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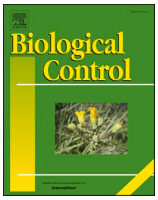

Discovery of novel non-live bacterial bioinsecticides for mosquito control through a high-throughput screening pipeline

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HIGHLIGHTS:

- **High Efficacy:** Three bacterial isolates exert 100% larval mortality within 24 h, demonstrating potential as biocontrol agents.
- **Broad-Spectrum Activity:** Larvicidal actiivity observed against *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi.*
- **Taxonomic Diversity:** Larvicidal isolates represent a diverse range of genera, including *Enterobacter*, *Bacillus*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Chromobacterium*, *Pantoea*, *Staphylococcus*, and *Raoultella.*
- **Practical Advantages:** The larvicidal effect was retained in non-live, heat-inactivated bacterial biomass, indicating stability, safety, and ease of deployment.
- **Enhanced Formulation:** Pellet-formulated bacterial biomass enhances larval effect via improved larval exposure.

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ABSTRACT

The growing threat of insecticide resistance and ecological harm from synthetic insecticides underscores the urgent need for new sustainable mosquito control strategies. In this study, we implemented a systematic high-throughput screening (HTS) of non-live bacterial cultures to identify novel bacterial strains with potent mosquito larvicidal activity. From 48 environmental samples collected in a tropical environment on the southeast coast of Puerto Rico, 486 bacterial isolates were obtained, of which 30 exhibited significant killing-activity as non-live desiccated cultures, against *Aedes aegypti* larvae. Notably, three isolates achieved 100% larval mortality within 24 h. Further bioassays confirmed dose-dependent efficacy across *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi*, indicating broad-spectrum larvicidal activity. Taxonomic analysis revealed diverse genera, including *Enterobacter*, *Bacillus*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Chromobacterium*, *Pantoea*, *Staphylococcus*, and *Raoultella.* Crucially, the larvicidal effect was retained in non-live bacterial biomass after heat inactivation, highlighting practical advantages with regard to stability, safety, and deployment over live biocontrol agents. Comparative assays of different formulations further indicated that delivering bacterial biomass in pellet form can enhance larval mortality compared to suspended powder in the larval breeding water, likely due to improved retention and localized concentration of active metabolites. Collectively, this study establishes a robust discovery pipeline for environmentally friendly, microbe-derived non-live bioinsecticides to support integrated mosquito vector management.

1. Introduction

Vector-borne diseases continue to pose a formidable threat to global public health, accounting for an estimated 700,000 deaths annually (WHO, 2024). While arboviral infections such as dengue, chikungunya, and Zika are expanding their geographical reach, with their incidence rising in both endemic and non-endemic regions (Guerrero et al., 2025; Lim et al., 2025) malaria remains a significant threat, especially in

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urbanizing regions of sub-Saharan Africa (Merga et al., 2025). The emergence of *Anopheles stephensi*, a major urban malaria vector formerly restricted to South Asia, in African cities such as Djibouti, Ethiopia, Sudan, and Nigeria underscores this new risk (Emiru et al., 2023; Waymire et al., 2024). Unlike traditional rural vectors, *An. stephensi* thrives in artificial container habitats, making larval interventions in urban areas a strategic countermeasure to prevent outbreaks (Emiru et al., 2023; Lehmann et al., 2023; Zhou et al., 2024). Similarly, *Culex quinquefasciatus*, a resilient vector capable of transmitting lymphatic filariasis (Wuchereria bancrofti) as well as West Nile, St. Louis encephalitis and other arboviruses, breeds in a wide range of habitats, including polluted water (Diaz et al., 2013; Nchoutpouen et al., 2019; Samy et al., 2016). Its adaptability makes larval-stage control particularly actionable and efficient (Rique et al., 2024). In the absence of effective antiviral treatments or widely available vaccines, current control efforts are primarily focused on interrupting transmission by targeting mosquito vectors, most notably *Aedes aegypti*, the principal vector for many arboviruses (Gupta et al., 2025; Paz-Bailey et al., 2025).

However, overreliance on chemical insecticides poses several critical limitations: they kill non-target insects, present substantial risks to human health and the environment, and their intensive use has contributed to the rapid development of resistance in mosquito populations (Hemingway and Ranson, 2000; Love et al., 2023; Minwuyelet et al., 2025). Consequently, these approaches are becoming unsustainable and insufficient in mitigating disease outbreaks (Hemingway et al., 2002; Messenger et al., 2023).

An alternative and complementary strategy that has gained traction is the use of larvicides to target the immature aquatic stages of mosquitoes. Larviciding offers several advantages: larvae are confined to well-defined aquatic habitats, making them easier to target with high precision and effectiveness (Okumu et al., 2025). Furthermore, larvicidal interventions often present fewer safety concerns and reduced environmental impact compared to broad-spectrum adulticidal spraying, as they can be applied in a more targeted manner to specific breeding sites (Fillinger and Lindsay, 2006; WHO, 2013). Nevertheless, currently available synthetic larvicides, including temephos (an organophosphate) and pyriproxyfen (an insect growth regulator), suffer from a host of drawbacks, such as toxicity to humans and non-target aquatic organisms, potential for the development of resistance in mosquito populations, and limitations in scalability or suitability for deployment in remote or resource-limited settings (Abe et al., 2014; Clifton and Lopez, 2025).

Given these concerns, there is growing interest in the development of eco-friendly, affordable, and effective alternatives to synthetic insecticides (Benelli, 2015). In this context, microbe-based larvicides have emerged as a promising solution. Entomopathogenic bacteria such as *Bacillus thuringiensis israelensis* (*Bti*) and *Lysinibacillus sphaericus* have demonstrated high efficacy against mosquito larvae and are already in widespread use for controlling important vectors such as *Aedes*, *Anopheles*, and *Culex* (Lacey, 2007; Lekakarn et al., 2015). Certain microbial metabolites also possess potent larvicidal properties, such as the polyketide compounds aureothin and allo-aureothin, derived from *Streptomyces distallicus*, exhibited strong *Ae. aegypti* larvicidal activity (Kim et al., 2022). Similarly, a non-viable preparation of *Chromobacterium species* Panama (*C. sp_P*) was found to possess significant larvicidal effects across multiple mosquito genera (Caragata et al., 2020).

These findings underscore the untapped potential of non-live microbial preparations in the development of sustainable mosquito control strategies. Despite existing advancements, further exploration of bacterial biodiversity is needed to uncover novel strains and bioactive compounds with mosquitocidal properties (Benelli et al., 2016a). To expedite the discovery of bacteria producing potent biocontrol agents, the development of high-throughput and robust screening methodologies, that specifically target bacteria that kill insect pests through molecules and not by infection is crucial. These methodologies enable the rapid and efficient evaluation of numerous microbial isolates from diverse natural sources. Natural environments, such as soils, water bodies, and plant surfaces, harbor diverse microbial communities, representing a wealth of untapped potential for these novel bioactive compounds (Wood et al., 2025).

