Research Article

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Natural products from *Xenorhabdus* and *Photorhabdus* show promise as biolarvicides against *Aedes albopictus*

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Abstract

BACKGROUND: In the perpetual struggle to manage mosquito populations, there has been increasing demand for the development of biopesticides to supplant/complement current products. The insecticidal potential of *Xenorhabdus* and *Photorhabdus* has long been recognized and is of interest for the control of important mosquitoes like *Aedes albopictus* which vectors over 20 different arboviruses of global public health concern.

RESULTS: The larvicidal effects of cell-free supernatants, cell growth cultures and cell mass of an extensive list of *Xenorhabdus* and *Photorhabdus spp.* was investigated. They were quite effective against *Ae. albopictus* causing larval mortality ranging between 52–100%. Three *Photorhabdus spp.* and 13 *Xenorhabdus spp.* release larvicidal compounds in cell-free supernatants. Cell growth culture of all tested species exhibited larvicidal activity, except for *Xenorhabdus sp.* TS4. Twenty-one *Xenorhabdus* and *Photorhabdus* bacterial cells (pellet) exhibited oral toxicity (59–91%) against exposed larvae. The effect of bacterial supernatants on the mosquito eggs were also assessed. Bacterial supernatants inhibited the hatching of mosquito eggs; when unhatched eggs were transferred to clean water, they all hatched. Using the easyPACId approach, the larvicidal compounds in bacterial supernatant were identified as fabclavine from *X. szentirmaii* and xencoumacin from *X. nematophila* (causing 98 and 70% mortality, respectively, after 48 h). *Xenorhabdus cabanillasii* and *X. hominickii* fabclavines were as effective as commercial *Bacillus thuringiensis* subsp. *israelensis* and spinosad products within 5 days post-application (dpa).

CONCLUSION: Fabclavine and xenocoumacin can be developed into novel biolarvicides, can be used as a model to synthesize other compounds or/and can be combined with other commercial biolarvicides.

Keywords: *Aedes albopictus*; larvicidal; *Xenorhabdus*; *Photorhabdus*; fabclavine; xenocoumacin

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1 INTRODUCTION

Arboviral infections are emerging at an unprecedented rate, infecting and killing thousands throughout the world. Numerous hematophagous arthropods such as mosquitoes, midges, ticks and sandflies transmit a majority of these infections.[1] *Aedes albopictus* (the Asian tiger mosquito) is an aggressive daytime-biting mosquito species that can vector over 20 different arboviruses of major global public health concern.[2–5] They include yellow fever, which leads to kidney and liver failure, and jaundice; Dengue Fever, which can give patients a characteristic skin rash;[6] Zika virus, which can cause birth defects like microcephaly during pregnancy; and chikungunya virus, which can leave victims with debilitating joint pains.[1,7,8] Among these diseases, Dengue has had the greatest impact with a 4-fold increase in incidence over the last 30 years. Annually, approximately 100 million infections, half a million cases of dengue hemorrhagic fever, and at least 40 000 deaths are reported in more than 100 resource-poor countries with most cases occurring in children aged 15 and under.[9] *Aedes albopictus* can also transmit filarial nematodes in the genera *Dirofilaria* and *Serratia* that affect domestic animals such as dogs.[3,10]

Aedes albopictus, originally native to tropical and sub-tropical regions of Asia, is spreading and is now widely distributed in at least 30 countries throughout the tropics, subtropics, and temperate regions of the world outside Asia.[4,5,11,12] This expansion has been significantly facilitated by the transport of its drought-resistant eggs in bamboo plants, used tires, and artificial containers during global trade and shipping activities, its tolerance of cold temperatures up to −10 °C in temperate regions in northern latitudes and its opportunistic feeding behavior on a wider host range including man, domestic and wild animals.[13–15]

There has been a perpetual struggle to manage mosquito populations to thresholds that impede transmission down through the ages. The main mosquito control method at present involves either killing adult and/or juvenile stages with pesticides (adulticides and larvicides, respectively) or the challenging task of emptying or elimination of *Ae. albopictus* breeding sites which are natural and artificial water-filled containers found around human dwellings.[16,17] Chemical-based control is highly efficient, provides quick results and is less costly; however, the effects are generally short-termed and have detrimental effects on human health, other non-target organisms and the environment.[18,19] In biological control, predators, parasites, pathogens, competitors of mosquitoes or their toxins can be used to control mosquito populations.[20–22] Only a few of these organisms are commercially produced and used on a large scale as difficulties in mass production limit the potential use of most bio-agents. Currently, *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) and *Lysinibacillus sphaericus* bacteria and spinosad toxin obtained from *Saccharopolyspora spinosa* are the only bacterial larvicidal products available to control mosquito larvae.[23–25] These larvicides are applied to mosquito breeding sites to kill larva before they develop into adults. Despite having been used extensively for many years, there are no reports of field or laboratory findings of mosquito resistance to Bti, which produces a cascade of parasporal toxins that work synergistically to enhance toxicity to mosquito larvae.[26,27] However, there are reports of resistance to *L. sphaericus*; its toxin targets a single receptor in larval midgut which increases risks of resistance.[23,28,29] For decades, there has been a significant and increasing demand for the development of biopesticides to supplant or complement current mosquito control products. This demand in biopesticides has been driven by several factors such as restriction and bans on several extant pesticide products, increased interest in ecofriendly vector and pest control practices and increased knowledge of biopesticides and their usage.[30,31] Several potential new substances are being investigated and have been reported in the literature as promising biopesticides from fungus, bacteria, and plants.[32,33]

