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Screening for new *Bacillus thuringiensis* (Caryophanales: Bacillaceae) strains effective against *Aedes aegypti* larvae (Diptera: Culicidae)

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ABSTRACT

Arboviruses transmitted by *Aedes aegypti*, such as dengue, Zika, and chikungunya, pose significant public health threats. The biological agent *Bacillus thuringiensis* (Bt) has been explored as an effective, safe, and economically sustainable method to reduce mosquito populations. This study aimed to identify Bt strains with potent larvicidal activity against *Ae. aegypti*, contributing to the development of effective bioinsecticides. We evaluated 13 Bt strains from the Embrapa Maize and Sorghum collection against *Ae. aegypti* larvae. Five replicates of each strain were prepared in 200 mL containers, each containing 18 mL of autoclaved distilled water, 2 mL of bacterial suspension (10^8 spores/mL), and 20 third-instar *Ae. aegypti* larvae. Mortality rates were monitored by counting live and dead larvae at 1, 24, 48 and 72 hours. PCR characterization was used to identify genes associated with toxicity for lepidopteran and dipteran pests in three strains. The bioassays confirmed the toxicity of the larvae, with strains 1644 and 1608A causing 100% and 95% mortality, respectively, within 24 hours. Strain 1656 showed 85% mortality up to 72 hours. Larvae exposed to Bt suspensions of strains 1644, 1608A, and 1656 lost agility, their tegument lost their shine, and the color became opaque and eventually darkened after death. Characterization of strain 1656 revealed the presence of *cry* and *vip* genes, which are associated with toxic activity in dipterans. The high toxicity of Bt strains 1644, 1608A, and 1656 underscores their potential as biocontrol agents against *Ae. aegypti* larvae.

Introduction

The *Aedes aegypti* mosquito (Linnaeus, 1762) (Diptera: Culicidae) has great epidemiological importance, contributing to the rise of various emerging and re-emerging infectious diseases globally (Dunford et al., 2016). Native to Africa, this species has established itself in tropical and subtropical regions worldwide due to its highly invasive behavior and close relationship with humans (Walker et al., 2018).

Over the years, the World Health Organization (WHO) has consistently highlighted the global increase in mosquito-borne infections. Notable diseases include dengue, Zika, chikungunya, and malaria, which are directly linked to the emergence and resurgence of invasive mosquito species. These species have become increasingly adapted to urban environments and are highly effective at spreading pathogens (Tabachnick, 2016). Despite significant efforts by vector control programs, these arthropods continue to pose a major threat to public health (Achee et al., 2019).

In Brazil, the arboviruses with the greatest impact on the public health system are the dengue, chikungunya, and Zika viruses. The co-endemicity of multiple arboviruses complicates clinical-epidemiological diagnosis and clinical management, as these diseases are directly influenced by the urbanization process and the territorial expansion of the vector (Wilke et al., 2017; Arduino et al., 2020; Magalhães et al., 2020). These arboviruses cause high rates of morbidity and mortality, significantly burdening the health system's economy by billions of dollars every year (Yang et al., 2020). Although the global distribution of dengue transmission is uncertain, it is estimated that at least 30% (up to 54%) of the world's population lives in areas at risk of dengue infection (WHO, 2009; Brady et al., 2012).

In an attempt to reduce the incidence of these diseases, vector control is essential. Effective strategies include chemical and mechanical control methods, which, when combined with social mobilization initiatives and environmental protection laws, help reduce *Ae. aegypti* infestations. The ideal strategy is to eliminate the vector in its immature

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stages (egg, larva, and pupa) and, consequently, its breeding sites. This approach is more effective because once *Ae. aegypti* reaches the adult stage, it can disperse across various environments (Campos et al., 2020), making control efforts impractical and potentially causing environmental damage.

The use of synthetic chemical compounds to reduce the population of mosquito vectors remains the most effective method for reducing the number of disease cases in more populous and poorer areas. However, the indiscriminate application of these synthetic insecticides (such as organochlorines, organophosphates, carbamates, and pyrethroids) can cause adverse effects on the health of humans and ecosystems, leading to their bioaccumulation in food, water, soil, and other environmental components. Another crucial element of the interaction between synthetic chemical insecticides and mosquito vectors is the emergence and resurgence of resistant populations caused by the intense and regular use of a single insecticide (Mossa et al., 2018; Badr, 2020). Previous studies have shown that resistance to insecticides like temephos and deltamethrin in larvae of *Ae. aegypti*, *Anopheles stephensi* Liston, 1901, and *Culex quinquefasciatus* Say, 1823 is associated with the overexpression of cytochrome P450 and esterase family genes (Vivekanandhan et al., 2021; Ramkumar et al., 2023).

