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Bacteria isolated from *Aedes aegypti* with potential vector control applications

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ABSTRACT

Highly anthropophilic and adapted to urban environments, *Aedes aegypti* mosquitoes are the main vectors of arboviruses that cause human diseases such as dengue, zika, and chikungunya fever, especially in countries with tropical and subtropical climates. Microorganisms with mosquitocidal and larvicidal activities have been suggested as environmentally safe alternatives to chemical or mechanical mosquito control methods. Here, we analyzed cultivable bacteria isolated from all stages of the mosquito life cycle for their larvicidal activity against *Ae. aegypti*. A total of 424 bacterial strains isolated from eggs, larvae, pupae, or adult *Ae. aegypti* were analyzed for the pathogenic potential of their crude cultures against larvae of this same mosquito species. Nine strains displayed larvicidal activity comparable to the strain AM65-52, reisolated from commercial BTi-based product VectoBac® WG. 16S rRNA gene sequencing revealed that the set of larvicidal strains contains two representatives of the genus *Bacillus*, five *Enterobacter*, and two *Stenotrophomonas*. This study demonstrates that some bacteria isolated from *Ae. aegypti* are pathogenic for the mosquito from which they were isolated. The data are promising for developing novel bioinsecticides for the control of these medically important mosquitoes.

1. Introduction

Aedes aegypti mosquitoes are the main vectors responsible for the transmission of arboviruses that cause diseases such as urban yellow fever, dengue, chikungunya, Zika fever, and others, which infect thousands of people around the world (Camara, 2016, Kraemer et al., 2019, Souza-Neto et al., 2019). Nowadays, chemical insecticides are the main vector control tools used against *Ae. aegypti*. Due to their continuous application in the field, for the management of agricultural and medical insect pests, chemical insecticides cause environmental pollution, food contamination, death of non-target organisms, and selection of naturally resistant insects (Amelia-Yap et al., 2018, Dusfour et al., 2019, Vontas et al., 2020, De Almeida Rocha et al., 2021).

Thus, the search for efficient and ecologically safe *Ae. aegypti* control agents has been intense. Identification, isolation, and characterization of environmental microorganisms and their metabolites with entomopathogenic activities have provided promising alternatives (Saldaña et al., 2017, Soares-da-Silva et al., 2017, Dahmana et al., 2020, Katak et al., 2023, 2021, De Oliveira et al., 2021). For example, the genera *Bacillus*, *Brevibacillus,* and *Lysinibacillus* harbor bacterial species useful for the control of insect vectors of diseases, such as mosquitoes of the genera *Aedes*, *Anopheles,* and *Culex* (Barbieri et al., 2021, Katak et al., 2021).

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These bacteria synthesize a wealth of molecules and virulence factors for mosquitoes, including the proteins Cry, Cyt, Vip, Mpp, Tpp, CpbB, chitinases, and others (Bravo et al., 2007, Palma et al., 2014, Marche et al., 2017, Crickmore et al., 2021), which are currently used in various insect biological control programs (Valtierra-de-Luis et al., 2020).

While mosquito-associated bacteria have been demonstrated to positively impact mosquito fitness, facilitate the acquisition of nutrients (Gaio et al., 2011), provide defense against pathogens (Oliver et al., 2003, Dong et al., 2009) and influence vectorial capacity by modulating the transmission of arboviruses (Weiss and Aksoy, 2011, Jupatanakul et al., 2014), until the writing of this article, no study focused on the bacteria present in *Ae. aegypti* with antagonistic interactions with the host mosquito. Here, we demonstrate that the mosquito microbiota should be further explored as a source of bacteria with entomopathogenic properties that can be developed into new products for the control of *Ae. aegypti*.