Xenorhabdus and *Photorhabdus* bacteria, members of the Morganellaceae family, are enteric bacteria found in the gastrointestinal tracks of *Steinernema* (Rhabditae: Steinernematidae) and *Heterorhabditis* nematodes (Rhabditae: Heterorhabditidae).[34–36] These nematode-bacterial complexes have convergently evolved to be insect pathogens that dwell naturally in mainland and insular soil environments worldwide; they are only absent or are yet to be isolated from Antarctica.[37,38] These bacteria produce a plethora of biologically active compounds as a defense/survival strategy, i.e., these compounds play an important role in the bioconversion of host cadaver, stimulation of nematode reproduction and growth, and inhibition of growth of various antagonistic or opportunistic bacterial, fungal, and protozoal microorganisms while host nematodes develop in insect cadavers.[39,40] The antimicrobial and insecticidal potential of these metabolites have long been recognized as up-and-coming sources of new pharmaceutical agents and biopesticides.[41–45] Several studies have demonstrated the larvicidal efficacy of cell-free bacterial supernatants (CFS) and/or bacterial cell suspensions of *Xenorhabdus* and *Photorhabdus* on different mosquito species but none has yet identified the bioactive natural product.[46–49]

This study investigated: (i) the larvicidal efficacy of cell growth cultures, cell free supernatants and bacterial cell (pellet) suspensions of an extensive list of *Xenorhabdus* and *Photorhabdus* bacteria against *Ae. albopictus* larvae, (ii) assessed the effect of bacterial supernatants on the eggs of *Ae. albopictus*, (iii) identified the novel larvicidal compound/s in the supernatants of *X. szentirmaii* and *X. nematophila* using mutants generated using the easyPACId biotechnological approach, and (iv) compared the effects of bioactive compound with other commercial products.

2 MATERIALS AND METHODS

2.1 Maintenance of *Aedes albopictus*

This mosquito was reared in 45 × 45 × 45 cm insect cages (Bugdoms) placed under insectary conditions at 27 ± 1 °C, 70% RH and under a 14D:10 L photoperiod. Cotton pads soaked in 10% sugary water were available *ad libitum* to adult mosquitoes. Female mosquitoes were regularly fed defibrinated sheep blood using an artificial blood feeder every 2–3 days and cylindrical containers with water and filter paper on the sides were provided for oviposition. Hatched larvae were fed daily with ground fish food flakes (Tetramin®).[50,51]

2.2 Preparation of bacterial cell suspension, cell free supernatant and growth culture

Twenty-nine different *Xenorhabdus spp*. and *Photorhabdus spp*. were used (Table 1). These bacteria were first streaked on Luria-Bertani (LB) agar from stock cultures and then a single colony was inoculated and incubated in LB broth (10 mL) on a rotary incubator at 28 °C and 150 rpm for 24 h. From this overnight pre-culture, 0.5 mL was transferred to a LB broth (50 mL) and incubated for a further 72 h. Afterwards this culture was divided into two parts: one served as growth culture used in the larvicidal assays and the other was centrifuged at 10 000 rpm at 4 °C for 10 min. The supernatant was transferred into another centrifuge tube and filtered through a 0.22 µm Millipore filter (Sartorius,

Table 1. *Xenorhabdus* and *Photorhabdus* wildtype bacterial species

	Bacterial species	Abbreviation
1	*Xenorhabdus bedingii* DSMZ 4764	*X. bed*
2	*X. bovienii* SS-2004	*X. bov*
3	*X. budapestensis* DSMZ 16342	*X. buda*
4	*X. cabanillasii* JM26-1	*X.cab*
5	*X. doucetiae* DSMZ 17909	*X. dou*
6	*X. eapokensis* DL20	*X. eap*
7	*X. ehlersii* DSMZ 16337	*X.ehl*
8	*X. griffinae* DSMZ 17911	*X. grif*
9	*X. hominickii* DSMZ 167903	*X. hom*
10	*X. indica* DSMZ 17382	*X. ind*
11	*X. innexi* DSMZ 16336	*X. inx*
12	*X. ishibashii* DSMZ 22670	*X. ishi*
13	*X. japonica* DSMZ 16522	*X. jap*
14	*X. kozodoi* DSMZ 17907	*X. koz*
15	*X. miraniensis* DSMZ 17902	*X. mira*
16	*X. stockiae* DSMZ 17904	*X. stock*
17	*X. szentirmaii* DSMZ 16338	*X. szen*
18	*X. thuongxuanensis* 30 TX1	*X. thou*
19	*X. vietnamensis* DSMZ 22392	*X. viet*
20	*X. koppenhoeferii* DSMZ 18168	*X. kop*
21	*X. nematophila* ATCC 19061	*X. nema*
22	*X. poinarii*	*X. poi*
23	*Xenorhabdus* sp. TS4	-
24	*Photorhabdus akhurstii* DSMZ 15138	*P. akh*
25	*P. asymbiotica* ATCC 43949	*P. asy*
26	*P. laumondii* TT01	*P. lau*
27	*P. thracensis* DSMZ 15199	*P. thr*
28	*P. namnaoensis* PB 45.5	*P. nam*
29	*P. kayaii* DSMZ 15194	*P. kay*

Goettingen-Germany).[52] The remaining bacterial pellets were re-suspended with sterile physiological saline and the turbidity was adjusted to OD_{600nm} = 1.0 by spectrophotometer. Hence, growth culture, cell-free supernatant (CFS) and re-suspended bacterial pellet (bacterial cell suspension) were ready for use in bioassays.[43,49]

2.3 The larvicidal efficacy of *Xenorhabdus* and *Photorhabdus spp.*

The efficacy of growth culture, CFS and bacterial cell suspension against 3rd-4th stage larvae of *Ae. albopictus* was evaluated in wells of a 24-well plates.[51,53] Each well had 10 mosquito larvae in 1 mL of distilled water with 50% of prepared growth culture, CFS or bacterial cell suspension. Distilled water was used as the negative control. Each treatment had six replicates (wells). The experiments were carried out at 24 ± 1 °C and larval mortality was assessed after 24 and 48 h. Dead larvae were touched with a fine tipped brush to confirm death. The experiment was conducted three times on different dates.