The urgent development of new active ingredients is essential to ensure continued safety, cost-effectiveness, and sustainability of mosquito and vector control. In this context, biological control has emerged as a promising alternative, using predators or pathogens to reduce vector populations (Bellows and Fisher, 1999; Benelli et al., 2016). Effective predators include fish species that consume mosquito larvae and pupae, various aquatic invertebrates that feed on the immature stages of mosquitoes, and carnivorous plants capable of capturing and digesting mosquito larvae (Couret et al., 2020; Ranathunge et al., 2021). Pathogens, such as entomopathogenic bacteria and fungi, release lethal toxins to mosquitoes, thus offering an effective control strategy (Samuels et al., 2016).

Given the serious impacts that chemical pesticides can cause, the biological control of *Ae. aegypti* larvae using the entomopathogenic bacterium *B. thuringiensis* (Bt) has gained prominence among the various tactics for integrated pest and insect vector management. During its sporulation phase, this bacterium produces protein inclusions known as δ-endotoxins, as well as other virulence factors such as β-exotoxins, hemolysins, enterotoxins, chitinases, and phospholipases (Bravo et al., 2005). In the vegetative growth phase, Vegetative insecticidal proteins (Vip) are produced and secreted into the culture medium as soluble proteins (Estruch et al., 1996). Some strains are also capable of producing secreted insecticidal proteins (Sip), as well as biostimulatory molecules, biofertilizers, and parasporin proteins that exhibit specific cytotoxicity against human cancer cells (Raddadi et al., 2007; Okumura et al., 2011; Santos et al., 2022).

In addition to *B. thuringiensis* var. *thuringiensis* (Bti), other Bt strains have a highly effective gene profile against mosquito larvae of significant health importance (Valtierra-de-Luis et al., 2020). The proteins produced by Bt strains are highly specific to target insects and are synthesized as protoxins. After ingestion by the insect, these protoxins are solubilized in the alkaline environment of the midgut and cleaved by the insect's proteases, activating them (Bravo et al., 2011). The active toxin then binds to specific receptors in the microvilli of the insect's midgut, causing the intestinal cells to rupture and consequently paralyzing the digestive system, leading to death by starvation and septicemia (Glare and O'Callaghan, 2000; Raymond et al., 2010).

In Brazil, Embrapa Maize and Sorghum has Bt strains that exhibit a wide range of mortality rates, from 0% to 100%, against the fall armyworm *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae). This study aimed to identify and select strains from this Bt bank that are toxic to *Ae. aegypti* larvae.

Materials and methods

This study was conducted at the Biological Control Laboratory of Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais (MG), in partnership with the Oswaldo Cruz Institute.

Aedes aegypti *eggs*

We used *Ae. aegypti* eggs provided by the Vector Insect Laboratory of the University of Vassouras, Rio de Janeiro (RJ). Filter papers containing the *Ae. aegypti* eggs were placed in plastic containers (44 cm x 30 cm x 8 cm) filled with distilled water preheated to 28 °C to stimulate larvae hatching. These containers were kept in a Biological Oxygen Demand (B.O.D.) incubator set at 28 ± 0.5 °C, 70 ± 10% RH, and a 12:12 light/dark photoperiod until the larvae reached the stage for use in the bioassays.

Preparation of bacterial suspensions

Bt strains (1120E, 1132C, 1132E, 1136B, 1145B, 1145C, 1148F, 1168C, 1608A, 1641, 1644, 1656, and S462A) were previously collected from soil samples and grain dust at different locations in Brazil, which now are part of the Embrapa Maize and Sorghum Microorganism Bank. Each strain was grown in a Petri dish containing commercial Luria Bertani (LB) medium enriched with mineral salts (0.002g FeSO4, 0.02g ZnSO4, 0.02g MnSO4, and 0.3g MgSO4) at 29 °C for 72 hours in a bacteriological oven to ensure the sporulation process and release of crystals. The bacterial content was then collected, transferred to Falcon tubes containing autoclaved deionized water, and diluted with a 0.05% Tween-20 emulsifier. Spores were counted using a Neubauer chamber under a phase-contrast optical microscope.

Selective bioassays against Aedes aegypti

Groups of 20 third-stage larvae were placed in 200 mL disposable cups, each containing 2 mL of each bacterial suspension (10^8 spores/mL) in 18 mL of autoclaved deionized water. Each treatment was carried out using five replicates (R1, R2, R3, R4, and R5) adapted from WHO (2005), was carried out in three repetitions. A strain of *B. thuringiensis* var. *thuringiensis israelensis* registered as BtiJAB from the Embrapa Maize and Sorghum Microorganism Bank was used as the positive control, while the negative control consisted of autoclaved deionized water without the bacterial suspension. The larvae were kept on their normal diet, based on brewer's yeast, at a rate of 0.3 mg per larva (Cabral et al., 2009). The treatments were kept in a B.O.D. incubator at 25 °C, 70 ± 10% RH, and a 12:12 light/dark photoperiod. Larval mortality rates were estimated after 1 hour, 24 hours, 48 hours, and 72 hours of exposure to the bacterial suspensions, according Lobo et al. (2018). The data were subjected to analysis of variance (ANOVA; $P \leq 0.05$), followed by factorial analysis using the Scott-Knott test ($p>0.05$), using the statistical software Sisvar Version 5.6.

