Bacteria Isolated from Mosquito Proliferation Sites Influence on the Oviposition Behavior in *Anopheles Arabiensis* (Diptera: Culicidae)

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ABSTRACT

The bacterial composition of mosquito oviposition sites significantly influences egg-laying preferences, making it a critical factor in developing innovative mosquito control strategies, particularly in malaria-endemic regions like Kwale County, Kenya. However, the specific bacterial profiles that affect *Anopheles arabiensis* oviposition preferences remain largely unstudied. This research investigated the role of bacteria isolated from mosquito proliferation sites in Kwale County and their impact on the oviposition behavior of gravid *An. arabiensis*. Water samples were collected from preferred (with mosquito larvae) and non-preferred (without mosquito larvae) oviposition sites. Bacteria were isolated and identified through culture-based methods and molecular characterization using the 16S rRNA gene, with phylogenetic analysis employed to examine genetic relationships among isolates. The Oviposition Activity Index (OAI) was calculated in controlled bioassays to assess the attractiveness or repellence of these bacteria to gravid *An. arabiensis*. The study identified 14 bacterial isolates, with *Enterobacter* species showing the highest attractiveness to mosquitoes, as indicated by a higher OAI compared to *Pluralibacter* species. Notably, *Bacillus pumilus* strain RGS9 demonstrated the lowest OAI among bacteria from preferred sites, while *Uncultured bacterium clone wp2*, identified in non-preferred sites, exhibited a negative OAI, indicating repellence. Phylogenetic analysis revealed distinct clades, with *Enterobacter sp. CDB3* and *Enterobacter sp. MLB32* forming closely related clusters with high bootstrap support, highlighting their strong genetic similarity. Conversely, *Pluralibacter gergoviae* and *Pluralibacter sp. strain SC* were less attractive, as reflected by their lower OAI values compared to *Enterobacter* species. For example, *Pluralibacter gergoviae* had an OAI of 0.48, lower than the 0.84 of *Enterobacter* species, but higher than *Uncultured bacterium clone wp2*, which showed negative OAI values, indicating complete repellence. Significant differences in bacterial compositions were observed between preferred and non-preferred sites. *Enterobacter* species were predominantly associated with preferred sites, while *Pluralibacter* species were more common in non-preferred sites. Statistical analysis confirmed these differences, with OAI values showing a statistically significant distinction ($p < 0.05$) between bacteria from the two site types. These findings underscore the critical role of bacterial composition in shaping mosquito oviposition behavior. The strong attractiveness of *Enterobacter* species suggests their potential application in targeted mosquito control strategies. For instance, bait stations infused with *Enterobacter* species could lure mosquitoes away from human populations into controlled environments where they can be trapped or exterminated. Such approaches could significantly reduce mosquito-borne disease transmission by lowering mosquito populations in areas of high human activity.

Keywords: Anopheles arabiensis (An. arabiensis), bacterial isolates, oviposition sites, microbial ecology, phylogenetic analysis (PA).

INTRODUCTION

Malaria remains a significant global health challenge, with millions of individuals affected worldwide. In fact, in 2020, there were 241 million malaria cases and 627,000 malaria-related deaths globally, with many cases

(95%) and deaths (96%) reported in the World Health Organization (WHO) African region [1]. Africa remains the most affected region, with 95% of malaria cases occurring there, followed by the Eastern Mediterranean and South East Asia regions, which account for 2.5% and 2% of cases, respectively [2]. Particularly devastating is the impact of malaria on young children, with about 467,000 children under five years old succumbing to the disease in the African region alone, representing 78.9% of all malaria-related deaths [3].

The incidence of malaria in the WHO African Region increased from 218 million cases in 2019 to 232 million cases in 2020, with deaths rising from 544,000 to 599,000 during the same period [4]. Despite a general decline in malaria cases since 2000, disruptions caused by the COVID-19 pandemic led to an increase in cases in 2020, although rates fell again in 2021 [4]. This fluctuation underscores the vulnerability of malaria control efforts to external factors and the importance of maintaining consistent interventions to combat the disease effectively. A critical aspect of malaria control is understanding the oviposition behavior of mosquito vectors, as this behavior significantly influences their reproductive success and population size [5].

Mosquitoes exhibit diverse oviposition behaviors influenced by various environmental factors such as temperature, substrate color, water type, and the presence of microorganisms [6]. The selection of oviposition sites is guided by a combination of olfactory, temperature, visual, and chemical cues that can either attract or repel mosquitoes [7].

Recent studies have identified compounds from sources like hay infusions, bacteria, and pheromones that act as attractants or repellents for mosquito oviposition. However, there is limited data on how bacterial attractants from larval habitats impact oviposition site selection, highlighting the need for further research in this area [8]. Understanding these microbial interactions is crucial not only for developing new control strategies but also for effectively targeting and managing mosquito breeding sites to reduce malaria transmission rates.

Malaria continues to pose a significant public health challenge in sub-Saharan Africa, with the Anopheles mosquito being the primary vector for disease transmission [9]. Control efforts face obstacles such as mosquito resistance and behavioral changes that undermine traditional strategies [10]. Understanding factors that influence mosquito behavior, particularly oviposition site selection, is crucial for developing effective vector control strategies [11]. While it is known that physical and chemical cues play a role in oviposition site selection, the impact of microbial ecology on mosquito attraction and oviposition behavior remains underexplored.

Existing studies suggest that bacteria and other microorganisms present in larval habitats emit chemical compounds that can either attract or repel mosquitoes, thereby influencing their oviposition site preferences [11]. However, there is a lack of specific data on the bacterial compositions that affect *Anopheles arabiensis* oviposition in malaria-endemic regions like Kwale County, Kenya. This gap in knowledge hinders the development of targeted interventions that utilize microbial attractants or repellent to disrupt mosquito breeding cycles effectively [9].

Hence, this study sought to delve into the microbial ecology of *An. arabiensis* oviposition sites and assess the potential contribution of bacterial isolates to mosquito attraction. By shedding light on these microbial interactions, this study provided insights that could underpin innovative vector control strategies and ultimately aid in curtailing malaria transmission.

Justification of the Study

Understanding the bacterial composition of mosquito oviposition sites is crucial for advancing vector control strategies, particularly in malaria-endemic regions like Kwale County, Kenya [12]. While traditional mosquito control measures such as insecticide-treated nets and indoor spraying target adult mosquitoes, they often fall short against species like *An. arabiensis*, which exhibit behavioral adaptations that reduce their effectiveness [13], [14]. Recent studies have highlighted the role of microorganisms in larval habitats emitting chemical cues that influence mosquito oviposition preferences [15]. However, there is a scarcity of specific data on how bacterial communities impact the egg-laying behavior of *An. arabiensis* [16], [17]. This study addresses this gap by characterizing bacterial isolates from mosquito proliferation sites and evaluating their impact on

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mosquito attraction. By exploring the relationship between microbial composition and oviposition behavior, the findings could lead to innovative control methods that target mosquito breading sites more effectively, offering a complementary approach to existing vector management strategies.