2.4 Identification of the larvicidal compound using different *Xenorhabdus spp.* Δhfq promoter exchange mutants

The bioactive larvicidal compound was identified using *Xenorhabdus szentimaii* and *X. nematophila* Δhfq pCEP-KM-xy mutants generated by the easyPACId approach (Bode *et al.*, 2019). These mutants were generated by first creating Δhfq mutant and then exchanging the native promoter regions of selected natural product biosynthetic gene clusters of these bacteria with L-arabinose inducible promoter pBAD by the integration of the pCEP-KM plasmid.[54–56] With these mutants we could selectively produce a desired single natural product compound class and directly conduct bioactivity analysis of the corresponding supernatant instead of laborious isolation of every single compound in the supernatant(s). Table 2 shows the *Xenorhabdus spp.* Δhfq as well as *Xenorhabdus spp.* Δhfq pCEP-KM-xy mutants generated (xy describes the locus of the first biosynthetic gene cluster).[55,57]

Table 2. *Xenorhabdus spp. Δhfq* pCEP-KM-xy *mutants* used in this study

Bacteria species	Mutant name	Produced compound name
X. szentirmaii	DSM 16338	Wild type
	Δhfq_ pCEP_KM_0346	GameXPeptide
	Δhfq Pcep-KM-5118	Pyrollizixenamide
	Δhfq PCEP 3663	Xenoamicin
	Δhfq_pCEP_KM_3397	Rhabdopeptide
	Δhfq_pCEP_KM_3460	Szentiamid
	Δhfq_pCEP_KM_3680	Xenobactin
	Δhfq_pCEP_KM_3942	Rhabduscin
	Δhfq pCEP-KM-1979	Diketopiperazin
	Δ*hfq* pCEP-KM-0377	PAX-short
	Δhfq_pCEP_KM_fclC	Fabclavine
	Δhfq_pCEP_KM_xfsA	Xenofuranone
X. nematophila	ATCC 19061	Wild type
	Δhfq_pCEP_kan_XNC1_2022	Xenotetrapeptide
	Δhfq_pCEP_kan_XNC1_1711	Xenocoumacin
	Δhfq_P_{BAD}_XNC1_xndA	Xenortide
	Δhfq_P_{BAD}_XNC1_2228	Rhabdopeptide
	Δhfq_P_{BAD}_XNC1_2713	Xenematide
	ΔPPTase_P_{BAD}_XNC1_isnA	Rhabduscin
	Δhfq_ΔisnAB_P_{BAD}_XNC1_2300	Xenortide
X. cabanillasii	JM26-1	Wild type
	Δhfq_128–129	Fabclavine
X. hominickii	DSM 179903	Wild type
	Δhfq_130–131	Fabclavine
X. budapestensis	DSM 16342	Wild type
	Δhfq_pCEP_fclC	Fabclavine
X. stockiae	DSM 17904	Wild type
	Δhfq_pCEP_fclC	Fabclavine

The CFS of the different *Xenorhabdus spp.* Δhfq promoter exchange mutants were obtained as described in Bode *et al.*[55] and Wenski *et al.*[58] Briefly a single mutant colony of the mutants was streaked on LB agar supplemented with a 50 μg mL^{-1} final concentration of kanamycin and incubated at 30 °C for 48 h. transferring into LB medium (10 mL) also supplemented with a 50 μg mL^{-1} final concentration of kanamycin and incubated at 150 rpm and 30 °C. Then, this overnight culture was inoculated into a fresh 20 mL LB with the final optical density (OD_{600nm}) adjusted to 0.1. After an hour incubation at 30 °C, these cultures were induced with 0.2% L-arabinose and incubated again for 72 h at 150 rpm and 30 °C.[45,55,58] Flask of non-induced mutants had no L-arabinose. The CFS were obtained by centrifugation at 10 000 rpm for 20 min in 50 mL Falcon tubes at 4 °C and filtration through a 0.22 μm Millipore filter (Thermo scientific, NY) to ensure total removal of bacterial cells.[42,59] The CFS were stored at −20 °C and used within 2 weeks.[60]

The same experimental design described above with 24-well plates was used to identify the bioactive larvicidal compound against 3rd-4th stage larvae *Ae. albopictus*. Each well had 10 mosquito larvae in 1 mL of water containing 50% of prepared cell-free

supernatant. Distilled water was used as the negative control. The experiments were carried out at 24 ± 1 °C and larval mortality was assessed after 48 h. Dead larvae were touched with a fine tipped brush to confirm death. There were six replicates per treatment and the study was repeated twice.

After identifying the bioactive compound/s, mutants of *Xenorhabdus* species (*X. hominickii, X. budapestensis* and *X. cabanillasii* and *X. nematophila*) in which different derivatives of this compound were assessed against mosquito larvae. Concentration effects ranging between 50–2.5% were also tested.

