 4: Mortality rates of 4th instar larvae of *Ae. aegypti* following exposure to varying concentrations of *B. subtilis* natural product

Concentration (ppm)	Mortality (%)
1000	12e ±0.50
3000	45d ±0.16
5000	63c ±0.25
7000	85b±0.12
9000	93a±0.22
control	0±0
LSD	11.5
P	0.0001

Values are mean ± S.E. *Means followed by similar letters in the same column reveal no significant differences between them at 0.05 significance level

Table 5: Mortality rates of 4th instar larvae of *Ae. aegypti* following exposure to various concentrations of commercial insecticide *B. thuringiensis*

Concentration (ppm)	Mortality (%)
0.1	14e±0.25
0.3	30d±0.12
0.5	63c±0.50
0.8	85b±0.22
1	92a±0.75
Control	0±0
LSD	9.63
P	0.001

Values are mean ± S.E. *Means followed by similar letters in the same column reveal no significant differences between them at 0.05 significance level

Table 6: Dose-mortality response of 4th instar *Ae. aegypti* larvae exposed to different concentrations of tested compound

Statical parameters	Tested compounds		Resistance ratio
	B. subtilis	***B. thuringiensis***	
LC_{50} (ppm)	3129	0.3504	
95% (F. L.)	2755.8- 3502.7	0.1926 - 0.5182	
LC_{90} (ppm)	9462.7	1.1124	8929
95% (F. L.)	8091.4- 11579.7	0.9448- 3.1843	
Slope	2.67±0.2201	2.55±0.2105	
Tabulated (Chi)^2	7.81	7.81	
Calculated (Chi)^2	6.1828	4.027	
P	0.0262	0.0029	
r	0.9839	0.9667	

*Tabular value of Chi square (χ^2) was greater than one calculated at 0.05 level of significance and 3 degrees of freedom. Therefore, the results were homogeneous and the toxicity line was a good representative of the results. • Used five replicates of 20 larvae/replicate. • The death rate in the witness was 0.0%.

found family internally was *Comamonadaceae*, while externally, *Bacillaceae*. *Delftia* sp. and *Elizabethkingia anophelis* were exclusively isolated from the pupal stage in both locations. Various *Bacillus* genera were isolated from the adult stage internally, including *B. tequilensis*, *B. subtilis* and *B. amyloliquefaciens.* Our findings are consistent with Kai *et al.* (2019), who identified diverse bacterial families such as *Acinetobacter, Delftia, Pseudomonas* and *Enterobacteriaceae*, and Terenius *et al.* (2012), who isolated various bacterial genera from *Ae. aegypti lab strains,* including *Bacillus, Elizabethkingia, Enterococcus, Klebsiella, Pantoea, Serratia* and *Sphingomonas*.

In the polymerase chain reaction (PCR) method, the DNA extracted from isolated bacterial specimens was discerned, revealing a length of 450 base pairs (bp) as depicted in Fig. 3, corroborating with the previous findings of Jin *et al.* (2024). Fig. 4 presents a phylogenetic tree delineating the evolutionary analysis of the isolated bacterial strains. Utilizing the MEGA software, this tree was constructed to elucidate the interrelatedness among the isolated bacterial entities on an evolutionary scale.

Regarding biological control, the efficacy of natural insecticide *B. subtilis* was compared with the commercial insecticide *B. thuringiensis. B. subtilis,* known for its potency against the larval stage of mosquitoes, exhibited significant larvicidal effects on *Ae. aegypti* larvae. As illustrated in Tables 4 and 6, the mortality rates of *Ae. aegypti* larvae exhibited a direct association with the concentration of the tested compounds. Specifically, mortality rates among strains treated with natural product *B. subtilis* ranged from 12% to 93%, and from 14% to 92%, while those treated with *B. thuringiensis* ranged from 14% to 92% and 12% to 93%, respectively. This finding aligns with previous studies demonstrating its effectiveness against mosquito larvae, including *Ae. aegypti* (Lacey *et al.*, 2015; Paulraj *et al.*, 2018; Elsayed *et al.,* 2020). The robust adaptability *B. subtilis* and notable antimicrobial properties make it a compelling candidate for eco-friendly larval control (Salazar *et al.,* 2023). On the other hand, *B. thuringiensis,* a widely used commercial insecticide, has been extensively studied and applied for mosquito control. It produces toxins harmful to mosquito larvae, offering an environmentally safer method for controlling *Ae.*