2. Materials and methods

2.1. Field collection of Aedes aegypti and laboratory breeding

Samples of *Ae. aegypti* larvae and pupae and breeding water were collected in the city of Manaus/Brazil (GPS coordinates −3.0726016 S, −59.9284962 W) in an area of high vector density, according to data from the local mosquito control program (Secretaria Municipal de Saúde do Amazonas - SEMSA). The collections were carried out in September 2020, with government authorization (SISBIO/74091-1/2020–2021) and the consent of the property owner who signed a free and informed consent form. Ten plastic trays (45 × 30 × 7.5 cm) with 1000 ml of sterile distilled water were placed at the collection site, monitored for ten days as per Silva et al. (2021). After this period, containers with larvae, pupae, and organic matter were moved to the laboratory (Laboratório de Controle Biológico e Biotecnologia da Malária e Dengue - LCBBMD) at INPA. Larvae and pupae were identified using entomological taxonomic keys (Forattini, 2002, Harbach, 2022, WRBU, 2022). The insectary-maintained conditions were 27 ± 2 °C, 80–90% relative humidity, and a 12/12 light/dark cycle. Field-collected larvae were kept in the laboratory with the same water and organic matter. Adult *Ae. aegypti* females were fed hamster blood (*Mesocricetus auratus* Waterhouse, 1839) and provided with a 10% sucrose solution. Hamsters were anesthetized following INPA's approved ethics protocol (029/2021). After the blood meal, eggs were collected for three days. Some eggs were used for bacterial isolation, while the rest were stored for 72 h and then immersed in containers with distilled water to stimulate larval hatching for bioassays. Lab-reared larvae followed similar procedures as field-collected ones, with fish food (Tetramin) added to their diet.

2.2. Isolation of bacteria

Bacterial strains were isolated as described by Rocha et al. (2021), with adaptations. Apart from eggs that were not prewashed, samples of other developmental stages (4th instar larvae, pupae, and adult females) of *Ae. aegypti* were washed for 1 min in 70% alcohol and then in sterile H_2O before being macerated in microtubes containing 1.5 ml of sterilized H_2O and vortexed for three minutes. For egg collection, the gravid females were placed in containers with sterilized filter paper and distilled water, so that bacteria derived from eggs originated from the egg laying females. Three replicates, 50 µl aliquots from all samples, were spread on Petri dishes containing Nutrient Agar (NA), Luria-Bertani Agar (LB) or ISP2 medium. Fluconazole (20 mg/ml) was added to the medium to prevent fungal growth. All Petri dishes were incubated at 29 °C for 24, 48 and 72 h. Negative control plates with only sterile water did not result in colonies.

2.3. Morphological and molecular characterization of bacteria

Bacterial colonies used in the selective bioassays were examined for size, shape, texture, elevation, color, and Gram stain at 1000X magnification. Genomic DNA from the nine bacterial strains in the quantitative bioassays was extracted using InstaGene™ Matrix (BioRad) following the manufacturer's instructions. DNA was quantified with a Thermo Scientific™ NanoDrop™ OneC Microvolume UV–Vis Spectrophotometer and adjusted to 150 ng/µl. Bacterial 16S rRNA genes were PCR-amplified using GoTaq® DNA Polymerase 2× (Promega) and primers 16S08F (5′-GYCCADACWCCTACGG-3′) and 16S08R (5′-CACGAGCTGACGAC-3′) (Arruda et al., 2021). The amplicon corresponds to a fragment of approximately 700 bp and comprises the variable regions V2 to V6 of the 16S rRNA gene. Each reaction consisted of 25 µl of the 2X master mix; 2 µl DNA (150 ng/µl); 21 µl of milli-Q H_2O and 1 µl (10 pMol) of each primer. The PCR program had an initial denaturation at 94 °C for 7 min, followed by 30 cycles of [94 °C for 45 s, 50 °C for 30 s, 72 °C for 60 s], followed by a final extension at 72 °C for 10 min. PCR products were evaluated on 1% agarose gel stained with UniSafe Dye 0.03% (v/v) and visualized under UV light. Sanger sequencing in both directions using the primers V2 and V6 (BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied) Sequencing Reaction and Ethanol/EDTA/Sodium Acetate precipitation reaction according to manufacturer's suggested protocol) was performed by capillary electrophoresis in a ABI3730xl Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed using the programs Chromas Lite and Geneious 4.8.3. Taxonomic assignments of the isolated bacteria were based on consensus sequence comparisons with 16S sequences in GenBank applying BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at the National Center for Biotechnology Information (NCBI) and the Ribosomal Database Project, RDP-II (https://rdp.cme.msu.edu/comparison/comp.jsp).