Genomic DNA extraction and molecular characterization

Bt strains were grown in an LB culture medium at 29 ºC for 16 hours. After incubation, genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA), following the

manufacturer's protocol. The quality of the extracted DNA was assessed after running the samples on a 1% agarose gel electrophoresis, and the quantification was performed using a Nanodrop device (ND-1000 V3.1.2 – Spectrophotometer). The samples were diluted to a concentration of 10 ng/µL and stored at -20°C (Shuhaimi et al., 2001). PCR reactions were carried out using specific primers to detect the presence of *cry*, *cyt*, and *vip* genes (Table 1). The reactions consisted of 10 ng of DNA, 0.5 µM of each primer, 5 µM of each dNTP, 1x buffer solution, 2 mM MgCl2, and 2 U of Taq polymerase (KAPA Biosystems, USA) in a total volume of 10 µL. The amplifications were performed in a Veriti® 96-Well Thermal Cycler under the following conditions: 94 ºC for 5 minutes; 35 cycles of 95 ºC for one minute, annealing at the temperature specific to each primer, and 72 ºC for one minute; final extension at 72 ºC for 10 minutes.

The PCR products were subjected to electrophoresis on a 1% agarose gel. A 1kb plus molecular weight marker (Invitrogen,USA) was used on the gel for band comparison.

Results

Pathogenicity of Bacillus thuringiensis

Among the 13 Bt strains tested, 1644, 1608A, and 1656 resulted in a mortality rate of over 70% for *Ae. aegypti* larvae. A Bti strain was used as the positive control in this study due to its proven efficacy against Culicidae of medical importance and its presence in various commercial products for mosquito control (Davidson and Sweeney, 1983; Mohammad, 2022). Consequently, the BtiJAB strain caused 100% mortality in *Ae. aegypti* larvae. Similarly, strain 1644 was capable of causing 100% mortality in immatures within 24 hours. Up to 72 hours, strains 1608A and 1656 killed 95 and 85% of the larvae, respectively. The other strains exhibited low efficiency in controlling the target insect, with average mortality ranging from 0 to 53% (Table 2).

During the evaluation, a distinct behavior was observed between the larvae in the control group and those treated with the BtiJAB strain (positive control) (Figures 1C and 1D), as well as strains 1644, 1608A, and 1656. The larvae contaminated by these Bt strains lost their agility, spending most of their time at the bottom of the plastic container without feeding, and rarely surfacing to breathe. The larvae slowed down their movements to a standstill, and their tegument changed color, becoming matte. The larvae became flaccid, and upon death, there was a darkening of the muscles and fatty body, indicating the onset of tissue deterioration. These changes may indicate the toxic effect caused by ingesting the spores and crystals of the Bt strains (Figures 1C, 1E, 1G).

This evaluation showed that the observed changes were dependent on the Bt strains tested. The *Ae. aegypti* larvae in the negative control group maintained a normal elongated and vermiform appearance, with a body visually divided into the head, thorax, and abdomen. The thorax was wider than the head and had tufts of bristles, which were also present on the abdomen (Figures 1A and 1B). According to Serra-Freire and Mello (2006), the general characters of the first body parts of *Ae. aegypti* larvae are globose, and the abdomen consists of 10 segments, with the eighth segment having a pair of spiracles located at the end of the siphon (tubular organ).

During exposure to the Bt strains, we observed that the *Ae. aegypti* larvae showed reduced agility, spending most of their time at the bottom of the container without feeding and rarely rising to the surface to breathe. Their movements gradually slowed to a complete stop, and their integument changed color, adopting a matte appearance. The larvae became flaccid, and, upon death, their muscles and fatty bodies darkened, initiating a process of tissue deterioration. This indicates the toxic effect resulting from ingesting the spores and crystals of the Bt strains (Figures 1E, 1F, 1G, 1H, 1I, 1J).

The main changes in larvae treated with Bt strains include a narrowing of the mesenteron, a decrease in body fat, thickening of the peritrophic membrane, leakage from the intestine, elongation of the cervix, and spacing of the anal papillae. The morphology of the anal papillae can affect the regulation of osmotic functions, influencing the survival of *Ae. aegypti* larvae (Chaithong et al., 2006). These observations are consistent

Table 1
Primers used for genomic DNA amplification of *Bacillus thuringiensis* strains.