In this study, we present a systematic and streamlined approach for the rapid screening and identification of heat-inactivated non-live bacterial strains with larvicidal activity from diverse environmental samples. Traditionally, the primary screening involves bioassays of live bacterial cultures against target insects, leading to the identification of bacterial isolates that either kill the insects through abiotic factors or entomopathogenic activity. While biopesticides with entomopathogenic action exist, their commercialization can often be complicated as it involves a live organism that frequently has a lower shelf-life, stability, and may also be scrutinized to a greater extent during the registration process. Our approach highlights the utility of a non-live bacterial high-throughput screening method for identifying potent isolates that have desirable biophysical properties, such as heat resistance, shelf-life, and non-pathogenic properties, for biopesticide development.

2. Material and methods

2.1. Ethics statement

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Animal Care and Use Committee (ACUC) of Johns Hopkins University, and the Institutional Ethics Committee. The Institutional Animal Care and Use Committee (IACUC) approved the protocol MO24H08. Mice were used for mosquito rearing.

2.2. Mosquitoes rearing

Three mosquito species were used in this study: *Aedes aegypti* Liverpool IB12 strain, maintained as a laboratory colony; *Anopheles stephensi* LISTON strain, obtained from the Johns Hopkins central insectary facility; and *Culex quinquefasciatus* JHB strain, procured from BEI Resources (NR-4305). All the mosquito lines were reared in the insectary of Johns Hopkins Malaria Research Institute under standard laboratory conditions at 27 ± 2°C and 80 ± 10% relative humidity with a 14:10-hour light/dark cycle. Larvae were maintained at a density of approximately 150 individuals per liter of deionized water from the first instar onward. *Ae. aegypti* and *Cx. quinquefasciatus* larvae were fed ground tropical fish flakes (TetraMin), while *An. stephensi* larvae were fed ground cat food pellets (Purina Cat Chow). Adult mosquitoes were housed in 25 × 25 × 25 cm metal cages and provided with a 10% sucrose solution for nutrition.

2.3. Sample collection and preparation

48 Natural samples, including Soil, tree bark, dried leaves, rhizomes, forest water, flower buds, plant compost, and damaged leaves, were collected from the southeast coast of Puerto Rico (Fig. 1). Samples were aseptically collected into sterile Eppendorf tubes and immediately transported to the laboratory within 24 h for further processing. Upon arrival, each sample was transferred to a sterile 50 mL Falcon tube and resuspended in 25 mL of sterile 1x Phosphate-Buffered saline (PBS) to disperse the microbial content within the PBS. To ensure thorough sample homogenization and maximize bacterial recovery, the samples were vigorously vortexed for 1 min. Subsequently, the suspensions were incubated at 4°C with gentle shaking overnight (Fig. 2A)

2.4. Establishment of bacterial cultures

To isolate individual bacterial strains, serial dilutions of each environmental sample were prepared in sterile 200 µl of 1x PBS in a 96-well plate. Four dilutions were prepared: A) undiluted, B) 1:10 dilution, C)

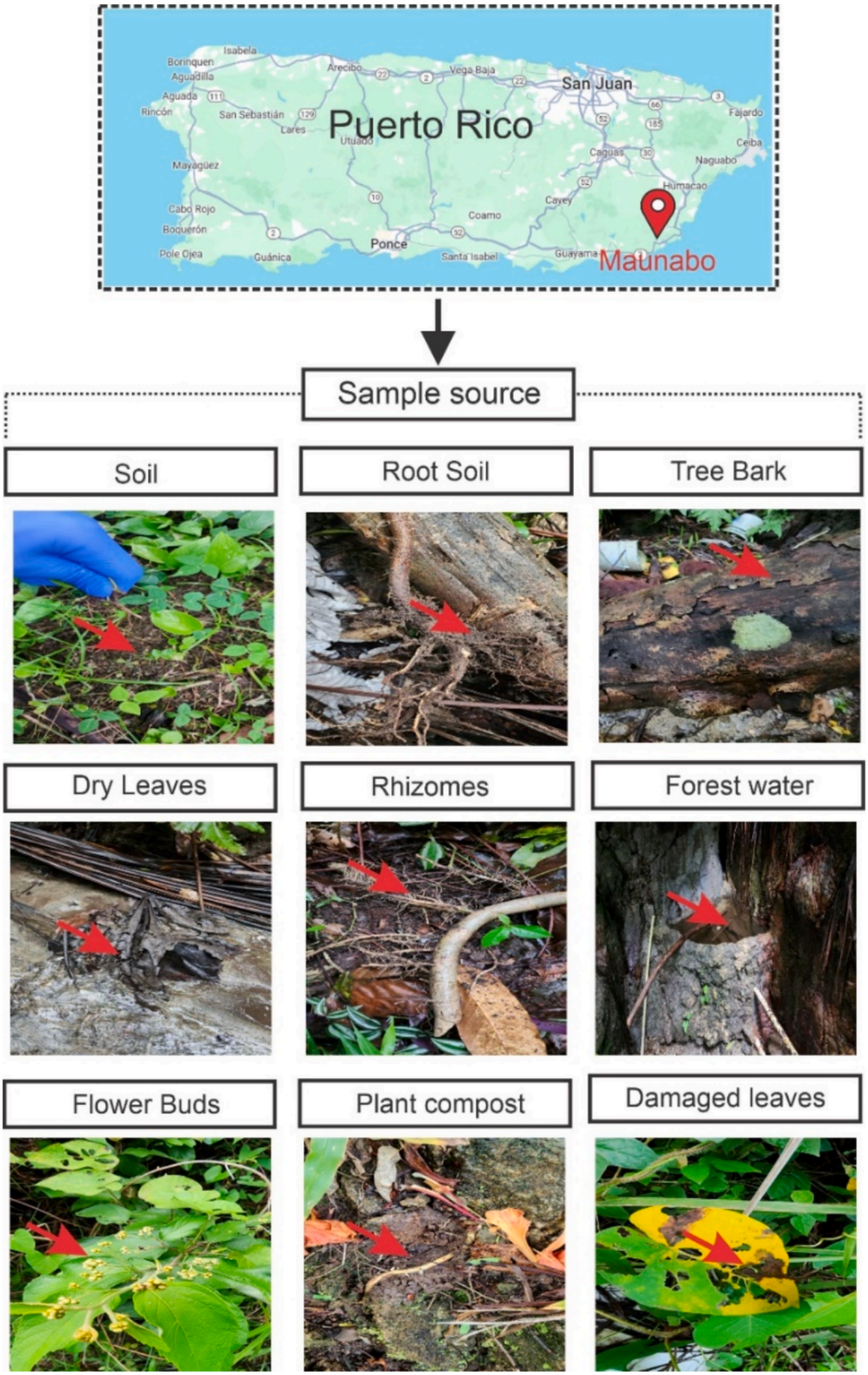

Fig. 1. Geographic location and sampling sources for isolation of natural bacteria in Puerto Rico. Environmental samples were collected from diverse ecological niches in Maunabo, Puerto Rico, as indicated on the map. Sampling sources included soil, root soil, tree bark, dry leaves, rhizomes, forest water, flower buds, plant compost, and damaged leaves. These sites represent distinct microhabitats targeted to capture a broad range of bacterial diversity. All samples were collected aseptically and transported to the laboratory for microbiological analysis.