MATERIALS AND METHODS

Study Site

Figure 1: Map of Study Site showing Mwakingeu village (4°30'19.6" S, 39°16'18.7" E) along the Lunga-Lunga – Ramisi road, in Pongwe/Kikoneni ward, Lungalunga constituency, Kwale County, Kenya. The study site, located in a malaria-endemic region, is highlighted with key features like nearby roads, water bodies, and elevation changes. The boundaries of Lungalunga constituency (2,803.80 km²) and Pongwe/Kikoneni ward (223.50 km²) are marked, alongside important ecological features influencing mosquito breeding.

This study was conducted in Kwale County, Kenya, which is a malaria endemic region focusing on Mwakingeu village along the Lunga-Lunga – Ramisi road (4°30'19.6" S 39°16'18.7" E). The site is located in the Pongwe/Kikoneni ward within the Lungalunga constituency, which spans an area of approximately 223.50 km² and hosts a population of about 41,000 people. The larger Lungalunga constituency covers 2,803.80 km² and is home to approximately 153,000 people. Kwale County, characterized by a hot and humid climate, experiences two rainy seasons annually, creating ideal conditions for mosquito proliferation. The county has the highest malaria prevalence in Kenya's coastal region, with rates ranging from 20-30% [18]. The climate in Kwale County features hot, muggy weather year-round, with average temperatures ranging between 22°C and 34°C, relative humidity between 70% and 80%, and annual rainfall between 900mm and 1500mm. The topography varies from sea level to 462 meters above sea level. Notable mosquito species include *An. gambiae* and *An. funestus*, present throughout the year with population peaks following seasonal rains [18]. In recent years, *An. gambiae* s.s. populations have declined, while *An. arabiensis* populations have increased, possibly due to ecological changes [19]. Kwale County is predominantly inhabited by the Digo and Duruma communities, with smaller populations of Kambas and others, especially in urban areas. The residents primarily engage in subsistence farming, cultivating crops such as cassava, coconut, mangoes, and corn, and raising livestock like sheep, goats, and cattle. Traditional homes are constructed using poles and sticks to create a framework filled with mud and thatched with palm leaves [19]. This study site is significant due to its high malaria prevalence, diverse mosquito vector populations, and the ecological dynamics influencing vector abundance and distribution, making it an ideal location for investigating the bacterial characterization of *An. arabiensis* larval habitats and implications for malaria control.

Sampling Technique and Procedure

Determination of Larval Habitat and Sample Collection

Prior to sampling, a reconnaissance survey was conducted to identify potential larval habitats of *Anopheles arabiensis* mosquitoes before the onset of the long rain season (April - July). During this survey, prospective

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breeding sites were marked using GPS coordinates for precise location recording, and their positions were stored for systematic sampling. The marking process involved recording the GPS coordinates of each site using a handheld GPS device, ensuring accuracy for future reference. Potential breeding habitats were classified based on visual inspection of habitat size. Small habitats, such as hoof prints and rain puddles, were defined as having a diameter of one meter or less, while large habitats exceeded one meter in diameter. Sampling was conducted during the heavy rainy season in April, specifically targeting both small and large breeding sites. Sampling was restricted to this period to maximize the chances of identifying active larval habitats, as these are more likely to be present during the rainy season. The presence of *An. arabiensis* larvae in each habitat was confirmed using a 350 mL dipper (BioQuip® Products) attached to a 1.2-meter pole. The dipper was gently lowered at a 45° angle below the water surface to collect larvae while minimizing disturbance. It is important to note that while reconnaissance was completed prior to the rains to map potential breeding grounds, actual larval sampling was only conducted during the heavy rains. This was deemed appropriate for capturing peak breeding activity, although future studies could explore additional sampling during the dry season to assess habitat persistence and year-round breeding dynamics.

Dipping was performed at three-minute intervals, with at least five dips per site to ensure comprehensive sampling. Sites yielding fewer than ten larvae per dip were classified as non-preferred oviposition sites. Larval morphology was observed in situ to ensure accurate identification. Larvae and larval water samples were collected for subsequent mosquito rearing, microbiological analysis, and bioassays. For small habitats like hoof prints, approximately 500 mL to 1 liter of larval water was collected, as larger volumes were unnecessary [20]. Sampling of small habitats from several locations was pooled where necessary to obtain sufficient material for analysis. The study used a two-stage clustered sampling technique. The study area was divided into six clusters, based on preliminary surveys, with three designated as oviposition sites and three as non-oviposition sites, classified by the presence or absence of larvae. Within each cluster, samples were collected using a simple random sampling technique, with three larval water samples collected per site.

To determine an appropriate sample size, we referred to previous studies that sampled similar habitats and mosquito species, while adjusting for the variability in larval density across different breeding sites. Data from repeated samples were analyzed for significant deviations, with means calculated at a 95% confidence level when substantial variation was observed. The larval density was calculated by averaging the number of mosquito larvae collected across all dips per habitat. Larvae were separated from culicine larvae, counted, and recorded as early or late-stage specimens. Both larvae and water samples were transported in a cooler box to maintain low temperatures during transit, ensuring that the larvae remained viable and water samples preserved for microbiological analysis. The data were analyzed using descriptive statistics, with comparisons made between oviposition and non-oviposition sites. Statistical tests, including t-tests were used to assess significant differences in larval density and microbiological composition.

Identification of Anopheles arabiensis Mosquito Larvae

To differentiate *Anopheles gambiae s.s.* from *Anopheles arabiensis*, allele-specific (AS)-PCR analysis was employed after larvae were successfully reared to adulthood for accurate species identification. Prior to the PCR procedure, larvae were reared under controlled conditions to ensure correct developmental stages for identification. PCR conditions included 25 µl of 2X Premix E, 1 µl (20 ng) of universal forward primer (5' GTGTGCCCCTTCCTCGATGT), 1 µl (20 ng) of *An. arabiensis* reverse primer (5' AAGTGTCCTTCCATCCTA), 1 µl Epicentre PCR enzyme mix, and DNA grade water to reach a 50 µl reaction volume. The reaction protocol involved an initial 80°C denaturation for 1 minute, followed by 30 cycles of 94°C denaturation for 30 seconds, 50°C annealing for 30 seconds, and 72°C extension for 30 seconds, with a final extension at 72°C for 4 minutes, as described by [21]. PCR products were then separated on 2% agarose TBE gels, stained with ethidium bromide, and visualized using a UV transillumination gel documentation system. The expected DNA bands (315 bp for *An. arabiensis*) were compared to a 400 bp reference ladder [22].

Larval Rearing in the Lab

Larvae collected from field sampling were sorted by developmental stage and transferred into rearing

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containers, each filled with filtered larval water from their respective collection sites. The containers were made from clear plastic plates (15 cm length, 7 cm width, and 5 cm height, manufactured by KENPOLY). The larvae were fed daily with Goldfish flake fish food (Supa LTD, UK). Pupae that emerged were transferred to a separate container placed inside a 30 cm by 30 cm by 30 cm rearing cage to prevent the adult mosquitoes from escaping. Adult mosquitoes were provided with 6% glucose solution for nourishment. After 3-4 days, blood-feeding was performed using bovine blood obtained from the Kwale main slaughterhouse, ensuring compliance with ethical protocols. The blood was prepared by collecting it in sterile containers and storing it at 4°C until use. Ethical approval for the use of bovine blood in mosquito rearing was obtained from National Commission for Science, Technology and Innovation (NACOSTI). As *Anopheles arabiensis* is zoophilic, blood feeding was critical for inducing gravid conditions in the adult females.