Target genes	Primers sequences (5'-3')	Tm (°C)	Fragment size (bp)	Reference
*cry*1Aa	TGTAGAAGAGGAAGTCTATCCA	53	272	Ceron et al. (1995)
	TATCGGTTTCTGGGAAGTA			
cry 1Ab	CGCCACAGGACCTCTTAT	55	232	Valicente et al. (2010)
	TGCACAACCACCTGACCCA			
*cry*1B	CTTCATCACGATGGAGTAA	55	367	Ceron et al. (1994)
	CATAATTTGGTCGTTCTGTT			
*cry*1C	AAAGATCTGGAACACCTTT	58	130	Ceron et al. (1994)
	CAAACTCTAAATCCTTTCAC			
*cry*1D	ATATGGAGTGAATAGGGCG	55	235	Ceron et Al. (1995)
	TGAACGGCGATTACATGC			
*cry*2Ac	ACAGCAGTCGCTAGCCTTGT	55	475	Fagundes et al. (2022)*
	CAAATTGTGGATTGCCGTTA			
*cry*2Ad	ACGATATCGCCACCTTTGTC	53	282	Fagundes et al. (2022)*
	AGGTGTTCCTGAAGGGCTTT			
*cyt*1	CCTCAATCAACAGCAAGGGTTATT TGCAAACAGGACATTGTATGTGTAATT	52	477	Ibarra et al. (2003)
*cyt*2	ATTACAAATTGCAAATGGTATTCC TTTCAACATCCACAGTAATTTCAAATGC	50	356	Ibarra et al. (2003)
*vip*1	TTATTAGATAAACAACAACAAG AATATCAATCTATTMGNTGGATHGG	48	585	Hernández-Rodríguez et al. (2009)
	GATCTATATCTCTAGCTGCTTTTT CATAATCTSARTANGGRTC			
*vip*2	GATAAAGAAAAAGCAAAAG AATGGGRNAARRA	48	845	Hernández-Rodríguez and Ferré (2009)
	CCACACCATCTATATACAGT AATATTTTCTGGDATNGG			
*vip*3	TGCCACTGGTATCAARGA	48	1621	Hernández-Rodríguez and Ferré (2009)
	TCCTCCTGTATGATCTACAT ATGCATTYTTRTTRTT			