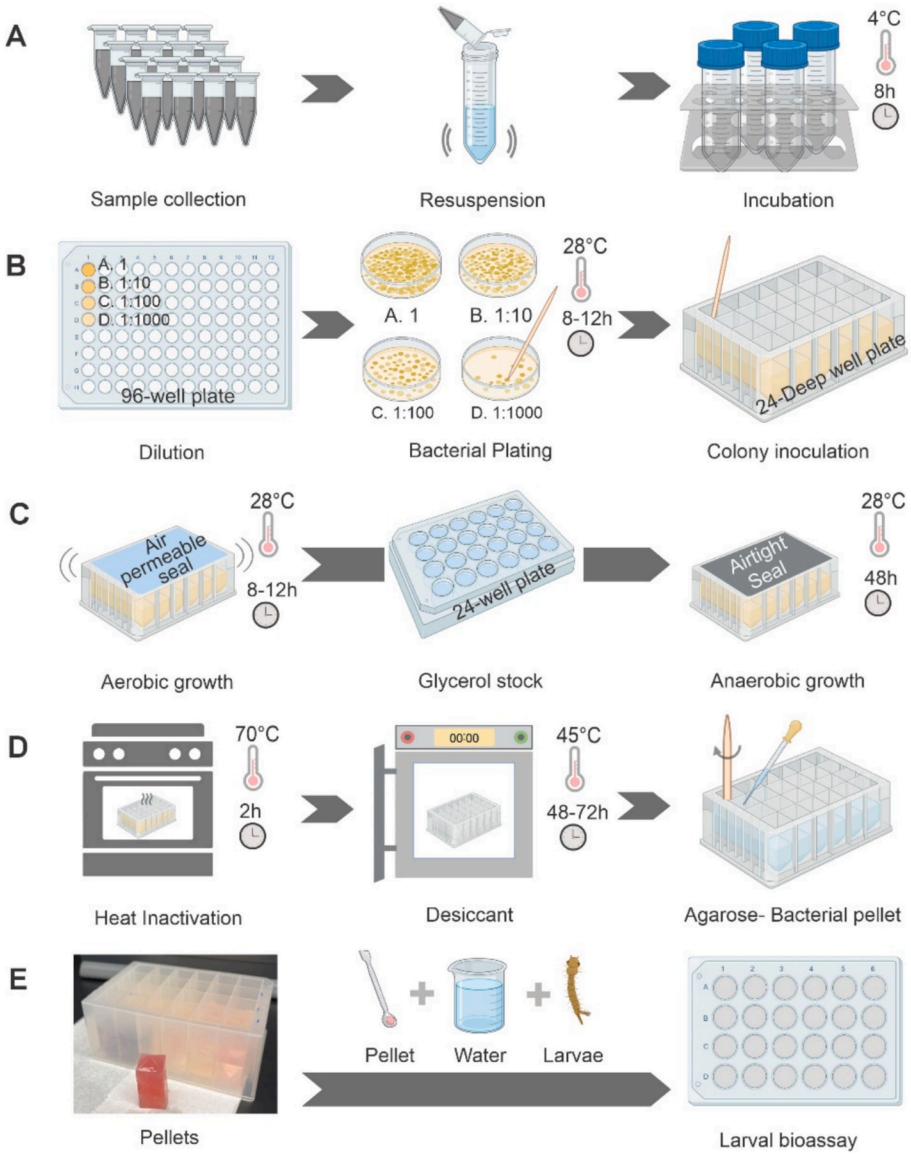

Fig. 2. High-throughput pipeline for bacterial isolation, biomass preparation, and larval bioassay. (A) Environmental samples were collected, resuspended in sterile phosphate-buffered saline (PBS), and incubated at 4°C for 8 h. **(B)** Serial dilutions (1, 1:10, 1:100, and 1:1000) were plated onto LB agar to isolate individual bacterial colonies, which were then cultured in 24-deep well plates. **(C)** Isolates were grown under aerobic and anaerobic conditions, and glycerol stocks were prepared for long-term storage. **(D)** Bacterial cultures were heat-inactivated at 70°C for 2 h, air-dried at 45°C for 48–72 h, and embedded in agarose to form a biomass pellet. **(E)** Pellets containing bacterial biomass embedded in agarose were suspended in water and used in a 24-well plate larval bioassay to evaluate insecticidal activity.

1:100 dilution, and D) 1:1000 dilution. One hundred microliters of each dilution were plated onto Luria-Bertani (LB) agar plates and incubated at 28°C for 24 h. From each sample (across all dilutions), up to twelve distinct colonies were randomly selected for further analysis, with priority given to pigmented colonies due to their potential for producing bioactive secondary metabolites. Notably, five environmental samples, specifically those collected from root-associated soil and rhizomes, produced pigmented colonies (yellow, red, and purple), which were prioritized for downstream screening. Each selected colony was inoculated into an individual well of a sterile 24-deep-well plate (Axygen, cat. no. 2026–07-07) containing 5 mL of LB broth (Fig. 2B). The plates were sealed with AeraSeal™ breathable seals (Excel Scientific, cat no. Z721573-50EA) and incubated at 28°C with shaking at 150 rpm for 16–18 h for aerobic growth. Following aerobic growth, 200 μL of each culture was transferred to a sterile 24-well culture plate (Thermo Fisher Scientific) containing an equal volume of 50% sterile glycerol. These glycerol stocks were stored at −80°C for long-term preservation. The original bacterial culture plates containing the remaining bacterial cultures were resealed with airtight sealers (GeneMate VWR, cat no. T-3025-8B) and incubated at 28°C for 2 days without shaking to allow for anaerobic growth (Fig. 2C).

2.5. Preparation of non-live bacterial biomass

Following anaerobic incubation, the bacterial culture plates were subjected to heat treatment at 70°C for 2–3 h to inactivate the live bacteria. Subsequently, the plates were air-dried at 45°C to reduce the culture volumes by 80–90%. To prepare the non-live bacterial biomass for subsequent larvicidal activity assays, an equal volume of melted 2% agarose prepared in the supernatant of 200 mg/ml fish food dissolved in distilled water was added to each well and mixed with bacterial culture, allowing the bacterial biomass to solidify within the agarose matrix

(Fig. 2D). These solidified agarose-bacteria small chunks were used to test the mosquito larvicide activity.