2.4. Assays for mosquito larvicidal activity of bacterial cultures

Individual colonies (bacterial strains) were inoculated in 5 ml of the same culture medium from which they were isolated, and these initial cultures were kept in a shaker incubator at 29 °C and 180 rpm for 24 h. Fifty microlitres of the initial cultures were inoculated in 50 ml of the same medium and incubated under the same conditions for 72 h. The optical densities of the bacterial cultures were monitored and the values at the stationary phase, 72 h, are shown in Table 1. Bioassays were performed according to WHO guidelines (2005), as described by Katak et al. (2021). Bioassays were carried out in three replicates, each with three cups and each cup containing 9 ml of distilled water, 1 ml of bacterial culture, and fish food (Tetramin). Ten third-instar larvae of the first generation of *Ae. aegypti* from the specimens collected in the field were placed in each cup. Larval mortality was determined at 24, 48 and 72 h after exposure to bacterial cultures. No mortality was observed at any time in negative controls without bacteria. The strain AM65-52 of *B. thuringiensis* subsp. *israelensis*, reisolated from the product VectoBac® WG, free from the additives present in the formulation, was tested as a positive control. Strains resulting in 100% mortality were further investigated and lethal concentrations (LC_{50} and LC_{90}) were determined.

2.5. LC_{50} and LC_{90} determination

2.5.1. Bacterial cultures

Bacterial strains were inoculated in 2 ml of the culture medium from which they were isolated and kept in a shaker incubator at 30 °C and 180 rpm for 24 h. Fifty µl of each culture were transferred to 100 ml of fresh medium, followed by incubation at 30 °C and 180 rpm for 72 h. Biological assays were carried out as described by WHO (2005) and Katak et al. (2021). These tests were carried out in five replicates, each containing 150 ml of water, 20 third-instar larvae and 1000, 900, 800, 750, 700, 650, 600, 550, 500, 400, 350, 300, 250, 200, 125, 100, 75 or 50 µl of bacterial culture. Dead larvae were counted at 24, 48, and 72 h

Table 1
Characterization of isolated bacteria with pathogenicity against *Ae. aegypti* larvae.

Strain	Culture medium/sample	OD600 nm (72 h)	Gram stain	Catalase activity	16S Seq. Identity %	Best match GenBank acc. n° Genus
L65	ISP2/Larvae	1.15	+	+	99.01	OP692702 - *Bacillus* sp.
L21	LB/Larvae	2.6	–	+	98.58	OP692703 - *Stenotrophomonas* sp.
L31	LB/Larvae	2.0	–	+	98.87	OP692704 - *Stenotrophomonas* sp.
L39	LB/Larvae	2.5	–	+	99.00	OP692705 - *Enterobacter* sp.
L41	LB/Larvae	2.7	–	+	99.29	OP692706 - *Enterobacter* sp.
P12	NA/Pupae	1.25	+	+	98.86	OP692707 - *Bacillus* sp.
P15	NA/Pupae	2.7	–	+	99.00	OP692708 - *Enterobacter* sp.
P18	NA/Pupae	2.1	–	+	99.29	OP692709 - *Enterobacter* sp.
L47	NA/Larvae	2.7	–	+	99.01	OP692710 - *Enterobacter* sp.

after exposure. Negative and positive controls were included as described above, using strain AM65-52 as active strain. Data from concentrations that caused between 10% and about 95% mortality of mosquito larvae were used for statistical analyses. LC_{50} and LC_{90} were evaluated using Probit, with $p \leq 0.05$ (Finney, 1971), using the statistical software Polo Plus 1.0 statistical software (LeOra Software, Berkeley, CA, USA) (Robertson et al., 2017). Lethal concentrations and confidence interval (95% CI) were analyzed using the Lilliefors normality test (K samples), analysis of variance (ANOVA), Tukey's multiple comparison tests ($p \leq 0.05$) and Student's *t*-test with BioEstat 5.3 software for Windows (Ayres et al., 2007).