*Unpublished data

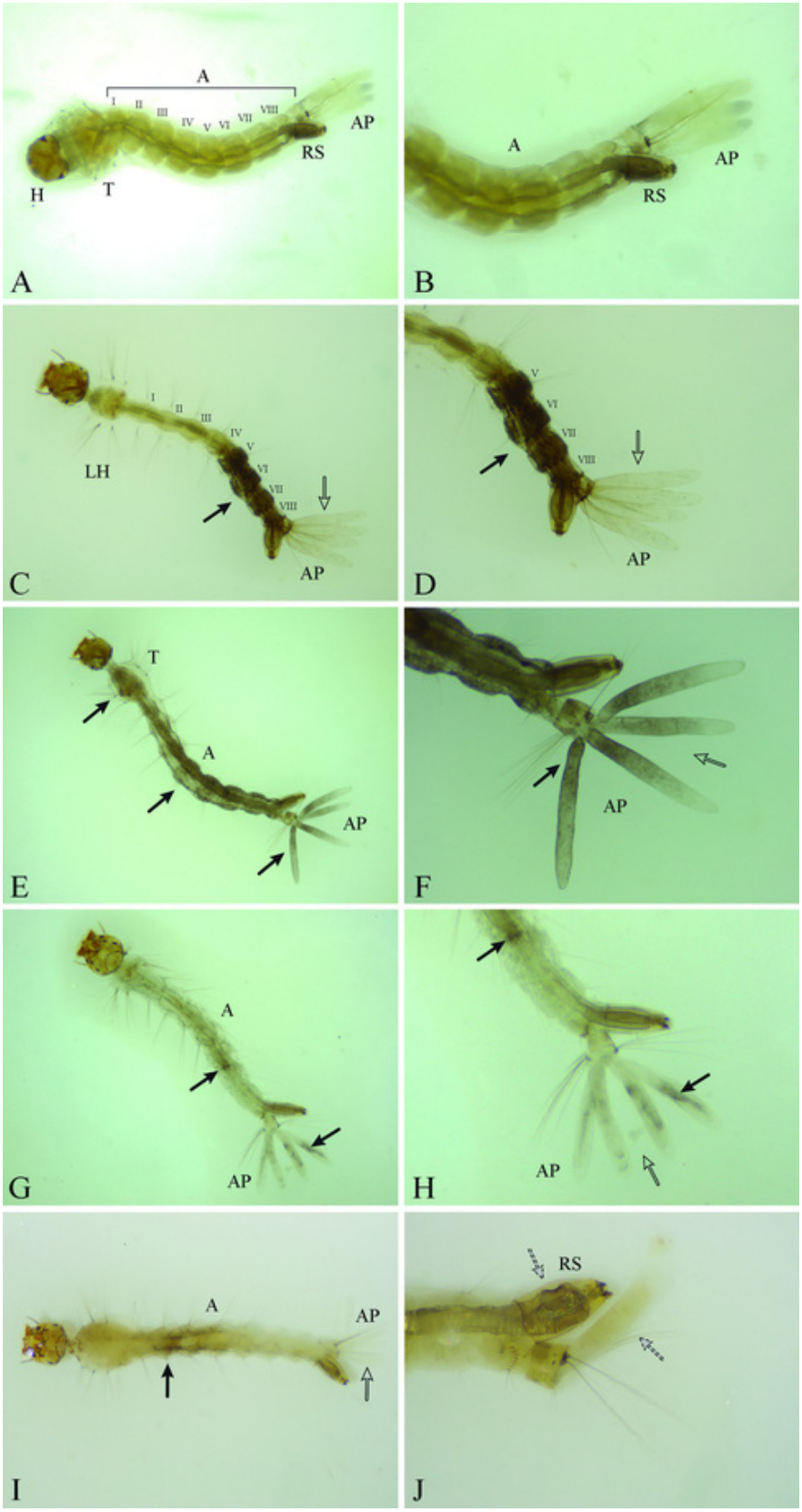

Figure 1. Light photomicrographs of the morphological aspects of 3rd instar Ae. aegypti larvae. Larva from the control treatment showing highlighted structures, with no alterations to the head, segments of the abdomen, respiratory siphon and anal papilla (A and B). Larva exposed to the standard BtiJAB strain with changes in the segments of the abdomen (dark pigmentation) (black arrow), loss of external hairs, and slight spacing in the anal papillae (hollow arrow) (C and D). Larva exposed to strain 1644, showing a strong darkening in the trunk region and along the extremities of the body, accentuating the darkening in the anal papillae (black arrows), the toxic effect of this strain also induced spacing of the anal papillae (hollow arrow) (E and F). Larva treated with strain 1608A showing darkening of the intestine and extremities of the body (black arrows), in addition to the translucent tracheal system and spacing of the anal papillae (G and H). Larva exposed to strain 1656 showing a damaged intestinal tract (black arrow), spacing of the anal papillae (hollow arrow), partial extrusion of the intestinal contents, a desiccated larva and the exuvia of the instar attached to the respiratory siphon region (dotted arrow) (I and J). Note: A, abdomen; H, head; T, thorax; RS, respiratory siphon; AP, anal papillae; LH, lateral hairs.

Table 2
Gene content of *Bacillus thuringiensis* strains and mortality percentage (%) of *Aedes aegypti* larvae after application of suspensions of 1.5 x 10^8 crystal spores/ml.

Treatments	Genes													Mortality (%)			
	cry cyt										*vip*			Time after application (h)			
	cry1Aa	*cry1Ab*	*cry*1B	*cry*1C	*cry*1G	*cry*2Aa	*cry*2Ac	*cry*2Ad	*cyt*1	*cyt*2	*vip*1	*vip*2	*vip*3	1	24	48	72
Water[A]	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0
*Bti*JAB[B]	-	-	-	-	-	-	-	-	-	-	-	-	-	100	100	100	100
1644* [C]	+	-	-	-	-	-	-	-	-	-	-	-	-	0	100	100	100
1608A* [C]	-	+	-	-	+	+	+	+	-	-	-	-	-	0	95	95	95
1656 [C]	+	-	-	-	-	+	-	-	-	-	+	-	+	0	45	30	10
S462A [C]	-	-	+	-	-	-	-	-	-	-	-	-	-	0	0	0	5
1132C [C]	-	-	-	-	-	-	-	+	-	-	+	-	+	0	5	0	15
1145B [C]	-	-	+	-	-	-	-	-	-	-	+	-	+	0	5	0	5
1148F [C]	-	-	-	-	-	-	-	-	-	-	-	-	+	0	5	0	0
1641* [C]	-	-	-	-	-	-	-	-	-	-	+	+	+	0	0	0	0
1138G [C]	-	-	+	-	-	+	-	-	-	-	+	-	+	0	0	0	0
1438 [C]	-	-	+	-	-	-	-	+	-	-	-	-	+	0	0	0	0
1136B [C]	-	-	-	-	-	-	+	-	-	-	+	-	+	0	0	0	0
1132E [C]	-	-	+	-	-	-	-	-	-	-	-	-	+	0	0	0	0

(+) presence of the gene; (-) absence of the gene. [A]Autoclaved distilled water used as a negative control. [B]Strain used as negative control. [C]Strains used in the selective bioassays.
*Data published: Valicente et al. (2010); Carvalho et al. (2020); Pinheiro and Valicente (2021).

with those reported by Fujiwara et al. (2017) and Lobato Rodrigues et al. (2021), who, in addition to observing lethargic movement of Culicidae larvae exposed to natural substances extracted from plants, noted the shortening and darkening of the abdomen and morphological changes in the anal papillae of *Ae. aegypti*.