2.6. High-throughput larval bioassay

High-throughput larval bioassays were conducted in 24-well plates using three-day-old *Ae. aegypti* larvae. Two milliliters of distilled water were added to each well, followed by the introduction of five 2nd instar *Ae. aegypti* larvae. A small piece of each solidified bacterial-agarose pellet was carefully transferred using a spatula to the corresponding well containing larvae (Fig. 2E). Non-live *E. coli* −agarose chunks served as negative controls, while C. sp_P (*Chromobacterium* sp. Panama) −agarose chunks were used as positive controls. Larval mortality was assessed at 24- and 96-hours post-exposure. Colonies demonstrating 80–100% larval mortality in two independent experiments were considered potent larvicidal candidates.

2.7. Identification of bacterial isolates using 16Ss rRNA sequencing

Bacterial 16S ribosomal RNA (rRNA) genes were amplified and sequenced to identify the bacterial isolates exhibiting the most potent larvicidal activity. Genomic DNA was extracted from the bacterial isolates using the QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's instructions. The 16S rRNA gene, a highly conserved genetic marker for bacterial identification, was amplified using universal primers 16s27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16s1492R (5′-ACGGYTACCTTGTTACGACTT-3′), which target the V1-V9 regions to generate a ~ 1500 bp amplicon (dos Santos et al., 2019). The amplification procedure included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. The PCR products were outsourced for sequencing to Quintara Biosciences (https://www.quintarabio.com), where Sanger sequencing was performed using their standardized protocols. The resulting sequences were compared to reference sequences in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLAST) to identify the closest phylogenetic relatives of the bacterial isolates based on sequence similarity and alignment scores.

2.8. Phylogenetic analysis

To understand the evolutionary relationships among the identified bacterial isolates, phylogenetic analysis was performed. 16S rRNA gene sequences of the closest relatives identified through NCBI-BLASTn searches were retrieved from the GenBank database. These reference sequences, along with the query sequences (16S rRNA gene sequences of the isolated bacteria), were aligned using the MAFFT v7.520 multiple sequence alignment tool (Katoh and Standley, 2013). To ensure accurate identification, 16S rRNA sequences of each isolate were queried against a manually curated 16S rRNA database, and the top 50 hits from a BLAST search were selected for further analysis. Maximum-likelihood phylogenetic trees were constructed and visualized using MEGA 11.0.13 based on the best-fit nucleotide substitution model, with 10,000 bootstrap iterations to assess branch confidence (Kalyaanamoorthy et al., 2017).

2.9. Bacterial culturing and non-live bacterial pellet formulation

To further evaluate the larvicidal activity of the most potent bacterial isolates (those exhibiting 80–100% mortality in the initial high-throughput screen), large-scale bacterial cultures were prepared. A total of 15 isolates were selected based on their phylogenetic diversity and strong larvicidal efficacy. These isolates were retrieved from glycerol stocks, originally prepared during bacterial culture establishment, and inoculated in 5 mL of LB broth. Cultures were incubated overnight at 28°C with shaking at 150 rpm. Subsequently, 1 mL of this overnight culture was used to inoculate 1 L of fresh LB broth for large-scale cultivation. The 1-liter cultures were incubated at 28°C for 24 h with shaking for aerobic growth, followed by 48 h of static incubation in sealed flasks to enable anaerobic growth. After incubation, the bacterial cultures were then transferred to silicon rubber trays (10″ x 11″ x 0.78″) that had been cleaned and wiped with ethanol. The trays were placed in an oven at 70°C for 2–3 h to inactivate the live bacteria. Subsequently, the cultures were air-dried at 40°C for 3–5 days until completely dry. The resulting non-live bacterial biomass was scraped from the trays and ground into a fine powder using an electric blender. To prepare the non-live bacterial pellets, the powdered biomass (at final concentrations of 200 mg/mL and 400 mg/mL) was mixed with larval food (supernatant of 200 mg/mL fish meal dissolved in distilled water) and an equal volume of 2% melted agarose. This mixture was poured into small, semi-spherical, non-sticky silicone molds (cavity size 0.86″ x 0.47″ x 0.6″) (Amazon, USA; ASIN: B084VS3RTZ). After solidification, each bacterial-agarose pellet (containing 0.5 ml of agarose non-live bacterial biomass mix) was removed from the mold and used in subsequent mosquito larvicidal bioassays (Fig. 3). The powdered non-live bacterial biomass was stored in airtight containers along with silica gel desiccant to minimize moisture and preserve sample integrity.

2.10. Larval bioassay of Scaled-Up bacterial isolates

Larval bioassays were performed to assess the larvicidal activity of non-live bacterial agarose pellets at two concentrations, 200 mg/mL and 400 mg/mL, against three mosquito species: *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi*. For each assay, 50 s-instar larvae were introduced into 250 mL of distilled water containing two bacterial-agarose pellets (equivalent to 1 mL of agarose mixture). Larval mortality was recorded 24 h post-exposure. Non-live *E. coli* and C. sp. P agarose pellets served as negative and positive controls, respectively. To evaluate the efficacy of powdered bacterial biomass independently of the agarose matrix, a larval bioassay was conducted using three randomly selected isolates from the 15 chosen for large-scale culture. For this comparison, 400 mg of non-live bacterial powder was directly added to 50 mL of distilled water containing 50 second-instar *Ae. aegypti larvae.* Mortality was recorded 24 h after exposure.

3. Results

3.1. High-throughput screening and larval bioassay

A total of 486 bacterial isolates were obtained from 48 diverse environmental samples, including soil, water, and plants from Puerto Rico (Fig. 1). The larvicidal activity of these isolates was systematically evaluated against second-instar *Aedes aegypti* larvae using a high-throughput screening (HTS) assay. For each isolate, a non-live bacterial pellet was prepared by heat-inactivating cultures at 70°C for 2–3 h, followed by air-drying at 40°C. The dried biomass was then mixed with 2% agarose (prepared in fish food supernatant as an attractant) and allowed to solidify. A small piece of each agarose pellet was transferred into a 24-well plate containing five larvae in 2 mL of distilled water.

Among the 486 isolates tested, 30 demonstrated potent larvicidal activity, consistently inducing 80–100% mortality within four days of exposure. The 30 larvicidal isolates originated from various environmental sources, including dry leaves (n = 8), root soil (n = 7), general soil (n = 6), rhizomes (n = 4), flower buds (n = 4), and plant compost (n = 1) (Table 1). Remarkably, three isolates—28.08 (*Pseudomonas*) and 28.04 (*Pantoea*) (from flower buds) and 29.01 (*Pseudomonas*) (from soil) achieved 100% mortality within the first 24 h in two independent experimental replicates. *E. coli*-agarose pellets served as a negative control and caused no larval mortality, whereas the positive control using *Chromobacterium* sp. Panama-agarose pellets resulted in 100% mortality within 24 h.