2.5.2. Bacterial metabolites

Bacterial strains were inoculated into 1000 ml of medium as in 2.4. After 120 h of incubation, cultures were filtered through a 0.22 μM Millipore Membrane. The filtrate for each strain was partitioned in a separation funnel with a mixture of ethyl acetate (AcOET) and isopropanol (iPr-OH) 9:1 volume/volume (v/v) three times, each time using 300 ml of the solvent mixture (Rakhmawatie et al., 2021). The pooled solvent fractions were concentrated in a rotary evaporator (Tecnal®), under reduced pressure with a vacuum pump and at 45 °C. Dried extracts were weighed and stored in a desiccator with activated silica.

Bioassays followed the criteria established by Dulmage et al. (1990) and WHO (2005) and were conducted under controlled conditions of temperature, humidity, and photoperiod, as mentioned above. Bioassays were carried out in triplicate, in 150 ml plastic plates containing 120 ml of distilled water, 20 third instar larvae, powdered larval food (Teklad Global 18%) and concentrations of 0.01 to 250 μg/ml of extracted bacterial metabolites (De Oliveira et al., 2021). All metabolites were solubilized in dimethyl sulfoxide (DMSO; Thermo Fischer Scientific), and the DMSO concentration was normalized to 1% (v/v) in all experiments. Mortality readings were recorded 24, 48 and 72 h after exposure to bacterial extracts (Danga et al., 2014). The DMSO solvent was used as a negative control, and Temephos (Pestanal Sigma-Aldrich) as a positive control. LC_{50} and LC_{90}, statistical analyses were conducted as in 2.4.

3. Results

3.1. Selective bioassays for mosquito larvicidal activity

A total of 424 bacterial strains were isolated from *Ae. aegypti*, 88 from eggs, 166 from larvae, 72 from pupae, and 98 from adult female mosquitoes. We examined the pathogenic potential of these 424 bacterial strains against *Ae. aegypti* larvae, and found nine strains that, under our assay conditions, caused 100% mortality in 24 h (Fig. 1).

3.2. Taxonomic assignment of active strains

The 16S rRNA gene of the nine strains pathogenic to *Ae. aegypti* larvae included two *Bacillus* representatives, five *Enterobacter*, and two *Stenotrophomonas*, with over 98.5% identity with sequences in the NCBI database. However, only genus-level classification was possible for these isolated lineages due to the limited resolution of the 16S rRNA locus (Table 1).

3.3. LC_{50} and LC_{90}

3.3.1. Bacterial cultures

In quantitative bioassays, the pathogenicity of bacterial cultures from the nine most active strains against *Ae. aegypti* larvae (Fig. 1 and Table 1), measured as larvae mortality, was generally comparable to strain AM65-52 cultures. For most assays, the estimated lethal concentration values and their 95% confidence intervals for the nine strains were not significantly different ($p > 0.05$) from those of strain AM65-52 cultures. However, there was a significant difference ($p < 0.00045$) between the LC_{50} values obtained for P18 (123 μl, SE: 2.4 ± 0.15) and strain AM65-52 (182 μl, SE: 2.2 ± 0.2) at 48 h, when P18 was more efficient. The estimated LC_{90} values for all tested strains were not statistically different from strain AM65-52 at 24 h of exposure. In contrast, statistically significant differences in LC_{90} were found at 48 h (L41 and L47) and 72 h for L41 (356 μl, SE: 4.5 ± 0.3), P12 (418 μl, SE: 2.9 ± 0.5), and L47 (389 μl, SE: 9.1 ± 0.8) compared to strain AM65-52 (261 μl; SE: 4.5 ± 0.3) (Table 2).