Detection of cry, cyt, and vip genes

The PCR reactions revealed that the 1644 strain, which proved to be efficient in controlling *Ae. aegypti*, amplified *cry*1Aa, *cry*1B, and *cry*1C genes. Strain 1608A contained *cry*1Ab, *cry*1G, *cry*2Ab, *cry*2Ac, and *cry*2Ad genes, while strain 1656 showed amplifications for *cry*1A, *cry*1Aa, *cry*1Ac, *cry*2Aa, *cry*2Ad, and *vip*3 genes (Table 2).

Discussion

The crystalline Bt inclusions, especially the δ-endotoxins, are present in several commercial bioproducts that effectively target major noctuid pests in the agricultural sector worldwide. In the specific case of the entomopathogenic bacterium *B. thuringiensis* var. *israelensis*, its use is particularly notable for controlling mosquito vectors (Priest, 1992; Lacey, 2007). Given the broad spectrum of action of its proteins, its efficacy, and the specificity compared to chemical control methods, using *B. thuringiensis* as a biological agent is considered an excellent tool for the integrated management of insect vectors in public health.

Floore (2006), Benelli et al. (2016), and Hegazy et al. (2022) described the key points in the use of major control strategies against mosquitoes in recent decades, including microbial control, particularly when *Bacillus*-based products are used on the larval stages of the target insects. This relevance has driven the search for new strains with high toxic activity against certain species of the suborder Nematocera, which transmit arboviruses and/or cause diseases in animals and humans.

Xinmin Ma et al. (2023) and Fatima et al. (2023) have also demonstrated that selective bioassays using Bt suspensions containing spores and crystals are highly viable in laboratory conditions. Furthermore, integrating this methodology with other management tactics enhances the high efficacy of this biological agent, particularly against mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles*.

Habib and Andrade (1998) described the main symptoms of Bt infection in agriculturally important caterpillars, including appetite loss and food abandonment at the onset of bacteriosis. Affected caterpillars experience regurgitation and diarrhea, loss of shine in the integument, and matte coloration due to changes in the internal tissues and hemolymph. They also show a loss of agility and, in some cases, notable flaccidity in the body. After paralysis and death, the dead caterpillars' skin color can change from cream to black. This symptomatology in lepidopterans has guided and encouraged the use of the bacterium Bti to control vectors of major epidemiological importance, including aquatic dipteran larvae.

In susceptible Culicidae and Simuliidae larvae, the Bti crystals are also ingested in the form of protoxins, which, after being solubilized by proteases in the midgut, become activated (toxic) and interact with receptors present on the apical microvilli of the intestinal epithelium (Soberón et al., 2007; López_Molina et al., 2020). This binding results in the formation of pores in the cell membrane, disrupting the ionic balance of the tissue membrane and causing alterations and/or lesions in the intestinal epithelium as well as other organs and systems. This process ultimately leads to the insect's death (Ben-Dov, 2014; Silva-Filha et al., 2021).

In the present work, the occurrence of these characteristic symptoms was verified, along with the presence of deformities in the anal papillae, which is due to the insecticidal behavior of Bt strains in Culicidae and some plant extracts potentially used in dipteran control (Valotto et al., 2011; Soonwera et al., 2022). After 24 hours of treatment with strains 1644 and 1608A, external morphological anomalies in the anterior mesenteric region of the larvae showed a high degree of destruction, as noted by Viana et al. (2020) and Bibi et al. (2020) in *Aedes* larvae treated with natural plant substrates and red algae.

Generally, the symptoms reported in Diptera larvae infected by Bt are similar to those observed in Lepidoptera larvae, including persistent disturbances in the digestive system, such as regurgitation or diarrhea (Glare and O'Callaghan, 2000). This is followed by effects on the larval integument, with a gradual loss of coloration until it reaches a dark brown hue. Additionally, infected larvae can lose their agility and, in some cases, become unresponsive to touch stimuli, becoming flaccid and ultimately experiencing a total loss of movement.

Small variations in the stages of the mode of action can occur depending on the Bt infection and the susceptibility of the target insect

(Bravo et al., 2013; Pacheco et al., 2023). Therefore, the external changes observed in this study, especially in the anal papillae region, are crucial for understanding the characteristic symptoms of Bt infection in *Ae. aegypti*.