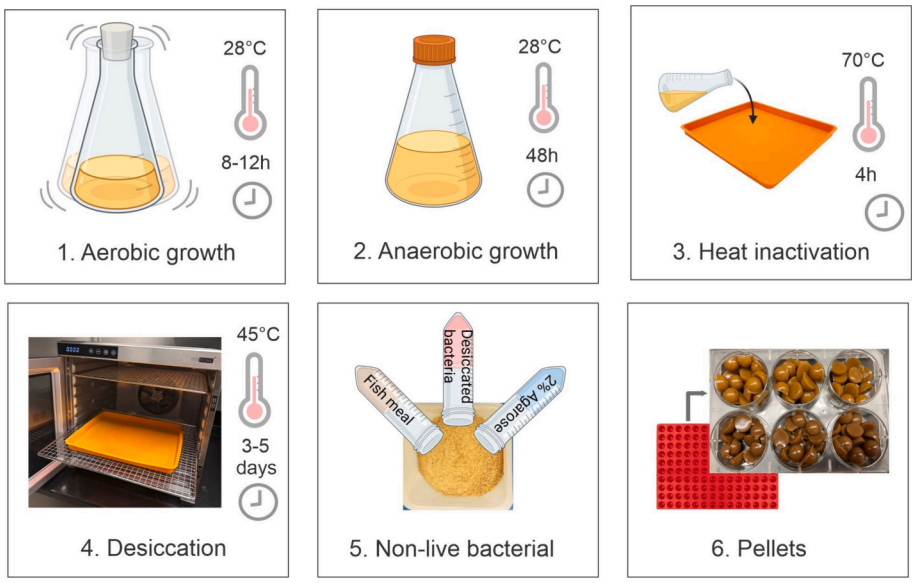

Fig. 3. Scale-up of bacterial biomass production. Schematic representation of the production process for bacterial biomass and agarose pellet formation. Bacterial isolates were cultured aerobically (28°C, 8–12 h) and anaerobically (28°C, 48 h), followed by heat inactivation (70°C, 4 h) and air-drying (45°C, 3–5 days). The desiccated bacterial biomass was mixed with fish meal and agarose to form bacterial-agarose pellets.

Table 1
Larvicidal activity of bacterial isolates achieving ≥ 80% larval mortality in microplate bioassays. Taxonomic classification was determined using 16S rRNA gene sequencing.

S. No	Sample Source	Sample code	Genous	% Mortality (EXP-I)		% Mortality (EXP-II)	
				Day 1	Day 4	Day 1	Day 4
1	Root soil	11.12	*Enterobacter*	0	100	60	80
2	Root soil	01.01	*Enterobacter*	20	100	20	100
3	Root soil	23.07	*Enterobacter*	80	80	80	80
4	Soil	05.12	*Enterobacter*	0	80	80	80
5	Dry leaves	06.01	*Enterobacter*	80	100	40	100
6	Dry leaves	10.12	*Enterobacter*	80	80	100	100
7	Dry leaves	10.04	*Enterobacter*	17	83	40	100
8	Soil	03.04	*Bacillus*	0	100	100	100
9	Soil	03.05	*Bacillus*	0	100	0	60
10	Soil	05.03	*Bacillus*	60	80	40	100
11	Soil	09.10	*Bacillus*	0	100	40	80
12	Dry leaves	10.11	*Bacillus*	0	80	0	80
13	Rhizomes	38.04	*Bacillus*	29	86	40	100
14	Root soil	37.12	*Serratia*	20	80	100	100
15	Rhizomes	38.01	*Serratia*	20	100	100	100
16	Root soil	39.02	*Serratia*	60	100	80	100
17	Rhizomes	39.03	*Serratia*	20	100	80	100
18	Rhizomes	39.12	*Serratia*	80	100	80	100
19	Dry leaves	10.10	*Acinetobacter*	0	80	80	100
20	Dry leaves	06.07	*Acinetobacter*	0	80	60	80
21	Dry leaves	06.09	*Acinetobacter*	0	100	40	100
22	Dry leaves	06.12	*Acinetobacter*	60	80	40	80
23	Flower Buds	28.08	*Pseudomonas*	100	100	100	100
24	Soil	29.01	*Pseudomonas*	100	100	100	100
25	Plant compost	18.02	*Chromobacterium*	20	80	80	80
26	Root soil	37.10	*Chromobacterium*	17	100	80	100
27	Flower Buds	28.07	*Pantoea*	60	80	100	100
28	Flower Buds	28.04	*Pantoea*	100	100	100	100
29	Flower Buds	28.02	*Staphylococcus*	100	100	80	100
30	Root soil	37.11	*Raoultella*	43	86	40	100

3.2. Identification of bacterial isolates

The taxonomic identities at the genus level of the 30 potent larvicidal bacterial isolates were determined through 16S rRNA gene sequence analysis using the NCBI BLASTn tool. This analysis revealed a diverse set of bacterial genera, summarized in Table 1. Genus-level identification was based on top BLAST hits with 50–80% sequence similarity. A total of nine genera were represented among the isolates, including *Enterobacter* (7 isolates), *Bacillus* (6), *Serratia* (5), *Acinetobacter* (4), *Pseudomonas* (2), *Chromobacterium* (2), *Pantoea* (2), *Staphylococcus* (1), and *Raoultella* (1).

To validate and visualize the evolutionary relationships of these isolates, maximum-likelihood phylogenetic trees were constructed based on 16S rRNA gene sequences. Each isolate was aligned with the top 50 closest relatives retrieved from the GenBank database, and

phylogenetic trees were generated using MEGA 11.0.13 with 10,000 bootstrap replicates. The resulting trees confirmed the genus-level affiliations and Bootstrap support values indicated at each node to show the reliability of the clustering (Supplementary Fig. S1).

3.3. Scale-up of selected bacterial isolates

To further validate the larvicidal efficacy against a broader range of mosquito species, fifteen isolates were strategically selected from the 30 potent larvicidal isolates identified during the initial high-throughput screening (Table 2). The selection was based on multiple criteria to ensure both phylogenetic diversity and potent larvicidal activity. First, taxonomic representation was prioritized by selecting at least one isolate from each major bacterial genus identified through 16S rRNA gene sequencing. This approach provided broad phylogenetic coverage while minimizing redundancy from isolates likely to act through known bioactive factors. The final panel comprised isolates from *Enterobacter* (5), *Bacillus* (2), *Serratia* (2), *Pseudomonas* (1), *Chromobacterium* (2), *Pantoea* (1), *Staphylococcus* (1), and *Raoultella* (1). One isolate initially classified as *Acinetobacter* was later re-identified as *Enterobacter* based on phylogenetic tree analysis, and thus *Acinetobacter* was not represented in the final selection. Second, isolates that exhibited rapid larvicidal activity in the primary screen, specifically those causing high mortality within 24 h, were selected to identify early-acting candidates; these were isolates 3.04 (*Bacillus*), 10.12 (*Enterobacter*), 28.08 (*Pseudomonas*), and 28.02 (*Staphylococcus*). Third, isolates displaying distinct colony pigmentation, suggestive of the production of bioactive secondary metabolites, were also selected, including isolates 37.10 (*Chromobacterium*), 37.12 (*Serratia*), and 39.02 (*Serratia*). These selected isolates were then subjected to large-scale culturing to generate sufficient non-live bacterial biomass embedded in agarose matrices, which were subsequently tested in expanded larval bioassays against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi*.