Isolation of Bacteria from Larval Habitats

Around 1 liter of water was collected from each larval habitat during site visits, with habitats categorized as either preferred or non-preferred. The water samples were transported to the laboratory in a sterile 15-liter plastic bucket (Kenpoly). Water from each habitat category was combined separately across three replicates, and a 500 ml representative sample from each category was taken for bacteriological analysis. The sample was sieved to remove debris and filtered through sterile 11 µm filters (Whatman).

From the filtered sample, 1 ml was transferred to 9 ml of distilled water in a test tube for serial dilution, marking the first dilution. This process was repeated up to the tenth dilution, with fresh pipette tips used for each step. The first five dilutions (from 10^0 to 10^5) were plated by spreading 0.1 ml of each dilution onto sterilized glass Petri plates (Borosil®) using the pour plate method. The plates were incubated at 37°C for 24 hours, and those containing 30–300 colony-forming units (CFUs) were selected for further analysis. Distinct colonies were streaked onto individual TSA plates (Tryptone Soya Agar, Techno Pharmchem India) for purification. The TSA medium was prepared by dissolving 40 grams of powder in 1 liter of water, pre-heating, and autoclaving at 121°C for 15 minutes. Pure isolates were obtained using the quadrant streaking method, which diluted the organisms across the plate to generate discrete CFUs. These pure isolates were then used in bioassays.

Characterization of Bacterial Isolates

The bacterial isolates were analyzed for their physical features using standard microbiological techniques. The colonies were visually examined under natural light and with a hand lens to document their shape, size, color, margin, and opacity. Colony size was categorized as small, medium, or large, while their clarity determined whether they were translucent or opaque. The margin was classified as either smooth (entire) or filamentous for colonies with a thread-like edge. Colony shapes were identified as circular or irregular, and their surfaces were noted as smooth or rough. The elevation of the colonies was described as flat, raised, or umbonate. Gram staining was conducted to distinguish the isolates based on their cell wall structure. The process involved applying crystal violet dye, followed by iodine to form a complex that adhered to the cell wall. Ethanol was then used as a decolorizing agent. Bacteria that retained the purple stain were identified as Gram-positive, while those that appeared pink or red were classified as Gram-negative. The biochemical characterization of the isolates involved several tests [23]. The catalase test detected the presence of catalase enzyme, indicated by the production of oxygen bubbles from hydrogen peroxide. The citrate utilization test assessed the ability of bacteria to use citrate as a sole carbon source. Positive results were indicated by a color change from green to blue in the medium. Nitrate reduction, indole production, and carbohydrate metabolism tests were conducted to determine specific metabolic capabilities. The nitrate test checked for nitrate reduction to nitrite, and the indole test assessed the production of indole from tryptophan. The carbohydrate fermentation tests detected acid and gas production from carbohydrate metabolism.

The Triple Sugar Iron (TSI) test was employed to distinguish bacterial organisms based on their carbohydrate fermentation patterns and hydrogen sulfide production. Gas production and pH indicator color changes were noted. The Sulfur, Indole, Motility (SIM) test combined assessments of sulfur reduction, indole production, and motility. Positive results for sulfur reduction were indicated by blackening in the medium, while motility was assessed by diffuse growth and indole production was detected through a black precipitate[24]. The

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Motility Indole Urease (MIU) test determined urease activity, motility, and indole production. Alkaline pH changes from urease activity were indicated by a color shift from yellow to pink-red in the medium. The Methyl Red (MR) and Voges-Proskauer (VP) tests were performed to identify fermentative bacteria. The MR test detected mixed acid fermentation, while the VP test identified acetyl methyl carbinol production. The Nitrate test determined nitrate reduction, and the Catalase test confirmed catalase enzyme production. Finally, the Starch Hydrolysis test evaluated the ability of bacteria to hydrolyze starch, with clear zones around colonies indicating starch degradation.

Mannitol Salt Agar (MSA) was used for its ability to selectively and differentially support microbial growth. The high salt concentration in MSA allowed for the selection of salt-tolerant organisms, while mannitol served as the differential component. Organisms capable of fermenting mannitol produced acidic by-products, which lowered the medium's pH. This acidification caused the pH indicator, phenol red, to change color from red to yellow. A yellow color indicated a positive result (acidic pH), while a pink color represented a negative result (alkaline pH). For arabinose fermentation, a sterile needle was used to inoculate 24-hour bacterial cultures into Nesla tubes containing arabinose medium. The inoculated tubes were incubated at 37℃ for 48 hours. A positive result was indicated by a yellow color change in the medium, while a negative result was signaled by a magenta or hot pink coloration.

Molecular Characterization of the Isolates

DNA Extraction

Bacterial DNA was isolated from pure cultures following the [25] protocol. About 5 ml of saturated cell culture was spun down in an Eppendorf tube, and the pellet was resuspended in 500 µL of ice-cold solution containing lysozyme, incubated on ice for 10 minutes. Subsequently, 50 µL of 10% SDS buffer was added, and the mixture was incubated at 37℃ for 5-10 minutes until clear and viscous. Phenol (550 µL) was added, followed by mixing and centrifugation at 4℃ for 15 minutes. The aqueous layer was transferred to a new Eppendorf tube, with 0.1 volume of 3 M NaOAc added and mixed. Two volumes of 100% ethanol were added to the supernatant, mixed, and the sample was cooled at -80℃ for 5 minutes. Following centrifugation at 4℃ for 15 minutes, the supernatant was discarded, and the pellet was vacuum-dried and resuspended in 50-100 µL of water. The integrity of the genomic DNA was verified by resolving the extracts on a 0.8% agarose gel and visualizing with ethidium bromide staining.

The 16S rRNA gene was amplified in a 25 µL PCR reaction, which included 1 µL of template DNA, 5 µL buffer, 3 µL MgCl2, 0.5 µL nucleotide mix, 0.2 µL of each primer (27f: 5'-AGAGTTTGATCCTTGGCTCAG-3'; 1492r: 5'-GGTTACCTTGTTACGACTT-3'), 1.0 U of Taq DNA polymerase, and double-distilled water. The PCR cycling conditions consisted of an initial denaturation at 95℃ for 5 minutes, followed by 35 cycles of denaturation at 94℃ for 1 minute, annealing at 50℃ for 1 minute, and extension at 72℃ for 2 minutes, with a final extension at 72℃ for 7 minutes. PCR success was confirmed by running the products alongside control samples on a 1500 bp DNA ladder. Gel Electrophoresis To assess the quality of the PCR products, 3 µL of amplified DNA was subjected to electrophoresis on a 1% agarose gel at 75 volts for 40 minutes. Subsequently, the products were resolved on a 3% MetaPhor agarose gel using 1× TBE running buffer for 70 minutes. Ethidium bromide staining was used to visualize the DNA bands under UV light.