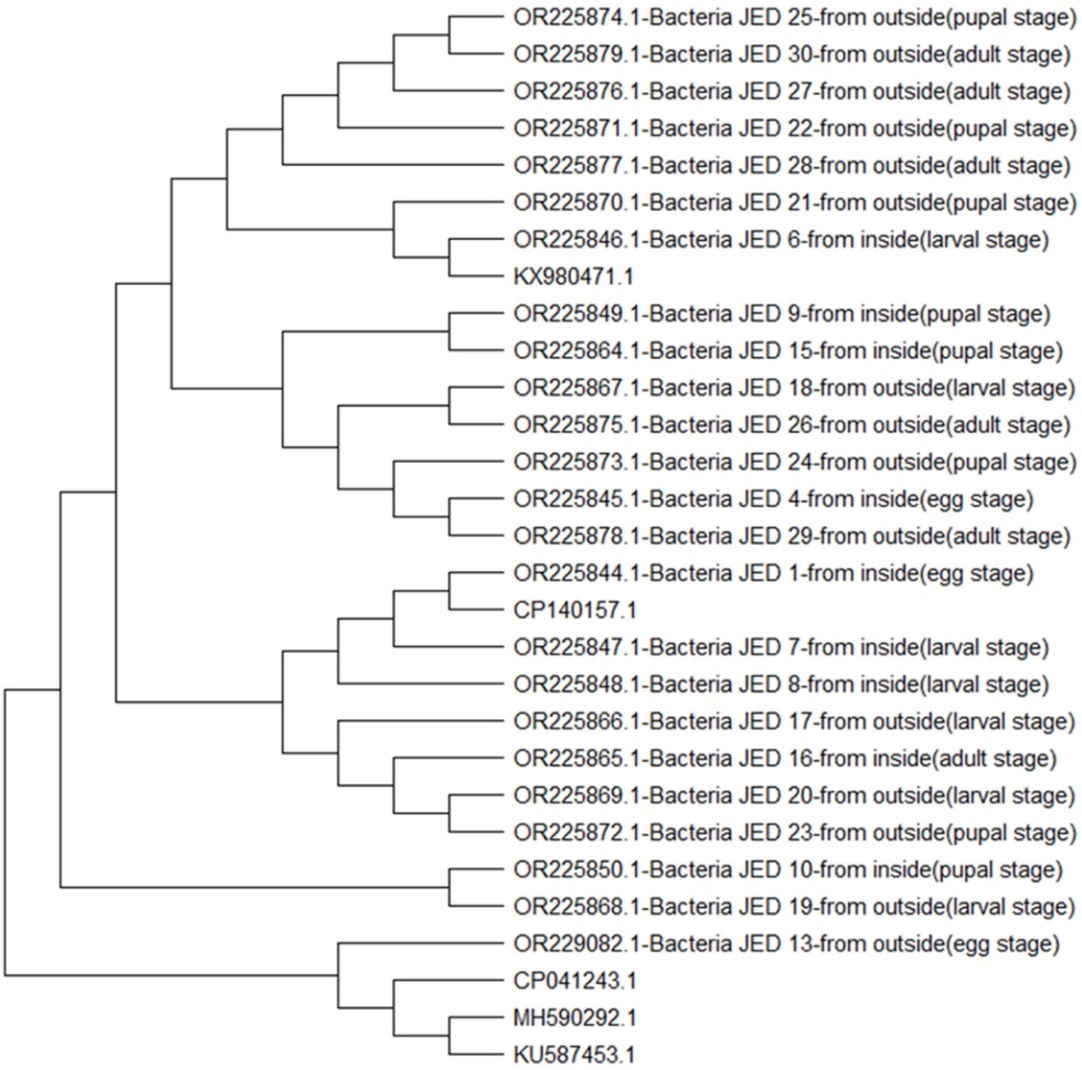

Fig. 4: Phylogenetic analysis of isolated bacterial strains. The tree was constructed using MEGA 11 software to depict the evolutionary relationships among the isolated bacteria.

aegypti populations (World Health Organization, 2005). The larvicidal efficacy of *B. thuringiensis* against *Ae. aegypti* larvae has been demonstrated in various settings, showcasing its effectiveness in reducing mosquito populations.

In comparison between these two bactericides, the LDP toxicity lines (Fig. 5 and 6) and LC_{50} and LC_{90} values (Table 5) clearly indicate difference in the sensitivity levels of 4th instar larvae exposed to various pesticide concentrations. After 24 hr of treatment, the concentrations required to kill 50% and 90% of larvae with *B. subtilis* were 2755.89 ppm and 8091.41 ppm, respectively (Table 6). This effect is caused by the strain's natural composition and weak nature. Whereas for *B. thuringiensis,* these concentrations were 0.3504 ppm and 1.1124 ppm because of its addition ingredients due to the presence