2.5 Comparing the effects of bioactive compound with other commercial products

This study was conducted to compare the efficacy of CFS obtained from *X. cabanillasii* Δhfq_128–129 and *X. hominckii* Δhfq_130–131 mutants with commercial larvicidal compounds of bacterial origin (see Table 3 for commercial products and their active ingredients). *X. cabanillasii and X. hominckii* emerged as one of the best performers in prior assays. Thirty 3rd to 4th stage *Ae. albopictus* larvae were transferred into 150 mL plastic containers with 50 mL clean (distilled) water or field collected water. The containers were treated with the recommended concentration of the commercial products and 50% CFS from *X. cabanillasii* Δhfq_128–129 and *X. hominckii* Δhfq_130–131 mutants. Setup was incubated at 27 ± 1 °C and larval mortality was assessed and recorded after 4, 24 and 48 h post application. This experiment was conducted thrice with three containers for each treatment.

The residual effects of these larvicidal compounds were also analyzed according to da Silva *et al.*[47] with some modifications. In these experiments, 30 mosquito larvae were dispensed into plastic containers (12 cm × 12 cm × 6 cm) with 300 mL distilled water. These containers were treated with the recommended concentration of the larvicidal compounds and 50% concentration of *X. cabanillasii* Δhfq_128–129 and *X. hominckii* Δhfq_130–131 supernatants. Larvae were added at 0, 3, 5, 7, 9, 11, and 15 dpa after treatment and larval mortality was assessed after every 24 h. After each assessment, larvae in each container were removed by sieving contents of the container and new healthy larvae were added; this was continued until no significant mortality was observed. These experiments were conducted thrice with three containers for each treatment.

2.6 Effects of cell-free supernatants of *Xenorhabdus spp.* on mosquito egg hatching

The effects of *Xenorhabdus szentirmaii* and *X. cabanillasii* bacterial CFS on *Ae. albopictus* eggs were evaluated in wells of a 24-well plates. Briefly, with six replicates per treatment, 10 mosquito eggs deposited on filter papers were transferred into wells using a fine brush. Then 1 mL of distilled water with 50% of CFS was added, just LB media was used in negative controls. Plates were incubated at 27 ± 1 °C for 5 days after which the number of hatched eggs was counted. This experiment was done twice.

2.7 Statistical analysis

Data on the effects of different *Xenorhabdus* and *Photorhabdus* bacteria, mutants, and control against *Ae. albopictus* were arcsine-transformed, analyzed using analysis of variance with bacterial species, incubation time, treatment type, assessment time as the main factors and their interactions taken into consideration; the means were separated using Tukey's test ($P < 0.05$). All analysis was done in SPSS program version 23.

3 RESULTS

3.1 The larvicidal efficacy of *Xenorhabdus* and *Photorhabdus spp.*

There were significant differences between the larvicidal activity of bacteria species (F = 276.894; df = 29; $P < 0.001$), treatment types (supernatant, bacterial growth cultures, bacterial cell suspensions; F = 1246.902; df = 2; $P < 0.001$), assessment time (F = 206.772; df = 1; $P < 0.001$) and their interactions (F = 3.427; df = 58; $P < 0.001$) on *Ae. albopictus* larval mortality. Application of bacteria as growth cultures had the highest effects (Table 4, Fig. 1).

Table 3. Commercial biopesticides used in this study

Commercial product	Active ingredient/s	Potency	Recommended concentration	Formulation type
Vectobac® 12AS	*Bacillus thuringiensis* subsp. *israelensis* AM 65–52	1200 ITU/mg	0.19 mL L^{-1}	SC
Vectomax® FG	*B.t.i* AM 65–52 & *Lysinibacillus sphaericus* ABTS 1743	50 ITU/mg	1.9 g L^{-1}	WDG
Serbate 15 C	Pyriproxyfen	-	0.66 mL L^{-1}	EC
Moskill 120C	Spinosad	-	3.3 mL L^{-1}	SC
Vectolex WDG	*L. sphaericus* ABTS 1743	650 ITU/mg	5 g L^{-1}	WDG

EC, emulsifiable concentrate; SC, suspension concentrate; WDG, water-dispersible granule.

Table 4. Analysis of variance data on the effects of different *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes albopictus*

Factors	df	F	*P*	Partial η2
Bacteria	29	276.894	0.000	0.724
Treatment type	2	1246.902	0	0.449
Assessment Time	1	206.772	0	0.063
Bacteria × Treatment	58	90.966	0	0.633
Bacteria × Assessment Time	29	3.127	0	0.029
Treatment × Assessment Time	2	2.449	0.087	0.002
Bacteria × Treatment × Assessment Time	58	3.427	0	0.061