After amplification, the 16S rRNA gene amplicons were purified and sequenced. Sequencing was carried out in a single direction using the 27f primer, targeting the gene's most variable regions. The resulting sequences were processed using BioEdit software (version 7.2) to remove low-quality regions. These sequences were then compared to the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) to identify the closest bacterial species based on 16S rRNA sequence similarity. A multiple sequence alignment of the obtained sequences and related sequences from GenBank was performed using the ClustalW algorithm. The aligned sequences were then used to construct a phylogenetic tree using the neighbor-joining method in MEGA X software. To ensure reliability, bootstrap analysis with 1,000 replicates was conducted. The phylogenetic tree provided valuable insights into the evolutionary relationships between the bacterial isolates and their closest relatives.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XII Issue XV January 2025 | Special Issue on Public Health

Potential Role of Bacterial Isolates in Oviposition Site Selection

To explore the role of bacterial isolates in oviposition site selection, a detailed bioassay was carried out. Field-collected larvae were fed Goldfish flake fish food (Supa LTD, UK) at their larval sites twice daily to support proper growth and development. When the larvae reached the pupal stage, pupae were collected in 10-cm-diameter Kenpoly plastic cups and transferred to mosquito cages overnight to allow adult emergence. The adult mosquitoes were provided with a 6% glucose solution soaked in cotton wool to maintain optimal health for oviposition trials. The timing of the trials was aligned with the peak egg-laying period identified during preliminary observations. Prior to the trials, mosquitoes were deprived of sugar for up to six hours and then offered bovine blood sourced from Kwale's primary slaughterhouse over two consecutive evenings. Only mosquitoes that had fully engorged on blood were selected for experimentation. Gravid females, identifiable by their swollen, pale white abdomens and between their sixth and seventh day of pregnancy, were used in the bioassays.

The trials were conducted in standard steel cages (30 x 30 x 30 cm) covered with fine cotton mosquito netting and placed on a galvanized metal base. Each cage included an insert sleeve for holding oviposition substrates, which consisted of stainless-steel cups (5 cm in diameter, 100 ml capacity) lined with white filter paper (Whatman) to prevent direct contact between the mosquitoes and the substrate. To avoid positional bias, the placement of the oviposition cups within each cage was systematically varied daily. Each cage contained five oviposition cups: one with distilled water (control), one with preferred oviposition water from the field, one with non-preferred oviposition water from the field, one with filtered water from preferred sites containing a specific bacterial isolate, and one with filtered water from non-preferred sites. During each trial, 20 female mosquitoes were exposed to the different oviposition substrates. The number of eggs laid in each cup was recorded for analysis. Data analysis was conducted using IBM SPSS Statistics Version 26. Descriptive statistics were calculated to determine the mean egg count for each condition. Independent-samples t-tests were used to compare the mean number of eggs laid between different substrates, with a significance level of 0.05 and 95% confidence intervals calculated for each mean.

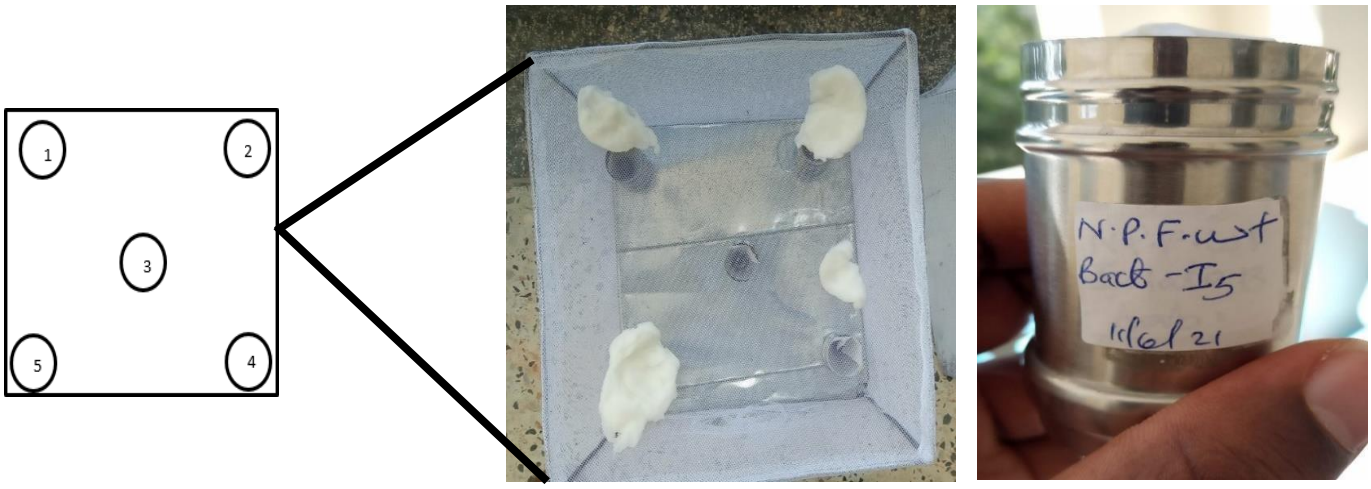

Figure 2:Arrangement of oviposition cups and 30 cm x 30 cm x 30 cm cage with 5-cm diameter oviposition cup.

RESULTS

Molecular Characterization of Bacterial Isolates

DNA Extraction

Genomic DNA was extracted from all 14 bacterial isolates and visualized using agarose gel electrophoresis. Figure 4 displays the results, where genomic DNA was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. The unmarked lane contains a 1500 bp ladder, while lanes 1 through 14 indicate the genomic DNA of the 14 bacterial isolates.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XII Issue XV January 2025 | Special Issue on Public Health

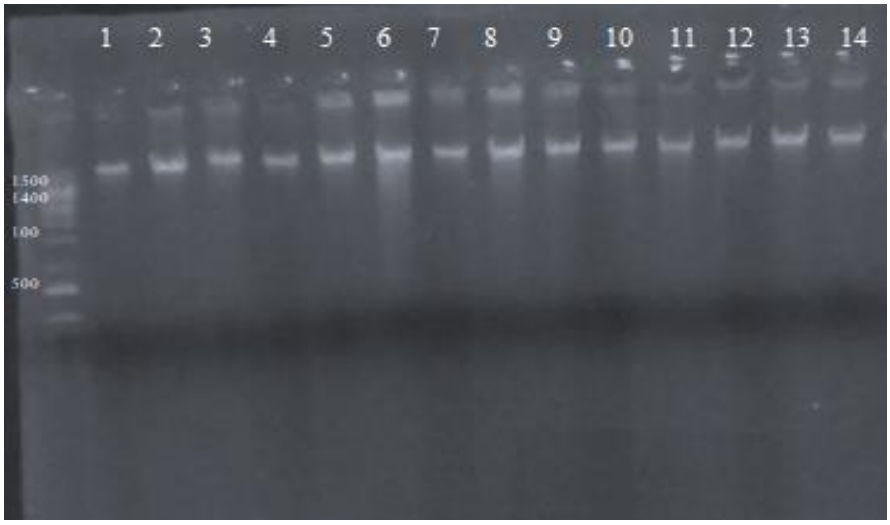

Figure 3: Agarose gel electrophoresis of genomic DNA from 14 bacterial isolates, with a 1500 bp ladder shown in the unmarked lane.