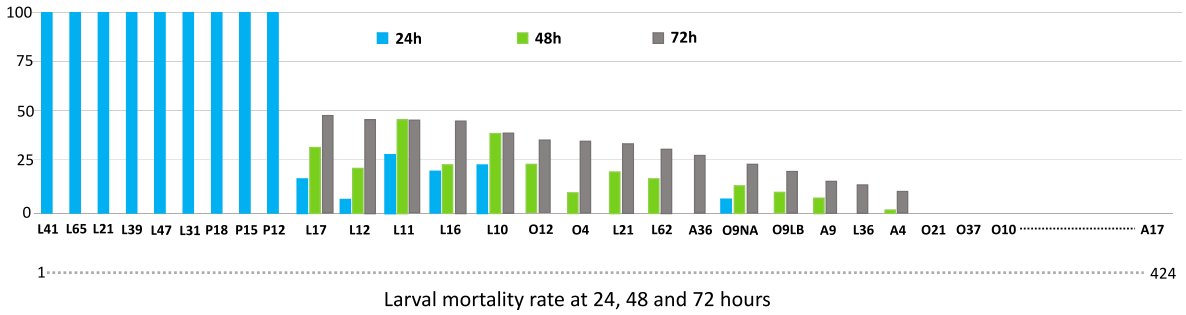

Fig. 1. Mortality of third instar *Ae. aegypti* larvae exposed to isolated bacterial strains. Mortality was assessed after 24, 48, and 72 h of exposure to bacteria. Among the bacterial strains isolated from *Ae. aegypti*, nine led to 100% mortality within 24 h, while fifteen other strains caused mortality below 50%. The remaining 415 strains showed no mortality during the evaluation period. All tests were performed in triplicate, and detailed results are available in Table S1.

Table 2
LC_{50} and LC_{90} values of bacterial cultures against *Ae. aegypti* larvae. Values expressed as microliters of 72 h bacterial culture per assay. The assays were carried out in five replicates, each containing 150 ml of water, 20 third-instar larvae and 1000, 900, 800, 750, 700, 650, 600, 550, 500, 400, 350, 300, 250, 200, 125, 100, 75 or 50 µl bacterial cultures. Dead larvae were counted at 24, 48, and 72 h after exposure to bacteria. LC_{50} and LC_{90} were evaluated using Probit, with $p \leq 0.05$. Statistical comparisons and confidence intervals (95 % CI) were analyzed using the Lilliefors normality test (K samples), analysis of variance (ANOVA), Tukey's multiple comparison test ($p \leq 0.05$) and Student *t*-test. For all variables in each column with the same letter (a.b.c), the differences between values are not statistically significant. LC = lethal concentration; CI = confidence interval; $\chi 2$ = chi-square; df = degrees of freedom; strain AM65-52 is a positive control.