PCR-based gene profiling with specific primers for *cry*, *cyt*, and *vip* genes is widely used in characterization studies of Bt strains. This technique allows for a better understanding of the specific pathogenicity of Bt proteins against different insect orders (Bravo et al., 1998; Gu et al., 2021).

Even though the pesticidal Bti proteins, which include the Cry4, Cry10, and Cry11 family genes specific to mosquitoes, are not present (Fernández et al., 2005; López-Molina et al., 2021), strains 1644, 1608A, and 1656 still demonstrated insecticidal activity against *Ae. aegypti*. This spectrum of susceptibility is due to the presence of genes from the Cry1 (*cry*1A, *cry*1B, *cry*1C, *cry*1G) and Cry2 (cry2Aa, *cry*2Ab, *cry*2Ac and *cry*2Ad) families. These findings corroborate the distribution of pesticidal activities of Bt proteins affecting the main species of the orders Lepidoptera, Diptera, and Coleoptera, as reported by Van Frankenhuyzen (2009, 2013). When evaluating these same genes, the author found toxicity to 23 dipteran species, including *Ae. aegypti*, a vector of significant human diseases, and highlighted the existence of cross-activity, with the Cry1 family being pathogenic to both Lepidoptera and Diptera.

Similar to this study, the presence of the *cry*1 and *cry*2 genes was frequent, occurring in around 80% to 100% of the Bt isolates molecularly characterized by Praça et al. (2004), Monnerat et al. (2007), and Boonmee et al. (2019). Another commonly observed factor was the co-occurrence of these two crystalline proteins in the genetic profile of Bt strains effective against various insects (Ben Dov et al.,1997; Adedayo and Uthman, 2021).

The classification by Schnepf et al. (1998) and Crickmore et al. (1998, 2021) highlights significant progress in understanding the toxic effects of Bt Cry proteins on various target organisms, including nematodes and human cancer cells. The continued investigation of its insecticidal activity on other invertebrates of agricultural, veterinary, or medical-sanitary importance is crucial. Our data align with the current nomenclature and reaffirm the potential action of Cry proteins on the main arbovirus vector.

Gómez et al. (2002) and Ibrahim et al. (2010) showed that different parasporal crystals are composed of single or multiple Cry proteins. Investigations into protein-receptor interactions provide a wealth of information on the spectrum of action and specificity of δ-endotoxins (Cry and Cyt proteins) with target insects. This is evident in other studies that have demonstrated the high degree of affinity of the Cry1A protein to the surface receptors and ion channels of the mesentera of lepidopterans, coleopterans, and dipterans. Such affinity has consequently led to high mortality rates of these insects under both experimental laboratory and field conditions (Gómez et al., 2014; Jneid et al., 2022; Liu et al., 2022).

Of the three strains (1644, 1608A, and 1656) that were effective against the larvae of *Ae. aegypti*, only strain 1656 amplified the *vip*3 gene. This finding aligns with Wang et al. (2020), who provided the first evidence that the Vip3Aa protein is toxic to *Ae. aegypti.* Furthermore, this toxicity may be associated with the presence of the *cry*1 and *cry*2 genes. The correlation between the occurrence of the *cry*1, *cry*2, and *vip* genes was previously reported by Hernández-Rodríguez and Ferré (2009) and Hernández-Rodríguez et al. (2009) when identifying and classifying *cry* and *vip* genes in a collection of 507 Bt strains from Spain and Bolivia, respectively.

We found that the Cry1 and Cry2 Bt family genes present in strains 1644, 1608A, and 1656 exhibit joint insecticidal activity against insects of the orders Lepidoptera and Diptera, which may have contributed to the toxicological response observed in *Ae. aegypti* larvae.

The 1644 strain, which proved to be efficient in controlling *Ae. aegypti*, presented the *cry*1B, *cry*1C, *cry*1D, and *cry*1Fb genes in a comparative analysis of *cry* genes carried out by Valicente and Lana (2008). The authors found that this strain also effectively controlled the fall armyworm *S. frugiperda*, one of the primary pests of economically important crops such as corn, soybeans, sorghum, and cotton, among others.

In the study by Carvalho et al. (2020), strain 1608A was found to contain the *cry*1Ab, *cry*1G, *cry*2Ab, *cry*2Ac, and *cry*2Ad genes. The authors examined the pathogenicity of Bt strains against several key pests of soybean crops in Brazil, including *Chrysodeixis includens* Walker, 1858 (Lepidoptera: Noctuidae), *Spodoptera cosmioides* Walker,1858 (Lepidoptera: Noctuidae), *Spodoptera eridania* Cramer, 1782 (Lepidoptera: Noctuidae), and *S. frugiperda*. The findings from both Carvalho et al. (2020) and our research indicate that strains like 1644 and 1608A hold significant promise for controlling insect pests and vectors across different orders.