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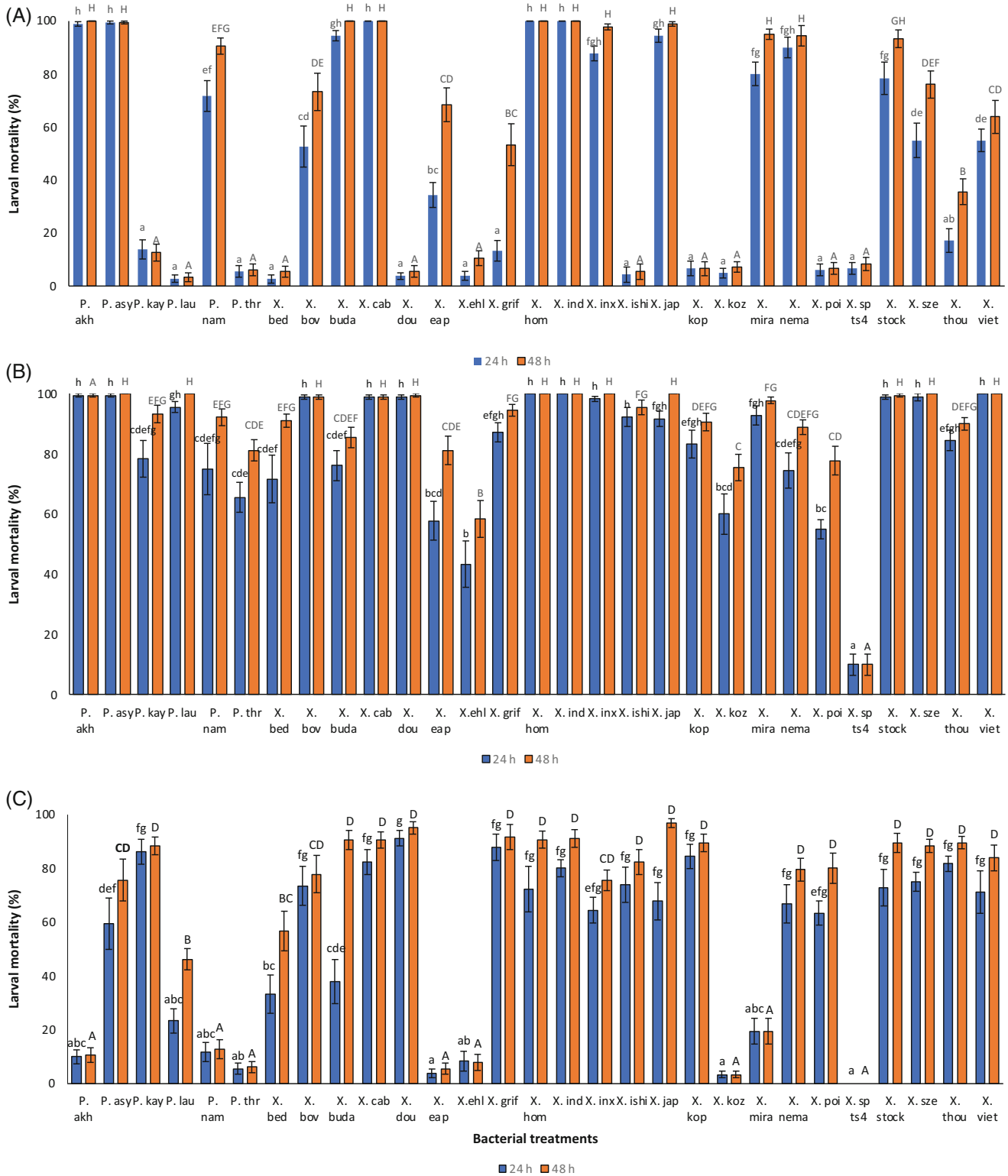

Figure 1. Mean larvicidal activity of cell-free supernatant (A), bacterial growth culture (B) and bacterial cell suspensions (C) of Xenorhabdus and Photorhabdus bacteria against *Aedes albopictus* larvae. Lower-case and upper-case letters above bars indicates no statistical difference for 24 h and 48 h mortality results, respectively ($P < 0.05$).

Comparison of the effects of the CFS showed that there was a significant difference among the species after 24 h (F = 132.509; df = 28, 521; $P < 0.0001$) and 48 h (F = 145.146; df = 28, 521; $P < 0.001$) with CFS obtained from three *Photorhabdus* species and 13 *Xenorhabdus* species killing 52–100% of *Ae. albopictus* larvae. The other species i.e., *P. kayaii*, *P. laumondii*, *P. thracensis*,

X. beddingii, *X. doucetiae*, *X. ehlersii*, *X. ishibashii*, *X. koppenoferii*, *X. kozodoi*, *X. poinarii*, *X. sp*. TS4, and *X. thuongxuanensis* presented less than 20% mortality at all points of assessment. Mortality in control was less than 10% (Fig. 1(A), Table 4).

Bacterial growth culture, which contains both bacterial cells and supernatants, from all tested species exhibited high larvicidal activity, except for *X. sp*. TS4. Twenty-eight of the tested bacteria displayed efficacy that ranged between 43 and 100% whereas, only *Xenorhabdus sp*. TS4 caused 10% mortality. There was a significant difference among the treatments after 24 h exposure ($F = 26.146$; $df = 28, 521$; $P < 0.001$). After 48 h exposure, generally more or less increase in efficacy was observed at all treatments and some of these differences were statistically significant ($F = 50.24$; $df = 28, 521$; $P < 0.001$) (Fig. 1(B), Table 4).

In the case of treatments with bacteria cell suspensions, some *Xenorhabdus* and *Photorhabdus* bacteria cells exhibited oral toxicity killing 59–91% of exposed larvae. Other bacterial species such as *P. akhurstii*, *P. namnoensis*, *X. eapokenensis* and *X. miraniensis* substantially presented less larvicidal activity compared to bacterial growth and CFS after 24 ($F = 34.705$; $df = 28, 521$; $P < 0.001$) and 48 h ($F = 79.669$; $df = 28, 521$; $P < 0.001$) (Fig. 1(C), Table 4).