3.4. Larval bioassay of Scaled-Up bacterial isolates

Larvicidal bioassays of the 15 selected isolates were performed as non–live bacterial agarose pellets at 400 mg desiccated biomass/mL pellet, and yielded high larvicidal activity (80–100% mortality) for 12 isolates against *Ae. aegypti*. Three *Enterobacter* isolates (6.01, 11.12, and 23.7) were less effective, causing 68%, 43%, and 18% mortality, respectively (Fig. 4A). Against *Cx. quinquefasciatus* and *An. stephensi*, 14 of 15 isolates produced 80–100% mortality; only isolate 23.7 (*Enterobacter*) showed negligible activity (0% and 3.5%, respectively) (Fig. 4B and 4C). At the lower dose (200 mg/mL), only isolate 28.8 (*Acinetobacter*) achieved > 80% mortality in *Ae. aegypti*, whereas ten isolates exceeded this threshold against *An. stephensi* and four against *Cx. quinquefasciatus*, indicating a clear dose-dependent response and mosquito interspecific differences with regard to susceptibility (Fig. 4A–C).

We also performed an assay where the non-live bacterial powder of three isolates (6.01 (*Enterobacter*), 10.12 (*Enterobacter*), and 37.12 (*Serratia*)) was directly resuspended in the *Ae. aegypti* larval breeding water instead of being provided through an agarose pellet. This treatment showed a similar larvicidal effect compared to pellet-based exposure for isolates 6.01 and 10.12 (*Enterobacter*) ($p > 0.05$). In contrast, isolate 37.12 (*Serratia*) exhibited minimal larvicidal activity when provided as a suspended powder but induced high mortality when delivered in pellet form ($p = 0.005$) (Fig. 5). This result suggests that the dispersal of active factors into the water is sufficient for larvicidal activity in some isolates (e.g., *Enterobacter*), whereas others (e.g., *Serratia*) may require a delivery matrix for larvicidal activity.

4. Discussion

The escalating challenge of mosquito-borne diseases necessitates the pressing need for innovative, eco-friendly vector control tools. This study established a systematic high-throughput screening (HTS) platform that effectively identified diverse bacterial isolates with potent larvicidal activity from varied natural habitats. Notably, our use of heat-inactivated bacterial preparations biased the screen toward strains producing heat-stable toxins or metabolites, rather than those relying on infection by live cells. This focus on thermostable larvicidal factors offers practical advantages for formulation and field application over live bacterial agents.

Our HTS methodology proved highly effective in streamlining the initial screening process. From 48 environmental samples, we isolated 486 bacterial strains, from which 30 exhibited significant larvicidal activity, including three isolates that caused 100% *Aedes aegypti* larval mortality within 24 h. The 24-deep well plate format enabled rapid, low-cost evaluation using minimal bacterial material and 2nd instar larvae, substantially reducing the time, labor, and resources typically required for large-scale bioassays. This approach aligns with the growing trend of

Table 2
Larvicidal activity of scaled-up bacterial isolates against three mosquito species at two concentrations.

Sample code	Bacterial strain	*Ae. aegypti*		*Cx. quinquefasciatus*		*An. stephensi*	
		400 mg/ml	200 mg/ml	400 mg/ml	200 mg/ml	400 mg/ml	200 mg/ml
10.12	*Enterobacter*	✓	X	✓	✓	✓	✓
6.01	*Enterobacter*	X	X	✓	✓	✓	X
10.04	*Enterobacter*	✓	X	✓	X	✓	✓
11.12	*Enterobacter*	X	X	✓	✓	✓	✓
23.07	*Enterobacter*	X	X	X	X	X	X
3.04	*Bacillus*	✓	X	✓	✓	✓	✓
9.10	*Bacillus*	✓	X	✓	X	✓	✓
37.12	*Serratia*	✓	X	✓	X	✓	✓
39.02	*Serratia*	✓	X	✓	X	✓	✓
28.08	*Pseudomonas*	✓	✓	✓	X	✓	X
18.02	*Chromobacterium*	✓	X	✓	X	✓	✓
37.10	*Chromobacterium*	✓	X	✓	X	✓	✓
28.07	*Pantoea*	✓	X	✓	X	✓	✓
28.02	*Staphylococcus*	✓	X	✓	X	✓	X
37.11	*Raoultella*	✓	X	✓	X	✓	X

✓80–100% mortality, X < 80% mortality.
Sample code refers to the selected bacterial isolates following large–scale screening.
Bacterial strain indicates the genus designation based on 16S rRNA sequencing.
Columns for *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi* show larval mortality for each isolate at the two tested concentrations: 400 mg/mL and 200 mg/mL. Data represent whether larval mortality reached the ≥ 80% threshold in a 24-hour exposure assay using agarose-embedded non–live bacterial biomass.