Similar to our study, Santos et al. (2012) and Wu et al. (2021) found different Cry proteins to be effective against *Ae. aegypti* larvae. This demonstrates the importance of also evaluating the potential of Bt strains tested on lepidopteran pests for use against culicids, as the proteins present in these Bt strains can be toxic to mosquito larvae.

Although most studies involving the selection of Bt strains for the control of *Ae. aegypti* highlight the pathogenicity of Bti against this vector, we did not perform a serological analysis of the subspecies of the Bt strains. This type of characterization does not consider the genes present in the Bt strains (Valicente, 2019). In the case of the BtiJAB strain, we are aware of its variety because it is a strain from the Embrapa Maize and Sorghum Microorganism Bank specifically used as a positive control in studies selecting strains against mosquitoes. Thus, we demonstrated that molecular characterization is an extremely useful tool for the integrated management of insect vectors and that strains 1644, 1608A, and 1656 presented toxic proteins to *Ae. aegypti* larvae and proved efficacious in bioassays.

In conclusion, the pathogenicity of the Bt strains was confirmed against third-stage *Ae. aegypti* larvae, with strains 1644, 1608A, and 1656 demonstrating high efficacy. Further studies are needed to explore these strains and their potential applications as public health tools to combat mosquitoes and mosquito-borne pathogens.

Conclusion

Bt is a highly effective tool for the biological control of insect pests and disease vectors. This study demonstrated the pathogenicity of Bt strains against third-stage *Ae. aegypti* larvae, with strains 1644, 1608A, and 1656 showing significant efficacy. These strains hold promise as potential sources for biopesticide formulations, offering a valuable solution to combat resistance issues in *Ae. aegypti* and other medically important Culicidae. Further research on these strains could enhance public health efforts to control mosquito-borne diseases.

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Data statement

The data that support the findings of this study are openly available in Mendeley Data at https://data.mendeley.com/datasets/vtcs5tmbp9/1. DOI: 10.17632/fscfhy6kdk.1.

Conflicts of interest

The authors declare no conflict of interest.

Author contribution statement

TAN conceptualization, investigation, methodology, software, formal analysis, data curation, writing - original draft, writing - review and editing.

ILSC methodology, investigation, formal analysis, data curation, writing - original draft and writing – review. KSC methodology, software, formal analysis, data curation and writing – review.

FHC validation, writing - review, supervision, project administration. MJPS investigation, data curation and writing – review. MMCQ conceptualization, writing - review, supervision, project administration and funding acquisition.

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Supplementary Material

The following online material is available for this article:

Figure S1 - PCR-amplified fragments for cry-specific genes from Bacillus thuringiensis strains. (A) cry1G gene and its positive control (C+): 1657 strain, (B) cry2Aa gene and its positive control (C+): 344 strain, C-: Negative control (sterile water). (C) cry2Ac gene and its positive control (C+): 1657 strain, C-: Negative control (sterile water). (D) cry2Ad gene and its positive control (C+): 1657 strain, C-: Negative control (sterile water). MM: Molecular marker 1Kb plus (Invitrogen, Tech-line, USA). NA: Samples not applicable to this work. The white arrows indicate the size of the amplified fragment.

Figure S2 - PCR-amplified fragments for the cyt genes of Bacillus thuringiensis strains efficient against Aedes aegypti. (A) cyt1 gene and its positive control (C+): Strain T14, (B) cyt2 gene and its positive control (C+): Strain T14, C-: Negative control (sterile water); M: 1Kb plus molecular marker (Invitrogen, Tech-line, USA). NA: samples not applicable to the present work. The white arrows indicate the size of the amplified fragment.

Figure S3 - PCR-amplified fragments for vip genes from Bacillus thuringiensis strains efficient against Aedes aegypti.(A) vip1 gene and its positive control (C+): HD-125 strain,(B) vip2 gene and its positive control (C+): 1657 strain, (C) vip3 gene and its positive control (C+): 1657 strain, C-: Negative control (sterile water), MM: Molecular marker 1Kb plus (Invitrogen, Tech-line, USA). NA: Samples not applicable to this work. The red arrows indicate the amplification products of the Bt strains, and the white arrows indicate the size of the amplified fragment.

Figure S4 - Larvicidal effect of Bacillus thuringiensis (Bt) 1644 strain on Aedes aegypti larvae: comparison between control group larvae and those exposed to the bacterial suspension of 1644 strain. The graphical abstract illustrates the spore-crystal complex of the 1644 Bt strain, as well as the external symptoms observed in the respiratory siphon and anal papillae of treated larvae, including a gradual loss of coloration until reaching a dark brown hue, in addition to the spacing observed in the anal papillae.