Interval	Strain	LC_{50} (CI 95 %)	$\chi 2$	df	Slope ± SE	LC_{90} (CI 95 %)	$\chi 2$	df	Slope ± SE
24 h	Strain AM65-52	250 (165–346) [a]	73.9	4	3.2 ± 0.18	528 (452–645) [a]	9.7	3	4.2 ± 0.38
	L65 - *Bacillus* sp.	–	–	–	20.4 ± 1.11	944 (888—1087) [a]	6.7	2	20.4 ± 1.11
	L21 - *Stenotrophomonas sp.*	381(359–399) [a]	11.5	4	10.3 ± 0.48	507 (483–542) [a]	10.3	4	10.3 ± 0.48
	L31 - *Stenotrophomonas* sp.	527 (320–607) [a]	0.9	5	1.0 ± 0.33	6776 (2933–3372) [a]	1.6	7	1.2 ± 0.26
	L39 - *Enterobacter sp.*	396 (361–425) [a]	3.9	8	2.9 ± 0.22	1078 (999–1193) [a]	3.9	8	2.9 ± 0.22
	L41 - *Enterobacter* sp.	397 (362–424) [a]	2.0	5	2.5 ± 0.27	1104 (1001–1001) [a]	0.8	4	3.2 ± 0.54
	P12 - *Bacillus* sp.	398 (301–449) [a]	9.8	4	2.7 ± 0.32	709 (668–818) [a]	10.4	3	10.2 ± 0.84
	P15 - *Enterobacter* sp.	274 (249–294) [a]	2.5	5	2.5 ± 0.22	701 (659–785) [a]	4.5	4	4.8 ± 0.61
	P18 - *Enterobacter sp.*	232 (213–248) [a]	2.1	4	2.6 ± 0.24	582 (517–725) [a]	9.0	4	3.8 ± 0.32
	L47 - *Enterobacter sp*	482 (346–522) [a]	4.5	2	9.4 ± 0.97	647 (620–682) [a]	9.5	4	10.1 ± 0.87
48 h	Strain AM65-52	182 (166–195) [a]	1.7	5	2.2 ± 0.16	505 (435–604) [a]	9.5	3	4.6 ± 0.40
	L65 - *Bacillus* sp.	–	–	–	–	863 (831–946) [a]	5.5	2	25.4 ± 1.71
	L21 - *Stenotrophomonas sp.*	353 (345–361) [a]	0.1	1	9.3 ± 0.87	–	–	–	–
	L31 - *Stenotrophomonas sp*	452 (181–556) [a]	0.3	3	1.0 ± 0.36	–	–	–	–
	L39 - *Enterobacter sp.*	373 (339–402) [a]	2.8	8	3.1 ± 0.23	955 (897–1036) [a]	2.8	8	3.1 ± 0.23
	L41 - *Enterobacter sp.*	337 (305–362) [a]	2.0	4	2.3 ± 0.28	856 (809–929) [b]	8.2	6	4.8 ± 0.40
	P12 - *Bacillus sp.*	301 (286–314) [a]	0.7	4	3.7 ± 0.27	650 (593–758) [a]	5.3	4	3.9 ± 0.36
	P15 - *Enterobacter sp.*	237 (220–252) [a, b]	3.5	7	2.7 ± 0.16	659 (591–854) [a]	7.5	4	3.3 ± 0.44
	P18 - *Enterobacter sp.*	123 (112–132) [b]	1.1	2	2.4 ± 0.24	372 (314–500) [a]	4.1	3	2.7 ± 0.23
	L47 - *Enterobacter sp*	353 (330–372) [a]	6.0	6	6.0 ± 0.34	574 (544–605) [b]	8.1	4	8.5 ± 0.84
72 h	Strain AM65-52	–	–	–	–	293 (261–343) [a]	14.5	4	4.6 ± 0.39
	L65 - *Bacillus* sp.	–	–	–	–	818 (798–866) [a]	2.8	2	31.9 ± 3.00
	L21 - *Stenotrophomonas sp.*	331 (300–354) [a]	7.4	2	12.7 ± 0.76	418 (385–511) [a]	7.4	2	12.7 ± 0.76
	L31 - *Stenotrophomonas* sp.	490 (268–572) [a]	24.5	6	2.5 ± 0.33	1032 (864–2230) [a]	85.0	6	5.0 ± 0.36
	L39 - *Enterobacter* sp.	361 (318–395) [a]	19.2	8	3.8 ± 0.22	789 (728–882) [a]	19.2	8	3.8 ± 0.22
	L41 - *Enterobacter sp.*	–	–	–	–	356 (301–391) [c]	3.7	4	2.9 ± 0.53
	P12 - *Bacillus sp.*	199 (126–254) [b]	10.0	3	1.9 ± 0.21	485 (418–690) [b]	8.6	3	4.4 ± 0.37
	P15 - *Enterobacter sp.*	178 (152–199) [b]	5.5	4	2.4 ± 0.19	–	–	–	–
	P18 - *Enterobacter sp.*	101 (93–112) [b]	1.8	2	2.2 ± 0.26	–	–	–	–
	L47 - *Enterobacter* sp.	175 (132–206) [b]	9.4	4	2.0 ± 0.18	428 (389–545) [b]	6.0	2	902 ± 0.83

3.3.2. *Bacterial metabolites*

Metabolic extracts from strains P18, L47, and L65 were tested for their efficacy in killing larvae of *Ae. aegypti*. The LC_{50} and LC_{90} values observed for P18 were not statistically different ($p < 0.05$) at 24 h and 48 h intervals when compared with the insecticide temephos (Table 3). The extracts from the L65 and L47 cultures were in general less effective, with values of LC_{50} and LC_{90} higher than those of the temephos or P18 extracts (Table 3).