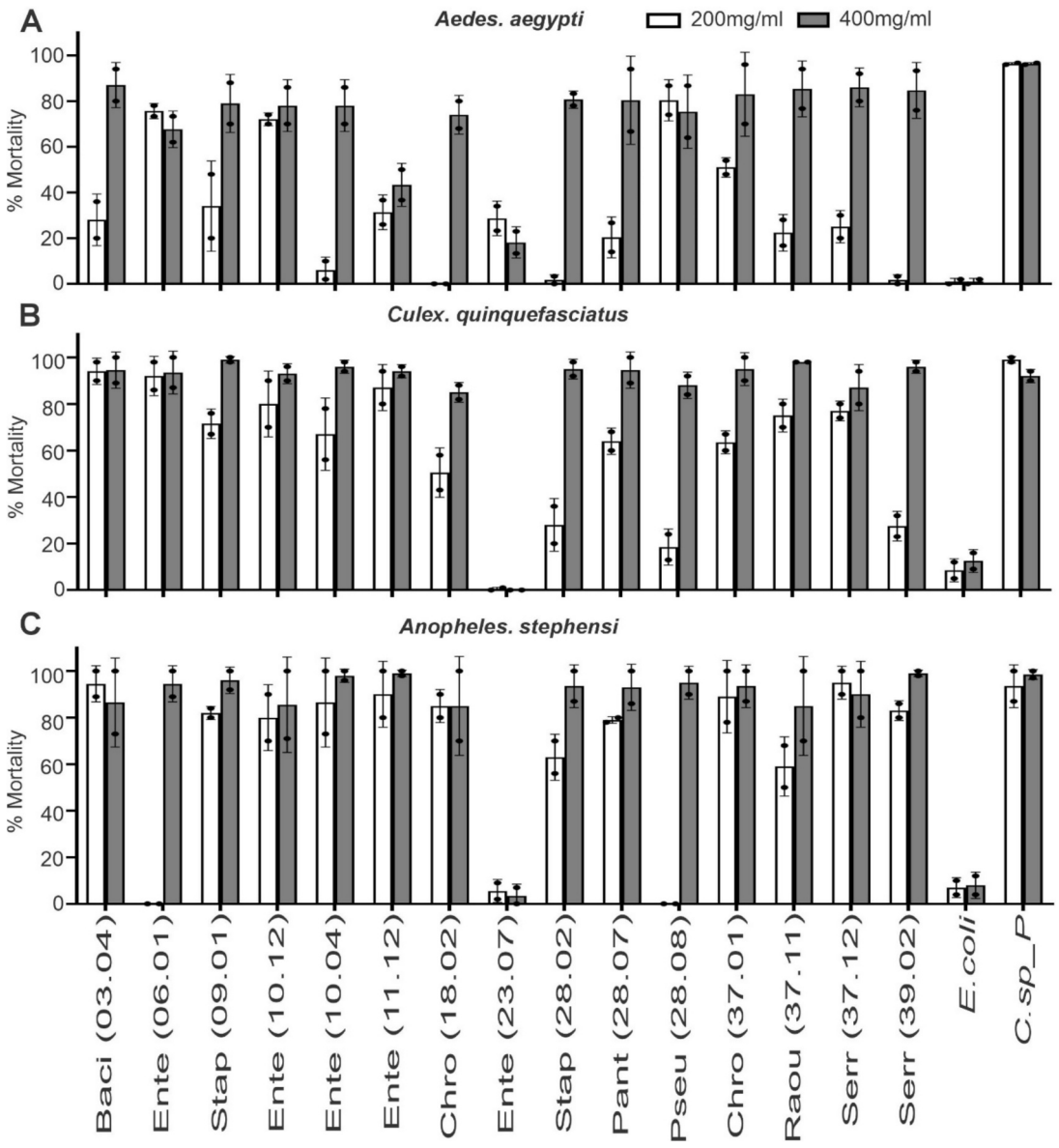

Fig. 4. Larvicidal efficacy of scaled–up non–live bacterial formulations. (A–C) Percent mortality of second–instar larvae of *Aedes aegypti* (A), *Culex quinquefasciatus* (B), and *Anopheles stephensi* (C) after 24 h exposure to non-live bacterial–agarose pellets at 200 mg/mL (open bars) and 400 mg/mL (filled bars). Data represent mean ± SD of two independent assays per isolate. *Bacillus* (Baci), *Enterobacter* (Ente), *Staphylococcus* (Stap), *Chromobacterium* (Chro), *Pantoea* (Pant), *Pseudomonas* (Pseu), *Serratia* (Serr), and *Raoultella* (Raou) isolates are indicated with their four-letter genus abbreviations followed by isolate codes in parentheses. *Escherichia coli* (*E. coli*)–agarose was used as a negative control; *Chromobacterium* sp. Panama (*C. sp_P*)–agarose served as a positive control.

applying HTS in insecticide discovery, offering a scalable, efficient platform for processing large microbial libraries (Buckingham et al., 2021; Pridgeon et al., 2009). By enabling the rapid screening of heat-inactivated bacterial and simplifying larval handling, our method enhances throughput and cost-effectiveness compared to traditional assays, underscoring its potential for accelerating the discovery of novel biocontrol agents.

Among the top 30 insecticidal microbial isolates identified in this study, a significant proportion originated from soil (43%), followed by decaying dry leaves (27%), rhizomes (13%), flower buds (13%), and plant compost (3%). While the presence of microbial insecticidal agents, particularly *Bacillus* spp., *Lysinibacillus sphaericus*, *Brevibacillus laterosporus* (Ebani and Mancianti, 2021), as well as entomopathogenic fungi such as *Metarhizium* and *Beauveria bassiana* (Majchrowska-Safaryan and Tkaczuk, 2021), has been well documented across diverse ecological niches, their broader ecological roles in plant-insect-microbe interactions are only beginning to be understood. Recent studies suggest that plants can modulate their root microbiomes by selectively recruiting beneficial microbes in response to insect herbivory (Grabka et al., 2022). These interactions not only strengthen plant defenses (Xu et al., 2024) but also enhance the likelihood of discovering novel insecticidal microbes in root-adjacent soils. The substantial recovery of potent isolates from root-associated soil in this study underscores the functional importance of these habitats as biological hotspots for insect-antagonistic microbes.

Additionally, dry leaf litter, often overlooked, represents a dynamic microhabitat where microbial competition and organic matter decomposition may favor the evolution of potent entomopathogens (Majchrowska-Safaryan and Tkaczuk, 2021). The identification of highly insecticidal strains from decomposing leaf material further supports the concept that environmental stress (Tuininga et al., 2009) and nutrient cycling processes contribute to the emergence of microbial traits beneficial for pest control. Collectively, these findings highlight the ecological richness and strategic value of root soil, rhizomes, and

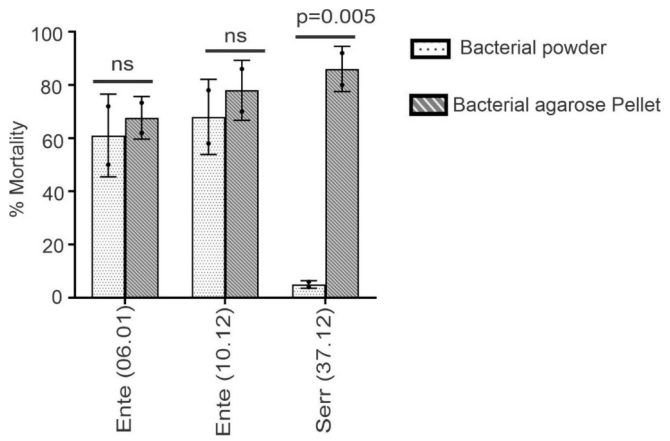

Fig. 5. Larvicidal activity of bacterial powders versus agarose pellets against *Aedes aegypti*. Bar graph comparing larval mortality (%) induced by three bacterial isolates, *Enterobacter* (Ente; 06.01 and 10.12), and *Serratia* (Serr; 37.12), formulated as non-live bacterial powders (dotted bars) or incorporated into agarose pellets (hatched bars) at 400 mg/mL. Data represent mean ± SD of two independent assays per isolate. Statistical differences were evaluated using an unpaired *t*-test; ns: not significant, $p = 0.005$.

decomposing plant matter as priority habitats for bioprospecting insecticidal microbes. Targeted exploration of these environments not only enhances the efficiency of microbial discovery pipelines but also aligns with the broader ecological paradigm of sustainable pest management.