3.2 Identification of the larvicidal compound using different *Xenorhabdus spp.* Δhfq promoter exchange mutants

Using the easyPACId approach, we were able to identify the bioactive compound by comparing the effects of a mutant strain with single gene in a blank Δ*hfq* background with that of the wildtype cells. Results showed clearly that the fabclavine-producing (*X. szentirmaii* Δhfq pCEP-KM-fclC) (Fig. 2(A)) and the xenocumacin-producing (*X. nematophila* Δhfq_pCEP_kan_XNC1_1711) (Fig. 2(B)) strains displayed larvicidal activity. There was a statistically significant difference among the compounds from the tested mutant strains of *X. szentirmaii* ($F = 178.205$; $df = 13, 280$; $P < 0.001$) (Fig. 2(A)) and X. *nematophila* ($F = 39.179$; $df = 11, 279$; $P < 0.001$) after 48 h (Fig. 2(B)).

Fabclavine produced by *X. szentirmaii*, *X. budapestensis*, *X. cabanillasii*, *X. stockiae*, *X. hominckii* and *X. bovienii* were also assessed against mosquito larvae. After 24 h, *X. hominickii* and *X. cabanillasii* displayed significantly higher effects (91–96%) than *X. budapestensis*, *X. szentirmaii* and *X. stockiae* ($F = 111.557$; $df = 6, 125$; $P < 0.001$) (Fig. 3). After 48 h, the efficacy of *X. szentirmaii* *X. budapestensis* and *X. stockiae* increased to 84, 81 and 37%,

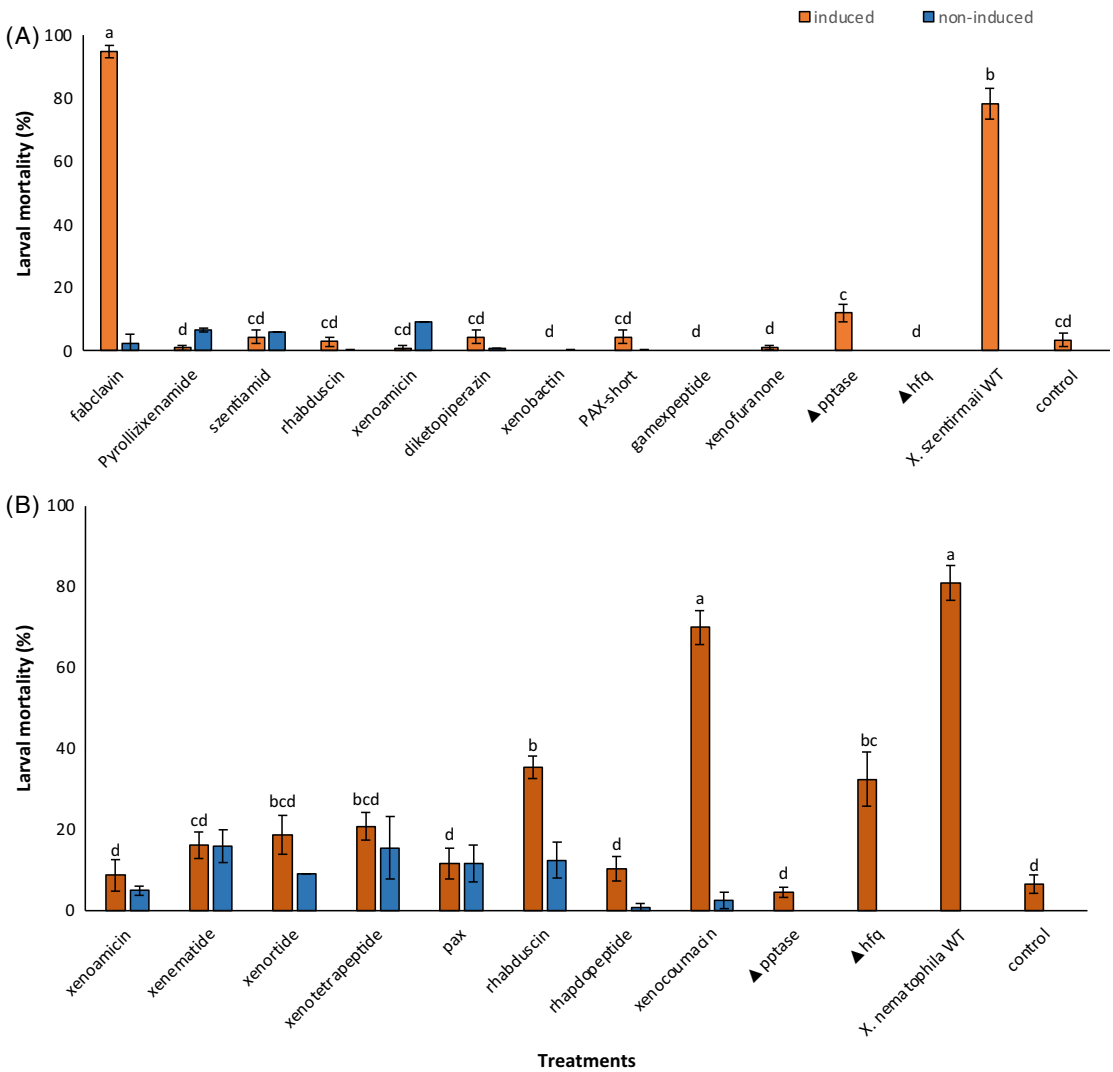

Figure 2. Larvicidal activity of cell-free supernatants obtained from induced and non-induced *Xenorhabdus szentirmaii* (A) and *Xenorhabdus nematophila* (B) Δhfq pCEP-KM-xy mutants against *Aedes albopictus* larvae after 48-h exposure. Same letter above bars indicates no statistical difference ($P > 0.05$).