PCR Amplification

The PCR amplification of the bacterial isolates resulted in single bands of 1,500 base pairs in size. This was achieved using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). As shown in Figure 5, the PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lane 1 indicates the 1500 bp ladder, lanes 2 and 4-14 indicate the 14 bacterial isolates, and lane 3 indicates the control.

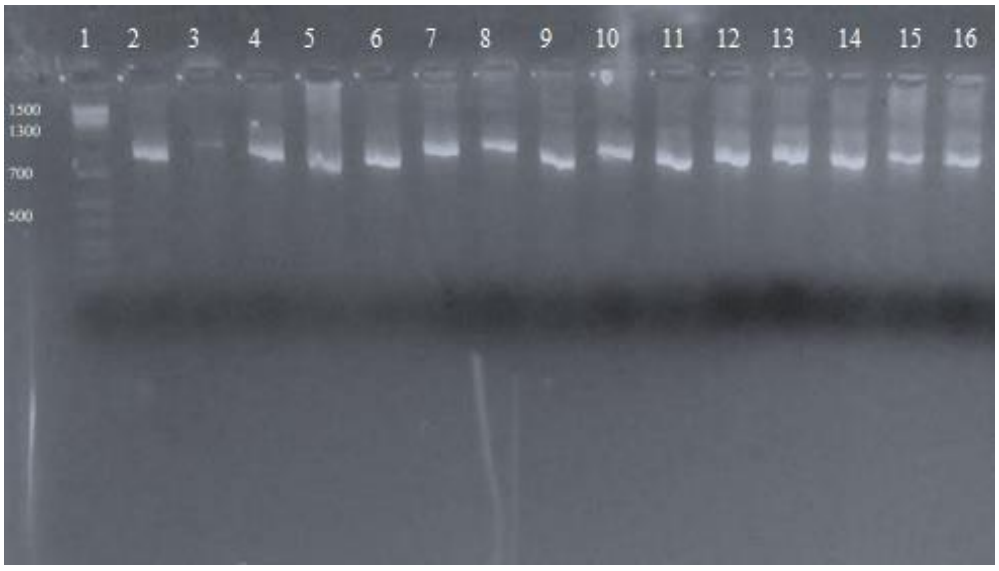

Figure 4: Agarose gel electrophoresis of PCR products. Lane 1: 1500 bp ladder; lanes 2, 4-14: PCR products from 14 bacterial isolates; lane 3: control.

Molecular Characterization

From the 14 isolates, sequences were obtained and compared with those in the NCBI GenBank. The isolates were sequenced using Sanger sequencing, which successfully identified 11 out of the 14 isolates. Analysis of the Sanger-sequenced DNA nucleotides (16S rRNA sequences) using the BLASTn program in MEGA X revealed that the isolates had percentage similarities ranging from 97.54% to 99.93% with nucleotide sequences available in the NCBI GenBank (Table 1). A higher percentage similarity was observed between isolate DPI2 and *Enterobacter cloacae* (99.93%), with GenBank accession number KT260438.1, while a lower percentage similarity was observed between isolate DNPI11 and *Pluralibacter gergoviae* (97.54%), with GenBank accession number LR699009.1. Table 1 provides detailed identities for all the isolates.

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Table 1: Molecular identification of selected bacteria isolates and similar sequences in the gene bank based on 16S rRNA gene sequencing

Isolate	Source	Org [d]	GB-A No [c]	% Sim[a]	E[b]
DPI5	Preferred	1	MH151217.1	99.73	0
DPI2	Preferred	2	ON153209.1	99.93	0
DPI5b	Preferred	3	HQ289884.1	97.57	0
DNPI11	Non-Preferred	4	NR_024641.1	97.54	0
DPI5c	Preferred	5	AB682278.1	99.80	0
DPI10	Preferred	6	MK491035.1	99.54	0
DPI13	Preferred	7	JQ765416.1	99.52	0
DNPI13	Non-Preferred	8	KX981861.1	99.64	0
DNPI14	Non-Preferred	9	KF739069.1	99.58	0
DNPI16	Non-Preferred	10	OM604768.1	99.91	0
DNPI16b	Non-Preferred	11	KC119223.1	98.97	0
DPI12	Preferred	12	KT835655.1	98.85	0
DPI12b	Preferred	13	KC119227.1	98.41	0
DPIC	Preferred	14	JQ765425.1	98.94	0

[a] percentages of similarity, [b] E-value, [c] GBA No. = GenBank Accession No., [d] Closest neighbour, 1= *Enterobacter ludwigii*, 2= *Enterobacter cloacae*, 3= *Enterobacter hormaechei*, 4= *Pluralibacter gergoviae* MGYG-HGUT 5= *Enterobacter gergoviae* NBRC 105706, 6 = *Bacillus pumilus* strain RGS9, 7= *Enterobacter bugandensis*, 8= *Pluralibacter gergoviae strain* FB2 9 = *Pluralibacter gergoviae* ATCC 33028, 10= *Pluralibacter gergoviae* strain PGBM32, 11 = *Pluralibacter gergoviae strain* ICB-CEG2, 12= *Enterobacter gergoviae* strain CICR-GV1, 13= *Enterobacter sp.* 24B, 14= *Enterobacter* sp. MLB32

The evolutionary relationships among the isolates were determined using the Neighbor-Joining method [26], as illustrated in Figure 6. The bootstrap consensus tree, inferred from 1000 replicates [27], represents the evolutionary history of the taxa analyzed. Branches with less than 50% bootstrap support were collapsed. The evolutionary distances were computed using the Tamura 3-parameter method [28] and are expressed as the number of base substitutions per site. This analysis involved 29 nucleotide sequences, with all positions containing gaps and missing data eliminated, resulting in a final dataset of 968 positions. Evolutionary analyses were conducted in MEGA11 [29].

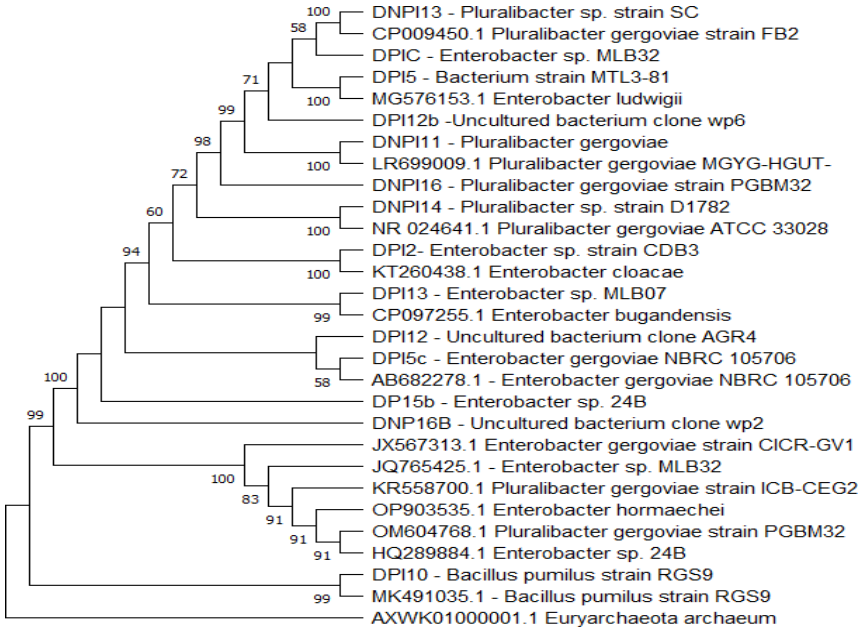

Figure 5:The evolutionary tree based on Neighbour-Joining method with evolutionary distances computed using the Tamura 3-parameter method.