Subsequent large-scale bioassays confirmed the larvicidal efficacy of 15 selected isolates, representing various bacterial genera, across multiple mosquito species, including *Ae. aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi*. Evaluating the larvicidal effect against diverse mosquito species is crucial for assessing the broad-spectrum potential and practical utility of novel bioinsecticides in integrated vector management programs, as different vectors may exhibit varying susceptibilities to control agents (Benelli et al., 2016b; Ghosh et al., 2012). A clear dose-dependent response was observed, with the 400 mg/mL concentration of desiccated, heat-inactivated bacterial biomass consistently inducing 80–100% mortality across most isolates and mosquito species. However, it should be noted that this dose standardization was based on biomass weight rather than on the concentration of specific larvicidal compounds. As such, comparisons between isolates in terms of potency must be interpreted with caution, since bacterial strains may differ significantly in the quantity and nature of bioactive molecules they produce. Notably, while *An. stephensi* larvae demonstrated high susceptibility to most isolates, *Ae. aegypti* larvae were generally less affected, particularly at the lower 200 mg/mL concentration. This differential susceptibility, with *Ae. aegypti* often displays greater resilience to various larvicidal agents, potentially due to its robust larval physiology and ability to thrive in diverse, often nutrient-rich breeding habitats (David et al., 2021; Thongwat et al., 2018). Such interspecies variation underscores the need to evaluate candidate larvicides against a range of mosquito vectors to develop broad-spectrum solutions.

Taxonomic identification revealed that the potent isolates belonged to diverse genera, including *Enterobacter* (7 isolates), *Bacillus* (6), *Serratia* (5), *Acinetobacter* (4), *Pseudomonas* (2), *Chromobacterium* (2), *Pantoea* (2), *Staphylococcus* (1), and *Raoultella* (1). These assignments were further supported by a phylogenetic tree constructed using 16S rRNA gene sequences, which confirmed the evolutionary relationships among the isolates and their genetic proximity to previously characterized insecticidal strains (supplementary Fig. 1). The identification of *Bacillus* and *Chromobacterium* species among the highly active isolates is particularly noteworthy. *Bacillus thuringiensis israelensis* (*Bti*) is a well-established and widely used mosquito larvicide, recognized globally for its specificity and environmental safety (Lacey, 2007). The presence of *Bacillus* strains in our collection suggests potential for new variants or alternative modes of action beyond the known Cry and Cyt toxins of *Bti* (Silva-Filha et al., 2021). Similarly, *Chromobacterium* sp. Panama (*C. sp_P*), used as a positive control in our study, is known for its potent insecticidal properties mediated by stable, non-live preparations (Caragata et al., 2020). The efficacy of our identified *Chromobacterium* isolates aligns with this existing knowledge, reinforcing the potential of this genus. The discovery of potent larvicidal activity from genera like *Pseudomonas*, *Serratia*, and *Enterobacter*, while some species within these genera are known for diverse metabolic capabilities, warrants further investigation into their specific bioactive compounds. Indeed, various *Pseudomonas* species have been reported to exhibit larvicidal or insecticidal effects through the production of toxins or secondary metabolites (Lalithambika and Vani, 2016; Mansour et al., 2023; Mayilsamy et al., 2025), and *Serratia marcescens* is a well-documented entomopathogen known to cause mortality in various insect pests, including some mosquito species, via proteases and other virulence factors (Ozgen et al., 2013; Sezen et al., 2001). Furthermore, *Enterobacter* species have also been shown to possess insecticidal activities, sometimes synergistically with other known biopesticides (Mi et al., 2023). The fact that the activity was maintained in non-live bacterial biomass strongly suggests the presence of stable secondary metabolites or toxins rather than relying on live infection. This characteristic offers distinct advantages for field application, aligning with the growing interest in leveraging non-live bacterial preparations as robust and stable bioinsecticides for mosquito control and other vector management strategies (Caragata et al., 2020; Tikhe et al., 2024).

The use of non-live bacterial biomass in our assays presents significant practical advantages for future vector control strategies. Unlike live microbial agents such as *Bti*, which can be susceptible to environmental degradation (e.g., UV radiation) and require careful handling for viability (Lacey, 2007), non-live preparations offer enhanced stability, potentially longer shelf-lives, and greater resilience to fluctuating environmental conditions. These attributes simplify storage, transport, and deployment across diverse climatic and resource-limited settings (Benelli et al., 2016a; Caragata et al., 2020). Additionally, the use of non-live biomass alleviates regulatory concerns associated with the release of live organisms, offering a safe and more predictable product profile that may accelerate commercialization and field adoption (Gassmann and Clifton, 2017). Furthermore, our comparison of agarose pellet and powder formulations revealed that pellet-based delivery enhanced larvicidal activity for certain isolates, most notably *Serratia* 37.12. This effect is likely due to more efficient exposure of larvae to bioactive compounds through ingestion of the pellet matrix, rather than relying solely on passive diffusion in water. Moreover, pellet formulations may offer a practical cost advantage, as effective activity through powder dispersion would require a higher amount of biomass to achieve similar efficacy. Similar matrix-based systems, such as carboxymethylcellulose gels (Prasad and Kalyanasundaram, 1991) and alginate beads (Khorramvatan et al., 2014), have shown prolonged efficacy and protection of bioactive agents. These formulations improve stability and interaction time with mosquito larvae.

While we successfully identified potent bacterial strains and demonstrated their larvicidal efficacy, the precise bioactive compounds and mechanisms of action remain to be elucidated. Future work will therefore prioritize the biochemical characterization and isolation of these active metabolites and undertake genomic analysis to identify biosynthetic gene clusters responsible for insecticidal activity. Additionally, comprehensive toxicological assessments against non-target organisms are crucial to confirm their environmental safety, as natural products can, in rare cases, have unintended ecological impacts (Benelli et al., 2016a). Finally, the efficacy observed under controlled laboratory conditions must be validated through semi-field and large-scale field trials to assess operational feasibility and performance under real-world environmental pressures, such as fluctuating water quality, temperature, and UV exposure, before broad implementation (Lacey, 2007;

WHO, 2005).

5. Conclusion

This study established a streamlined high-throughput screening pipeline for identifying novel bacterial strains with potent, larvicidal activity mediated by abiotic factors against major mosquito vectors. By exploring diverse environmental niches, particularly root-associated soil, decaying plant matter, and rhizosphere, we identified multiple candidates producing heat-stable insecticidal compounds. Several isolates exhibited rapid and broad-spectrum larval mortality, highlighting their potential as effective bioinsecticides. The advantages of non-live bacteria formulations, such as improved stability, ease of handling, and suitability for varied field conditions, further reinforce their value for practical application. Collectively, these findings emphasize the promise of microbial diversity as a source of environmentally sustainable and scalable mosquito control agents, offering new tools for integrated vector management amid growing concerns over insecticide resistance.

CRediT authorship contribution statement

Madhavinadha Prasad Kona: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vandana Vandana:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jenny Wang:** Methodology, Investigation. **Qiran Amy Dong:** Methodology, Investigation. **F. Elektra Maridaki:** Methodology, Investigation. **George Dimopoulos:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used Gemini to mine the literature and improve the text. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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