of bacterial supplements, which enhanced its efficacy beyond that of naturally isolated counterparts. A highly significant positive correlation was observed between the effects of the tested compounds and the larvae's response rate, with a correlation value (r) of 0.98 for the natural insecticide *B. subtilis* and 0.96 for the commercial insecticide *B. thuringiensis.* The significant correlation values of natural insecticide of *B. subtilis* and insecticide were P = 0.026 and P = 0.0029, respectively. The Chi-square values for *B. subtilis* and *B. thuringiensis* (Table 6) were approximately 6.1828 and 4.027, respectively, both below the tabular Chi-square value for significance α = 0.05 and degrees of freedom df = 3. This average of 7.8 suggests homogeneity among individuals and significant differences in the death rates of each compound based on the tested concentrations.

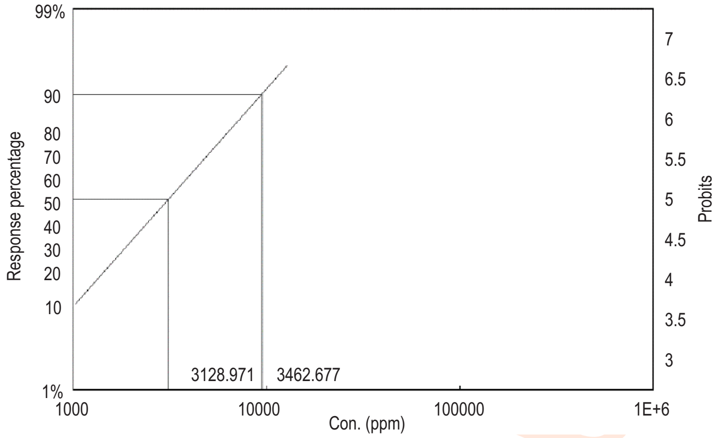

Fig. 5: Linear relationship between natural product concentrations of *B. subtilis* and the death percentage of instar larvae of *Ae. aegypti.*