Table 2 estimates the evolutionary divergence between sequences of the isolates. The number of base differences per site between sequences is presented. This analysis involved 14 nucleotide sequences, and all

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positions with gaps and missing data were eliminated, resulting in a final dataset of 2304 positions. Evolutionary analyses were conducted in MEGA11 [29].

Table 2:Estimates of evolutionary divergence between sequences

ISOLATE	DPI5	DPI2	DP15b	DNPI11	DPI5c	DPI10	DPI13	DNPI13	DNPI14	DNPI16	DNP16B	DPI12	DPI12b	DPIC
DPI5														
DPI2	0.060													
DP15b	0.057	0.033												
DNPI11	0.058	0.049	0.050											
DPI5c	0.053	0.039	0.028	0.054										
DPI10	0.250	0.242	0.238	0.253	0.232									
DPI13	0.049	0.039	0.037	0.050	0.025	0.238								
DNPI13	0.112	0.125	0.125	0.116	0.118	0.301	0.117							
DNPI14	0.049	0.041	0.039	0.054	0.030	0.235	0.030	0.120						
DNPI16	0.063	0.058	0.059	0.060	0.048	0.248	0.043	0.121	0.047					
DNP16B	0.061	0.047	0.035	0.059	0.028	0.229	0.039	0.122	0.038	0.059				
DPI12	0.056	0.032	0.027	0.049	0.033	0.237	0.033	0.121	0.039	0.057	0.037			
DPI12b	0.053	0.052	0.056	0.043	0.055	0.250	0.052	0.110	0.053	0.058	0.055	0.055		
DPIC	0.054	0.069	0.073	0.063	0.060	0.256	0.057	0.119	0.057	0.059	0.068	0.071	0.055	

DPI5 - Bacterium strain MTL3-81, DPI2 - Enterobacter sp. strain CDB3, DP15b - Enterobacter sp. 24B, , DNPI11 - Pluralibacter gergoviae, DPI5c - Enterobacter gergoviae strain: NBRC 105706, DPI10 - Bacillus pumilus strain RGS9, DPI13 - Enterobacter sp. MLB07, DNPI13 - Pluralibacter sp. strain SC, DNPI14-Pluralibacter sp. strain D1782, DNPI16 - Pluralibacter gergoviae strain PGBM32, DNP16B - Uncultured bacterium clone wp2, DPI12 - Uncultured bacterium clone AGR4, DPI12b - Uncultured bacterium clone wp6, DPIC - Enterobacter sp. MLB32

Potential Role of Bacteria in the Oviposition of *Anopheles arabiensis*

Bioassay Results

The bioassay assessed the potential role of various bacterial isolates in the oviposition behavior of *An. arabiensis*. The number of eggs laid by *An. arabiensis* per bacterial isolate was recorded daily, with three replicates per isolate. The mean number of eggs laid, along with the minimum, maximum, and standard deviation, were calculated for each bacterial isolate, as shown in Table 3. *Enterobacter sp.* strain CDB3 exhibited the highest mean egg count of 126, with a standard deviation of 11.53, indicating substantial variability in egg-laying activity. Conversely, *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2 had zero eggs laid, highlighting their lack of attractiveness as oviposition sites.

Table 3:Mean of the eggs laid per organism, minimum, maximum and standard deviations

Descriptive Statistics of the number of eggs laid per organism					
ORGANISM	N	Minimum	Maximum	Mean	Std. Deviation
Bacterium strain MTL3-81	3	43	58	51.00	7.550
Enterobacter sp. strain CDB3	3	115	138	126.00	11.533
Enterobacter sp. 24B	3	64	80	73.00	8.185
Enterobacter gergoviae NBRC 105706	3	61	78	68.00	8.888
Bacillus pumilus strain RGS9	3	30	40	35.00	5.000
Enterobacter sp. MLB07	3	33	40	37.00	3.606
Enterobacter sp. MLB32	3	44	55	48.00	6.083
Pluralibacter sp. strain D1782	3	16	28	22.00	6.000
Pluralibacter gergoviae	3	27	33	30.67	3.215
Pluralibacter sp. strain SC	3	19	27	23.00	4.000
Pluralibacter gergoviae strain PGBM32	3	0	0	0.00	0.000
Uncultured bacterium clone wp2	3	0	0	0.00	0.000
Uncultured bacterium clone AGR4	3	39	49	44.00	5.000
Uncultured bacterium clone wp6	3	33	42	37.00	4.583

There were three repetitions per organism and the data was analysed using SPSS version 26. The SD for Enterobacter sp. strain CDB3 is the highest at 11.53, indicating that the data points are more spread out and the number of eggs laid varies more for this organism. Based on the table, we can conclude that Enterobacter sp. strain CDB3 had the highest mean number of eggs laid and the highest SD, indicating that the number of eggs laid for this isolate was more variable.

Figure 6: Mean number of eggs laid per organism. Enterobacter sp. strain CDB3 showed the highest average with 126 eggs, while Uncultured bacterium clone wp2 and Pluralibacter gergoviae strain PGBM32 recorded zero eggs.

Figure 7 illustrates the number of eggs laid per organism, with Enterobacter sp. strain CDB3 having the highest mean of 126 eggs, while *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2 had the lowest mean counts of zero eggs. Bacteria from the preferred oviposition sites, such as *Enterobacter sp.*

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strains, resulted in higher egg counts compared to those from non-preferred sites. Notably, except for *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2, which showed zero egg counts, the t-statistics for other bacteria were significant at $p<0.05$, indicating a meaningful difference between the sample means and the hypothesized population mean. The oviposition activity index (OAI) was calculated to quantify the relative attractiveness of each bacterial isolate as an oviposition site compared to a control habitat with distilled water (mean egg count = 11). The OAI is given by:

$$OAI = \frac{\mathrm{NT} - \mathrm{NS}}{\mathrm{NT} + \mathrm{NS}}$$

where NT is the mean number of eggs laid in the treatment (bacterial isolate) and NS is the mean number of eggs laid in the control.

Table 4 below presents the OAI for both preferred and non-preferred oviposition site bacteria. *Enterobacter sp.* strain CDB3 had the highest OAI of 0.84, signifying high oviposition activity, whereas *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2 had OAI values of -1.00, indicating their extreme unattractiveness as oviposition sites. Figures 8 and 9 illustrate the OAI values for the preferred and non-preferred oviposition sites, respectively. *Enterobacter sp.* strain CDB3 showed the highest mean number of eggs laid and a high OAI, reflecting its strong attractiveness to *Anopheles arabiensis*. In contrast, *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2 were notably avoided, as evidenced by their zero egg counts and negative OAI values.