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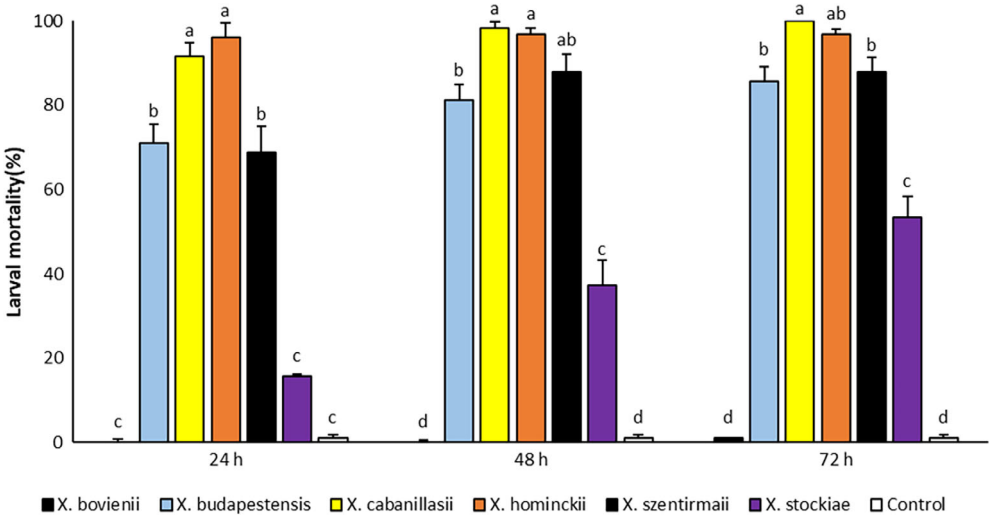

Figure 3. Larvicidal effects different fabclavine types from *Xenorhabdus spp*. on *Aedes albopictus* larvae. Same letter above bars indicates no statistical difference ($P > 0.05$).

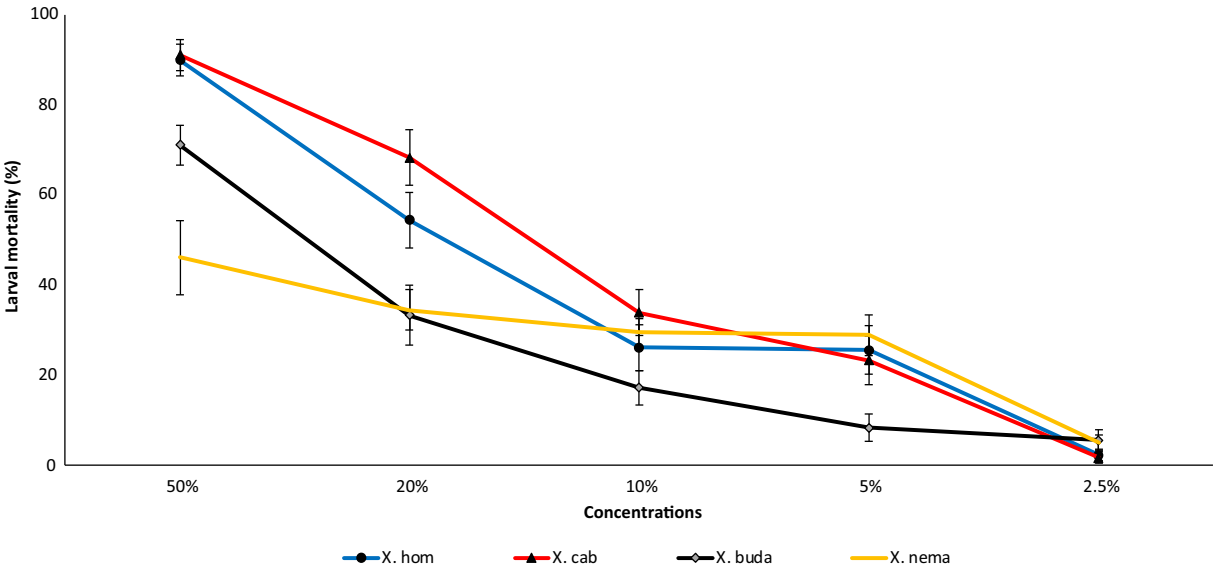

Figure 4. Larvicidal effects of xencoumacin from *Xenorhabdus nematophila* and fabclavines from *Xenorhabdus hominickii*, *Xenorhabdus budapestensis* and *Xenorhabdus cabanillasii* on *Aedes albopictus* larvae after 24 h.

respectively, whereas, a slight increment was observed for the other species, but there was still a significant difference among the strains ($F = 171.204$; $df = 6, 125$; $P < 0.001$) (Fig. 3).

Fabclavine from *X. hominickii*, *X. budapestensis* and *X. cabanillasii* and xencoumacin from *X. nematophila* were tested at lower concentrations ranging between 50–2.5%. There was a gradual decrease in the effects of the compounds as concentration decreased. Two-way ANOVA showed that there was a significant difference between the effects of compounds ($F = 15.097$; $df = 3$; $P < 0.001$), tested concentrations ($F = 135.751$; $df = 4$; $P < 0.001$), and their interactions ($F = 6.136$; $df = 12$; $P < 0.001$) on *Ae. albopictus* larval mortality (Fig. 4).