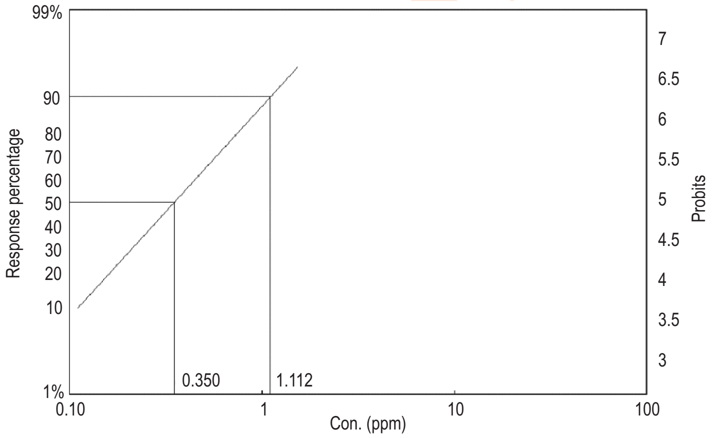

Fig. 6: Linear relationship between concentrations of commercial insecticide *B. thuringiensis* and the death percentage of instar larvae of *Ae. aegypti.*

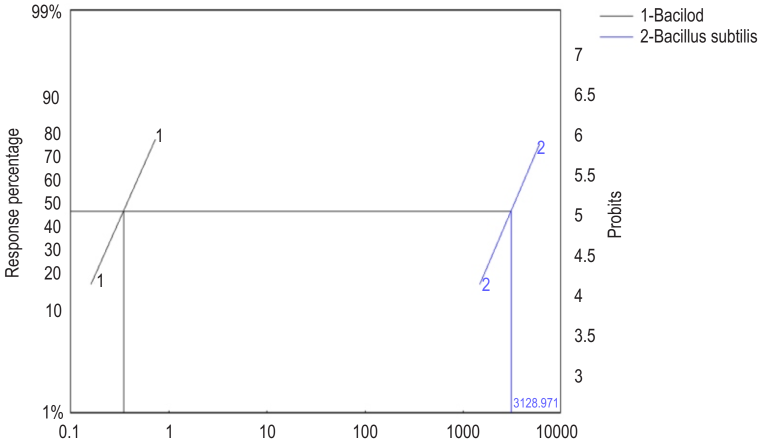

Fig. 7: Varying susceptibility levels of 4th instar *Ae. aegypti* larvae when exposed to different concentrations of both natural product *B. subtilis* and commercial insecticide *B. thuringiensis*.

Fig. 7 illustrates minimal disparity between the slopes of the toxicity lines for both compounds, with a slope of (2.6666 ± 0.2201) for the natural product *B. subtilis* and (2.5542 ± 0.2105) for the commercial insecticide *B. thuringiensis.* The relative resistance index (RR) values confirm a notable variation in the susceptibility level of *Aedes* mosquito larvae to the tested compounds. Specifically, the sensitivity level of the commercial insecticide *B. thuringiensis* was approximately 8939.917 times higher than that of *B. subtilis*. Comparing these two bactericides, studies indicate that *B. subtilis*, when used as a larvicide, can exhibit significant larvicidal effects on *Ae. aegypti* larvae, with reported lower sensitivity levels compared to its commercial counterpart, *B. thuringiensis* (Lacey *et al.,* 2015). The variance in sensitivity could stem from supplementary stimulants and additives present in commercial insecticides, augmenting their effectiveness, as suggested by Rezende-Teixeira *et al*. (2022). This highlights the complexity of formulation in commercial products, which may impact their potency and efficacy against target pests. Understanding these factors is crucial for optimizing insecticide performance and pest management strategies.

The current investigation a significant advancement in the comprehension of *Ae. aegypti* and their associated bacterial communities. Furthermore, this research includes the deposition of newly identified strains of *Ae. aegypti* and bacteria into the GenBank database. Through comprehensive molecular characterizations, insights into the genetic makeup of *Ae. ae gypti* and their associated bacteria have deepened. Moreover, environmentally sustainable bacterial isolates, such as *B. subtilis*, have been explored as potential agents for biological control against *Ae. aegypti* larvae, alongside commercial insecticides. Comparative analysis of these isolates revealed that *B. thuringiensis* demonstrated sensitivity to *Ae. aegypti,* approximately 8940 times higher than that of *B. subtilis,* suggesting its efficacy as a potent control agent. Nevertheless, the study underscores the necessity for further investigation. Future research endeavors should delve into the efficacy of isolated and identified bacteria from *Ae. aegypti* in biocontrol strategies. Continued investigations hold promise for yielding valuable insights, potentially facilitating the development of environmentally friendly and efficacious solutions to combat diseases transmitted by these mosquitoes.