4. Discussion

Arbovirus transmission patterns are influenced by multiple factors, including vectorial capacity (Valderrama et al., 2017). Thus, the control of *Ae. aegypti* is necessary to reduce the mortality caused by dengue, urban yellow fever, Zika, chikungunya viruses, and other human pathogens. The efficacy of chemical insecticides used for this purpose are compromised by the development of resistance in mosquitoes, prompting the search for new insecticides, especially biological ones (Amelia-Yap et al., 2018).

Table 3
LC_{50} and LC_{90} values of bacterial metabolites against *Ae. aegypti* larvae. LC_{50} and LC_{90} were evaluated using Probit and statistical analyzes were conducted as described in Table 2.

Interval	Extracts	LC_{50} µg/ml (CI 95 %)	$\chi 2$	df	Slope ± SE	LC_{90} µg/ml (CI 95 %)	$\chi 2$	df	Slope ± SE
	Temephos	42 (21–60) [a]	10.5	3	2.0 ± 0.14	184 (134–329) [a]	10.5	3	2.0 ± 0.14
24 h	P18 - *Enterobacter sp.*	41 (29–52) [a]	13.4	4	1.8 ± 0.10	199 (148 – 310) [a]	13.7	4	1.7 ± 0.10
	L65 - *Bacillus* sp.	–	–	–	–	–	–	–	–
	L47 - *Enterobacter* sp.	–	–	–	–	–	–	–	–
	Temephos	30 (14–42) [a]	6.3	3	2.4 ± 0.22	102 (81–144) [a]	6.3	3	2.4 ± 0.22
48 h	P18 - *Enterobacter sp.*	27 (16–36) [ab]	10.4	3	2.2 ± 0.14	103 (77–175) [a]	10.4	3	2.2 ± 0.14
	L65 - *Bacillus* sp.	177 (124–268) [b]	5.0	3	0.9 ± 0.09	–	–	–	–
	L47 - *Enterobacter* sp.	439 (271–1423) [ab]	14.5	3	1.3 ± 0.10	–	–	–	–
	Temephos	–	–	–	–	–	–	–	–
72 h	P18 - *Enterobacter sp.*	–	–	–	–	–	–	–	–
	L65 - *Bacillus* sp.	71 (13–125) [a]	31.9	3	1.5 ± 0.10	499 (243 – 1504)	31.9	3	1.5 ± 0.10
	L47 - *Enterobacter* sp.	191 (142–272) [a]	12.7	3	1.8 ± 0.10	–	–	–	–

In the last decade, there has been increased interest in research on insect microbiota. Bacteria associated with insect disease vectors have drawn special attention for their interactions with both insect hosts and the pathogenic organisms they transmit (Thongsripong et al., 2018, Da Silva Gonçalves et al., 2019, Caragata and Short, 2022). The complexity of the mosquito bacterial microbiota has been actively investigated and bacterial genera already detected in these insects include *Asaia, Bacillus, Enterobacter, Pseudomonas, Serratia, Proteobacteria* (Minard et al., 2015, Wang et al., 2018, Scolari et al., 2021). Studies on the diversity of cultivable bacteria associated with *Ae. aegypti* in the Brazilian Amazon are still scarce. Here, we explore the cultivable bacterial microbiota of the *Ae. aegypti* and we identified new entomopathogenic bacterial strains with activity against larvae of this same mosquito species.

Four hundred twenty-four bacterial strains isolated from *Ae. aegypti* eggs, larvae, pupae or adult females were used in this work. Nine were found to be pathogenic for larvae of this same mosquito, with a lethality of 100% within 24 h of exposure, under the experimental conditions described here. After sequencing the 16S rRNA, it became evident that these strains fall into the genera *Enterobacter* (five isolates), *Bacillus* (two isolates), and *Stenotrophomonas* (two isolates).