Table 4:Oviposition activity index of different types of bacteria compared to the given control.

Preferred oviposition site bacteria isolates		
Control habitat: Distilled water; no. of eggs laid =11		
Bacteria	**Mean No. of eggs laid**	**Oviposition Activity Index (OAI)**
Bacterium strain MTL3-81	51	0.65
Enterobacter sp. strain CDB3	126	0.84
Enterobacter sp. 24B	73	0.74
Enterobacter gergoviae strain: NBRC	68	0.72
Bacillus pumilus strain RGS9	35	0.52
Enterobacter sp. MLB07	37	0.54
Enterobacter sp. MLB32	48	0.63
Uncultured bacterium clone AGR4	44	0.60
Uncultured bacterium clone wp6	37	0.54
Non-preferred oviposition site bacteria isolates		
Bacteria	**Mean No. of eggs laid**	**Oviposition Activity Index (OAI)**
Pluralibacter sp. strain D1782	22	0.33
Pluralibacter gergoviae	31	0.48
Pluralibacter sp. strain SC	23	0.35
Pluralibacter gergoviae strain PGBM32	0	-1.00
Uncultured bacterium clone wp2	0	-1.00

The means were rounded off to enable calculation of OAI. Mean of the eggs was calculated using SPSS version 26 while the OAI was calculated using Microsoft Excel 2016 version.

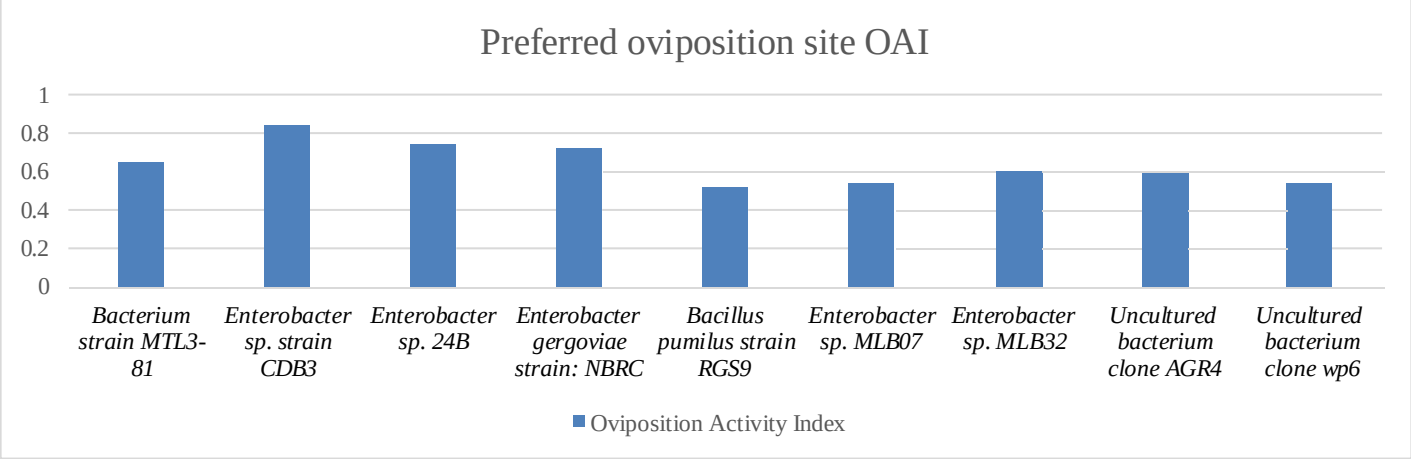

Figure 7: Oviposition Activity Index (OAI) in preferred sites. Enterobacter sp. strain CDB3 shows the highest OAI of 0.84, while Bacillus pumilus strain RGS9 has the lowest OAI among the preferred oviposition site bacteria.

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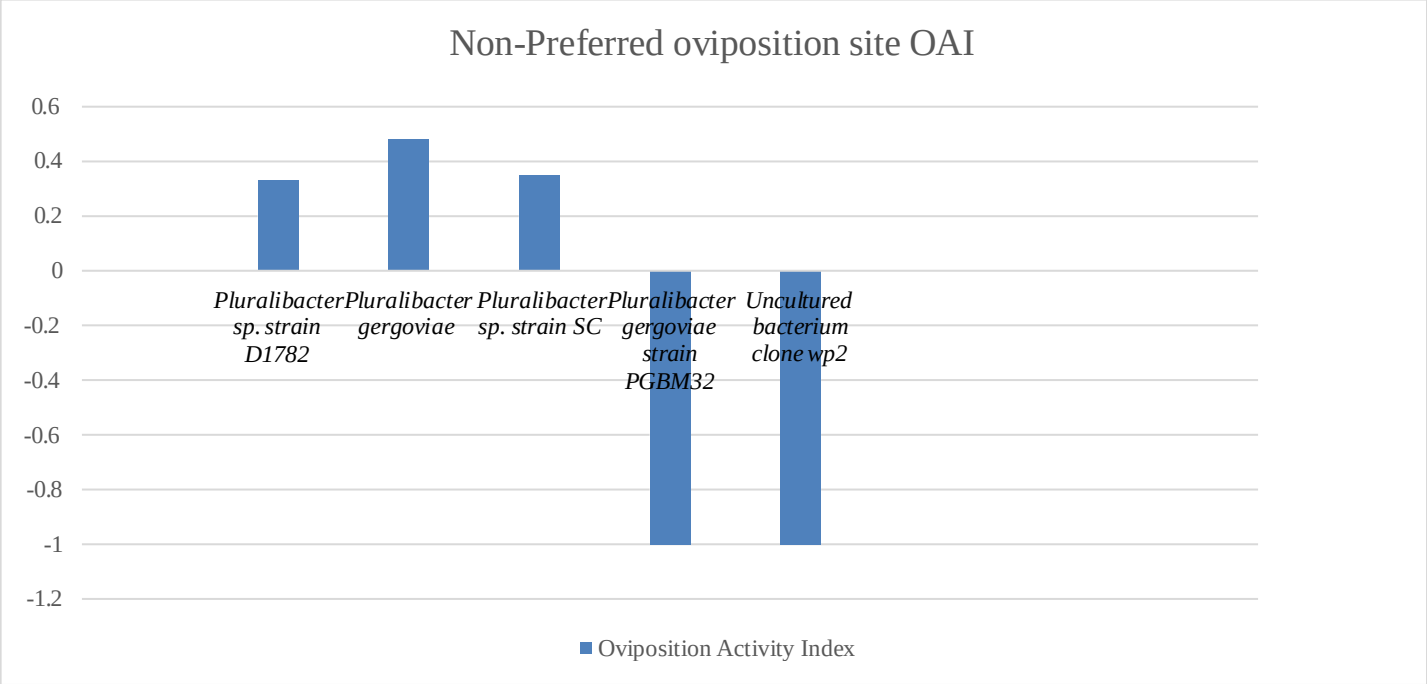

Figure 8: Oviposition Activity Index (OAI) in non-preferred sites. *Pluralibacter gergoviae* has the highest OAI of 0.48, while *Pluralibacter gergoviae* strain PGBM32 and Uncultured bacterium clone wp2 have negative OAI values, indicating low attractiveness for oviposition.