Acknowledgments

Special thanks are extended to the Microbiology Laboratory and the Dengue Mosquito Experimental Station (DMES) in King Abdul-Aziz University (KAU), Jeddah Province, Saudi Arabia, for providing the research equipment necessary as well as for cooperating with the authors over the course of the research period. The authors express their sincere gratitude to the Laboratory for its support during the entire research period.

Authors' contribution: S.E. Sharawi: Conceptualization and Data curation, Software, Writing original manuscript; **D.H. Kaneetah:** Conceptualization, Data curation, Methodology, Writing and editing; **J.A. Mahyoub:** Conceptualization, Data curation, Methodology, Writing – original manuscript; **H.M. Al-Solami, A.G. Alghamdi, Wafa Mohammed AL-Otaibi, T.S. Alghamdi, Naser A. Alkenani, M.A. Al-Shaeri, Manal E. Shafi and H.S. Alyahya:** Writing and editing.

Funding: There was no funding provided by any organization for this project.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that they have no financial or non-financial competing interests.

Data availability: The datasets generated during the current study are available in the NCBI database repository, accession numbers to datasets can be found in Table 3.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

References

Alahmed, A.M., K. Munawar, S.M. Khalil and R.E. Harbach: Assessment and an updated list of the mosquitoes of Saudi Arabia. *Paras. Vect.,* **12**, 1-9 (2019).

Alonso-Palomares, L.A., M. Moreno-García, H. Lanz-Mendoza and M.I. Salazar: Molecular basis for arbovirus transmission by *Aedes aegypti* mosquitoes. *Intervirology,* **61**, 255-264 (2019).

Alvarado, W.A., S.O. Agudelo, I.D. Velez and R.J. Vivero: Description of the ovarian microbiota of *Aedes aegypti* (L) Rockefeller strain. *Acta. Trop.,* **214**, 105765 (2021).

Bakr, E.: LDP Line Software, used for dose-response regression analysis in toxicological and biological studies according to Finney (1971).

Beaty, B.J., W.C. Black, L. Eisen, A.E. Flores, J.E. García-Rejón, M. Loroño-Pino and K. Saavedra-Rodriguez: The intensifying storm: Domestication of *Aedes aegypti*, urbanization of arboviruses, and emerging insecticide resistance. In: Global Health Impacts of Vector-Borne Diseases: Workshop Summary. Chapter 3. (Ed.: Alison Mack Rapporteur). National Academies Press*,* pp.111-142 (2016).

Bennett, K.L., C. Gómez-Martínez, Y. Chin, K. Saltonstall, W.O. McMillan, J.R. Rovira and J.R. Loaiza: Dynamics and diversity of bacteria associated with the disease vectors *Aedes aegypti* and *Aedes albopictus. Sci. Rep.,* **9**, 12160 (2019).

Bouzid, F., I. Gtif, S. Charfeddine, L. Abid and N. Kharrat: Polyphasic molecular approach to the characterization of methanogens in the saliva of Tunisian adults. *Anaerobe,* **85**, 102820 (2024).

Choudhary, J., M. Milind, N. Meena and B.N. Shringi: Sequence identification of toll like receptor 6 in dromedary camel (*Camelus dromedarius*). *Int. J. Adv. Biochem. Res.*, **8**, 395-398 (2024).

Ding, X., W. Lan, J. Li, M. Deng, Y. Li, Y. Katayama and J.D. Gu: Metagenomic insight into the pathogenic-related characteristics and resistome profiles within microbiome residing on the Angkor sandstone monuments in Cambodia. *Sci. Total Environ.,* **918**, 170402 (2024).