Species of *Enterobacter* are ubiquitously found in terrestrial and aquatic environments and as commensals in the intestinal tracts of humans and animals; however, they can cause opportunistic infections and become pathogens (Davin-Regli et al., 2019). Likewise, *Enterobacter spp*. have been found as inhabitants of the gut of many insect species, contributing to nutrition, protection from parasites and pathogens, modulation of immune responses, and communication (Engel and Moran, 2013, Oliver and Martinez, 2014) but, as evidenced in this and other works (Stathopoulou et al., 2021) *Enterobacter* strains may exhibit insecticidal activity against their hosts. Harikrishnan et al., (2023) described a strain of *Enterobacter cloacae* with larvicidal activity against *Culex quinquefasciatus*. The active metabolites identified in that study were rhamnolipid biosurfactants. Yoshida et al., (2001) identified an insecticidal molecule produced by *Enterobacter aerogenes* found in the saliva of *Myrmeleon bore* larvae.

In general, *Enterobacter* strains P18 and P15 had the lowest values of LC_{50} and LC_{90}, similar to those of strain AM65-52. Additional research is necessary to determine how strains isolated in our work cause the death of *Ae. aegypti* larvae. It is worth mentioning that to date there have been no reports on the biological activities of metabolic extracts from *Enterobacter* sp. strains against *Ae. aegypti* larvae.

Stenotrophomonas sp. has been found in association with many insects, including *Ae. aegypti* (Yadav et al., 2015). It was determined that some isolated strains of *Stenotrophomonas maltophilia* produce a rhamnolipid biosurfactant and a chitinase that have been investigated as potential biocontrol agents of insect pests (Deepali et al., 2014, Jabeen et al., 2018). Possibly, similar molecules produced by the strains isolated in this study are responsible for the observed larvicidal activity. The *Stenotrophomonas* strain L21 revealed to be more effective in killing mosquito larvae than *Stenotrophomonas* L31, indicating these two strains produce different quantities of larvicidal metabolites or have distinct mechanisms of pathogenicity.

The genus *Bacillus* contains several species of bacteria with entomopathogenic activity against insects of multiple orders, including Diptera, Lepidoptera, and Coleoptera (Lone et al., 2017, Falqueto et al., 2021). Products based on the *B. thuringiensis* subsp. *israelensis* (Bti) have been successfully applied around the world for at least three decades (Merritt et al., 1989, Regis et al., 2000, Dambach et al., 2020). These products, including the positive control used in this study, strain AM65-52, predominate in bioinsecticide markets worldwide, being ecologically safe based and specific to target organisms. In this study we identified two *Bacillus sp*. isolates (L65 and P12) with activity against *Ae. aegypti*.

The exploration and isolation of these microorganisms to control mosquito larvae can provide new products and active metabolites against disease vectors (Katak et al., 2023). Data obtained from the larvicidal activity of metabolic extracts from P18, L47 and L65 cultures showed efficacy against *Ae. aegypti* larvae. Although we have not investigated the mechanisms and molecules involved in the entomopathogenic capacity of the bacteria isolated and tested in this work, we hypothesize that the observed larvicidal activity is associated with the synthesis of toxic molecules during *in vitro* culture.

5. Conclusion

Nine bacterial strains isolated from *Ae. aegypti* showed larvicidal activity similar to that of the strain AM65-52 from commercially available VectoBac® WG product, against this vector mosquito. The data obtained are relevant for the development of new bacterial larvicides for use in vector control programs. The bacteria from strain P18, as well as their extracted metabolites, showed to be consistently active as larvicides, with activity comparable to strain AM65-52 and Temephos, respectively. More studies are needed to elucidate the mechanisms involved in the observed larval mortality.

CRediT authorship contribution statement

Juan Campos de Oliveira: Conceptualization, Data curation, Investigation, Methodology. **Ricardo de Melo Katak:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – original draft. **Veranilce Alves Muniz:** Investigation, Methodology. **Marta Rodrigues de Oliveira:** Data curation, Methodology, Supervision. **Elerson Matos Rocha:** Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **William Ribeiro da Silva:** Investigation, Methodology. **Edson Júnior do Carmo:** Methodology, Validation. **Rosemary Aparecida Roque:** Conceptualization, Formal analysis, Funding acquisition, Supervision. **Osvaldo Marinotti:** Formal analysis, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Olle Terenius:** Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Spartaco Astolfi-Filho:** Supervision, Validation, Writing – original draft.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108094.

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