Figure 9: Photos of *An. arabiensis* eggs on filter paper and in oviposition cups.

Figure 10 provides visual documentation of *An. arabiensis* eggs on filter paper and in oviposition cups, illustrating the physical appearance and distribution of the eggs during the bioassay. These findings suggest that the presence of certain bacterial strains significantly influences the oviposition behavior of *An. arabiensis*, with some bacteria acting as strong attractants and others as deterrents.

DISCUSSION

Phylogenetic Relationships and Bacterial Isolates from Oviposition Sites

The molecular characterization of the bacterial isolates from oviposition sites revealed distinct phylogenetic relationships and potential functional roles in mosquito oviposition preferences. The phylogenetic analysis, depicted in Figure 6, identified several clades among the isolates, indicating varying degrees of relatedness. *Pluralibacter* sp. strain SC showed a remarkable 99.52% similarity to *Pluralibacter gergoviae*, supported by a bootstrap value of 100, indicating a very close ancestral relationship. This high level of similarity suggests that these strains are closely related, a finding consistent with previous research indicating the genetic uniformity within *Pluralibacter* species [30]. *Enterobacter sp.* MLB32, with a bootstrap value of 58, diverges from this clade, reflecting its more distant relationship to the closely related strains in the first clade. The clades comprising Bacterium strain MTL3-81 and *Enterobacter ludwigii*, with bootstrap values of 100, demonstrate a

close genetic relationship. *E. ludwigii*, isolated in 2005, has been shown to play a role in mosquito population control [31]. This supports our observation of its significant similarity to MTL3-81, suggesting potential roles in mosquito attraction similar to *E. ludwigii's* effects on Aedes aegypti [32].

Among the 14 bacterial isolates, nine from preferred oviposition sites and five from non-preferred sites were identified. Bacterium strain MTL3-81, *Enterobacter sp.* strain CDB3, and *Enterobacter sp.* 24B are associated with preferred sites, whereas *Pluralibacter gergoviae* and its strains are linked to non-preferred sites. This distribution aligns with findings from previous studies, which suggest that bacterial communities in preferred oviposition sites may exhibit distinct characteristics influencing mosquito behavior [33]. The significant similarity of *Enterobacter sp.* strain CDB3 (99.3%) to *Enterobacter cloacae* is noteworthy. *E. cloacae*, a symbiont in Anopheles species' gut microbiota, enhances mosquito longevity and is proposed for malaria control [33], [34]. Our results suggest that *Enterobacter sp.* strain CDB3 may have similar effects on *Anopheles arabiensis*, highlighting its potential as a biocontrol agent. The high similarity of *Enterobacter sp.* 24B (97.57%) to *Enterobacter hormaechei*, an opportunistic pathogen with limited mosquito-related studies, points to its possible role in mosquito ecology. However, further research is needed to elucidate its interaction with mosquitoes. The identification of *Pluralibacter gergoviae* strains in non-preferred oviposition sites is a novel contribution of this study. While previous research has not linked *Pluralibacter* species to mosquito oviposition preferences, our findings suggest potential environmental or ecological roles that warrant further investigation.

Role of Bacteria in Oviposition

According to the findings, bacteria play a major impact in mosquito oviposition behavior; individuals from the species *Enterobacter* produce more eggs than those from the genus *Pluralibacter*. In particular, only three Pluralibacter strains—all of which are members of the Pluralibacter gergoviae—caused oviposition, while all *Enterobacter* isolates promoted egg laying. Nevertheless, compared to *Enterobacter* species, the egg count linked to Pluralibacter strains was noticeably lower. The regular egg-laying response that Enterobacter species evoke highlights the possibility that they have an impact on mosquito reproductive behavior. According to earlier research, *Anopheles gambiae* frequently have *Enterobacter* species, particularly *Enterobacter cloacae*, in their midgut after consuming blood [35]. According to this association, these bacteria might aid in nutrient digestion, which could increase the number of resources available in the mosquito's habitat. Given that blood meals are directly associated with subsequent egg laying, the availability of easily digestible food, made possible by these bacteria, may help to improve oviposition rates.

Conversely, the role of *Pluralibacter* species in mosquito oviposition remains underexplored. Our study indicates that only *Pluralibacter gergoviae* strains from this genus were involved in egg laying, and the number of eggs was comparatively lower than those elicited by *Enterobacter*. This suggests a potentially limited role of *Pluralibacter* in mediating oviposition compared to *Enterobacter*. The scarcity of research on *Pluralibacter* in this context points to a gap in understanding their ecological significance in mosquito habitats. Apart from *Enterobacter* and *Pluralibacter*, *Bacillus pumilus* was isolated from preferred oviposition sites, but its role in oviposition is less clear. Previous studies, such as those by [36], identified *Bacillus pumilus* associates in preferred oviposition sites but did not elucidate their specific role in egg laying. The absence of detailed studies on *Bacillus pumilus* in this context highlights the need for further investigation into its potential contributions to mosquito oviposition.

CONCLUSION

This work provides important new information about how bacterial isolates from oviposition sites affect mosquito behavior, specifically how they choose substrates for laying eggs. Different evolutionary links between bacterial strains were revealed by the phylogenetic analysis, which showed more divergence among some clades and close ties within others. Significant evolutionary proximity was shown by bacteria including *Pluralibacter sp.* strain SC and *Enterobacter sp.* strain CDB3, indicating a common ancestor and possible functional similarities. According to the results, bacteria belonging to the *Enterobacter* genus are more successful than those from the *Pluralibacter* genus at drawing mosquitoes for oviposition. Particularly, *Enterobacter* species that were primarily isolated from favored oviposition sites and showed strong

resemblance to known mosquito-associated bacteria included *Enterobacter sp.* strain CDB3 and *Enterobacter sp.* 24B. This suggests that these bacteria may play a role in enhancing the attractiveness of oviposition sites through their potential contributions to nutrient availability or other appealing factors. In contrast, *Pluralibacter* species, despite their presence in mosquito habitats, demonstrated lower oviposition activity, indicating that not all bacterial species have an equal impact on mosquito attraction.

The study emphasizes how specific *Enterobacter* species in oviposition sites have a major impact on mosquito behavior and attraction. This knowledge is essential for creating focused mosquito control plans. It might be feasible to improve the efficiency of mosquito traps or develop new attractants based on bacterial signals by taking use of the alluring qualities of particular bacterial strains. Furthermore, by designing interventions to interfere with mosquito breeding, knowledge of the bacterial profiles of favored oviposition sites can help lower mosquito populations and the spread of vector-borne illnesses. Future studies should concentrate on clarifying the precise processes by which these bacteria affect mosquito oviposition and investigating how they might be used in integrated pest management plans. Advancing our knowledge of bacterial influences on mosquito behavior can lead to more effective and environmentally sustainable mosquito control solutions.

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