Elsayed, S.A., J. Myamba and A.M. Mahande: Efficacy of *Bacillus subtilis* and *Bacillus thuringiensis* in controlling *Aedes aegypti* larvae under laboratory and semi-field conditions. *PLoS ONE,* **15**, e0243943 (2020).

Ferreira-de-Lima, V.H. and T.N. Lima-Camara: Natural vertical transmission of dengue virus in *Aedes aegypti* and *Aedes albopictus*: a systematic review. *Parasi. Vect.,* **11**, 77 pages (2018).

Gaio, A.D.O., D.S. Gusmão, A.V. Santos, M.A. Berbert-Molina, P.F. Pimenta and F.J. Lemos: Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (diptera: culicidae) (L.). *Parasi. Vect.,* **4**, 105 (2011).

Gupta, K., R. Dhawan, M. Kajla, S. Kumar, B. Jnanasiddhy, N.K. Singh, and L. Gupta: Molecular identification of *Aedes aegypti* mosquitoes from Pilani region of Rajasthan, India. *J. Vec. Borne Dise.,* **53**, 149-155 (2016).

Gusmão, D.S., A.V Santos, D.C. Marini, Jr. M. Bacci, M.A. Berbert-Molina, and F.J.A. Lemos: Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Trop.,* **115**, 275-281 (2010).

Herms, W.B.: Medical Entomology: With Special Reference to the Health and Wellbeing of Man and Animals. *New York Macmillan,* pp. 79-85 (1950).

Hoerauf, A. and R.U. Rao: The significance of *Wolbachia* endosymbionts in pathogen transmission, drug response and immunology: implications for the control of filarial infections. *Indian J. Medi. Microbiol.,* **25**, 261–268 (2007).

Jin, J., R. Yamamoto and K. Shiroguchi: High-throughput identification and quantification of bacterial cells in the microbiota based on 16S rRNA sequencing with single-base accuracy using BarBIQ. *Nat. Proto.,* **19**, 207-239 (2024).

Kai, S., Y. Matsuo, S. Nakagawa, K. Kryukov, S. Matsukawa, H. Tanaka, and K. Hirota: Rapid bacterial identification by direct PCR amplification of 16S rRNA genes using the MinION™ nanopore sequencer. *FEBS Open Bio.,* **9**, 548-557 (2019).

Kulkarni, A., W. Yu, J. Jiang, C. Sanchez, A.K. Karna, K.J. Martinez and J. Xu: *Wolbachia pipientis* occurs in *Aedes aegypti* populations in New Mexico and Florida, USA. *Ecol. Evolu.,* **9**, 6148-6156 (2019).

Lacey, L.A., D. Grzywacz, D.I. Shapiro-Ilan, R. Frutos, M. Brownbridge, and M.S. Goettel: Insect pathogens as biological control agents: Back to the future. *J. Inverte. Pathol.,* **132**, 1-41 (2015).

Lan, X., S. Wu, Q. Du and L. Min: The investigation of changes in bacterial community of pasteurized milk during cold storage. *Foods,* **13**, 451 (2024).

Matthews, B.J.: *Aedes aegypti*. *Trends* in *Genetics,* **35**, 470–471 (2019).

Paulraj, M.G., S. Ignacimuthu, M.R. Gandhi: Role of *Bacillus subtilis* as a potential biocontrol agent against *Aedes aegypti* and *Culex quinquefasciatus*. *Environ. Sci. Pollut. Res. Int.,* **25**, 34561–34569 (2018).

Pitt, W.J., W.R. Cooper, D. Pouchnik, H. Headrick and P. Nachappa: High-throughput molecular gut content analysis of aphids identifies plants relevant for potato virus Y epidemiology. *Insect Sci.,* (2024). DOI:10.1111/1744-7917.13327

Raharimalala, F.N., L.H. Ravaomanarivo and P. Ravelonandro: Biogeography of the two major arbovirus mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae), in Madagascar. *Parasi. Vect.,* **5**, 56 pages (2012).

Ramirez, J.L., J. Souza-Neto, R. Torres Cosme, J. Rovira, A. Ortiz, J.M. Pascale and G. Dimopoulos: Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Negle. Trop. Disea.,* **6**, e1561 (2012).

Ranasinghe, K., N. Gunathilaka, D. Amarasinghe, W. Rodrigo and L. Udayanga: Diversity of midgut bacteria in larvae and females of *Aedes aegypti* and *Aedes albopictus* from Gampaha District, Sri Lanka. *Parasi. Vect.,* **14**, 1-11 (2021).

Raoult, D.: *Wolbachia*: A bug's life in another bug. *Emerg Infect Dis.* **14**,1338 (2008).

Rezende-Teixeira, P., R.G. Dusi, P.C. Jimenez, L.S. Espindola and L.V. Costa-Lotufo: What can we learn from commercial insecticides? Efficacy, toxicity, environmental impacts and future developments. *Environ. Pollu.,* **300**, 118983 (2022).

Ricci, I., C. Damiani, A. Capone, C. DeFreece, P. Rossi and G. Favia: Mosquito/ microbiota interactions: from complex relationships to biotechnological perspectives. *Curr. Opini. Microbiol.,* **15**, 278-284 (2012).

Salazar, B., A. Ortiz, C. Keswani, T. Minkina, S. Mandzhieva, S. Pratap Singh and E. Sansinenea: *Bacillus* spp. as bio-factories for antifungal secondary metabolites: Innovation beyond whole organism formulations. *Micro. Ecol.,* **86**, 1-24 (2023).

Sohail, A., K.L. Anders, S.L. McGuinness and K. Leder: The epidemiology of imported and locally-acquired dengue in Australia, 2012–2022. *J. Tra. Medi.,* **31**, taae014 (2024).

Somia, E.S., I. Ullah, H.S. Alyahya and J.A. Mahyoub: Identification of *Wolbachia* new strains from *Aedes aegypti* mosquitoes, the vector of dengue fever in Jeddah Province. *BMC Microbiol.,* **23**, 287 (2023).

Syed, R., F. Manzoor, R. Adalat, A. Abdul-Sattar and A. Syed: Laboratory evaluation of toxicity of insecticide formulations from different classes against American cockroach (Dictyoptera: Blattidae). *J. Arthro. Borne Disea.,* **8**, 21-34 (2014).

Terenius, O., J.M. Lindh, K. Eriksson-Gonzales, L. Bussière, A.T. Laugen, H. Bergquist and I. Faye: Midgut bacterial dynamics in *Aedes aegypti*. *FEMS Microbiol. Ecol.,* **80**, 556-565 (2012).

Urdaneta-Marquez, L., C. Bosio, F. Herrera, Y. Rubio-Palis, M. Salasek, and W.C. Black: Genetic relationships among *Aedes aegypti* collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. *Ameri. J. Tropi. Medi. Hygi.,* **78**, 479-491 (2008).

World Health Organization: Guidelines for laboratory and field testing of mosquito larvicides. pp. 13-39 (2005). https://apps.who.int/iris/handle/10665/69101.

World Health Organization: Handbook for Integrated Vector Management. 67 pages (2011).

Yadav, K.K., A. Bora, S. Datta, K. Chandel, H.K. Gogoi, G.B.K.S. Prasad, and V. Veer: Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. *Parasi. Vect.,* **8**, 641 (2015).

Ye, Y.H., A.M. Carrasco, F.D. Frentiu, S.F. Chenoweth, N.W. Beebe, A.F. van den Hurk, C.P. Simmons, S.L. O'Neill and E.A. McGraw: *Wolbachia* reduces the transmission potential of dengue-infected *Aedes aegypti*. *PLoS Negl. Trop. Dis.,* **9**, e0003894 (2015).