3.3 Comparing the effects of bioactive compound with other commercial products

Except for *L. sphaericus*, the commercially available larvicidal products and fabclavines obtained from *X. cabanillasii* and *X. hominickii* mutants demonstrated larvicidal activity starting from 4 h after treatment in distilled water. Vectomax, Vectobac and spinosad caused 100%, Serbate caused 77% larval mortality whereas, fabclavines from *X. cabanillasii* and *X hominickii* mutants exhibited 17.8 and 30.9% larval mortality, respectively, at 4 h of treatments ($F = 348.928$; $df = 7112$; $P < 0.001$) (Fig. 5(A)). Mortality of fablcavine treatments increased up to >94 at 24 h and reached to 99–100% at 48 h post treatments. There was no significant difference between fabclavines and commercial larvicidal products at 48 h ($P < 0.05$). No or less than 2% mortality at control and *L. sphaericus* treatments after 24 and 48 h was observed.

Likewise, the larvicidal compounds differed significantly in their effects against *Ae. albopictus* larvae in field-collected water. Both fabclavines obtained from *X. cabanillasii* and *X. hominickii* caused 30% larval mortality whereas, Vectomax, Vectobac and Spinosad caused 100% mortality within 4 h ($F = 735.629$; $df = 7, 80$; $P < 0.001$) (Fig. 5(B)). After 24 h larvicidal mortality caused by

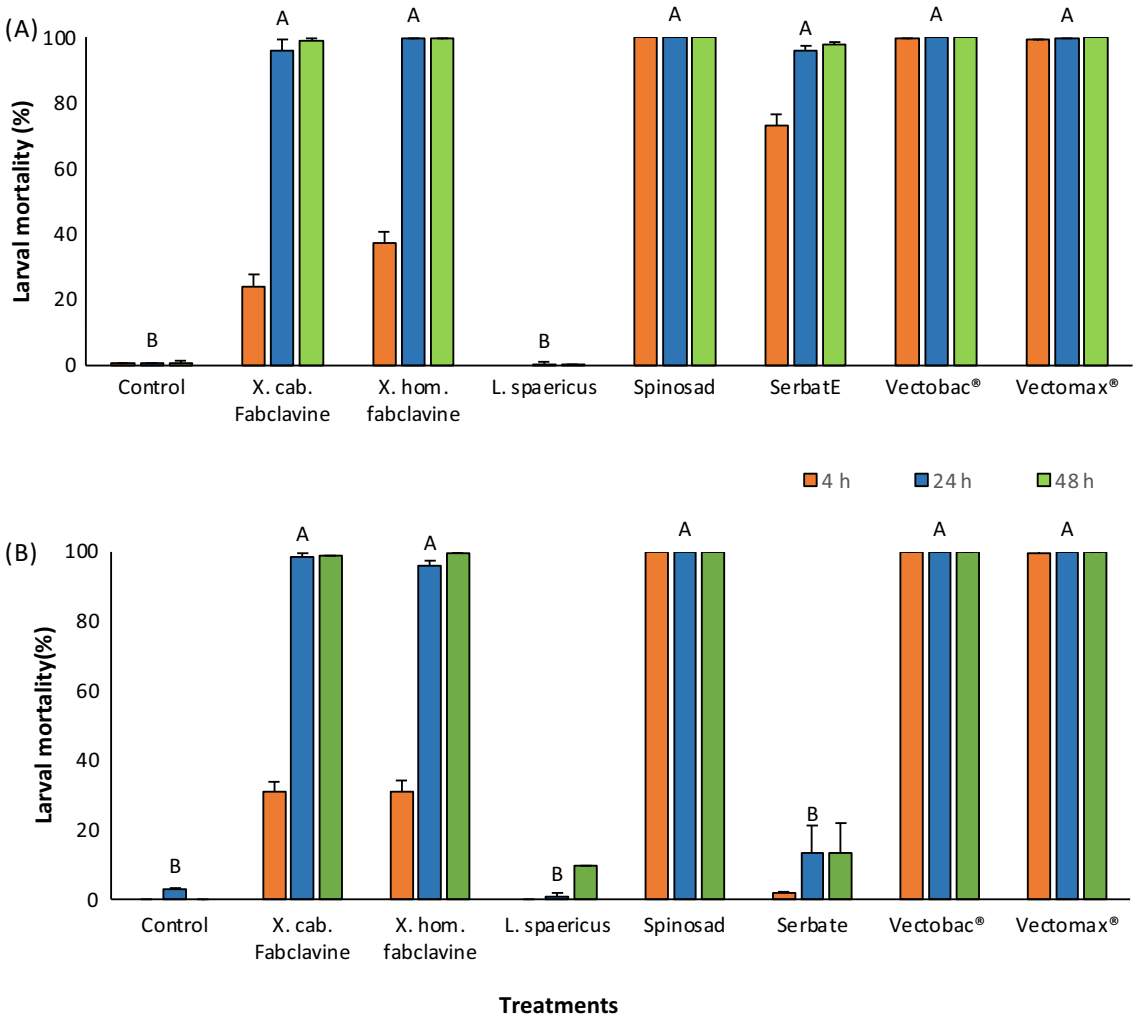

Figure 5. Comparison of the effects of fabclavine with commercial biolarvicides in clean (distilled) water (A) and field collected water (B). Same letter above bars indicates no statistical difference ($P > 0.05$).

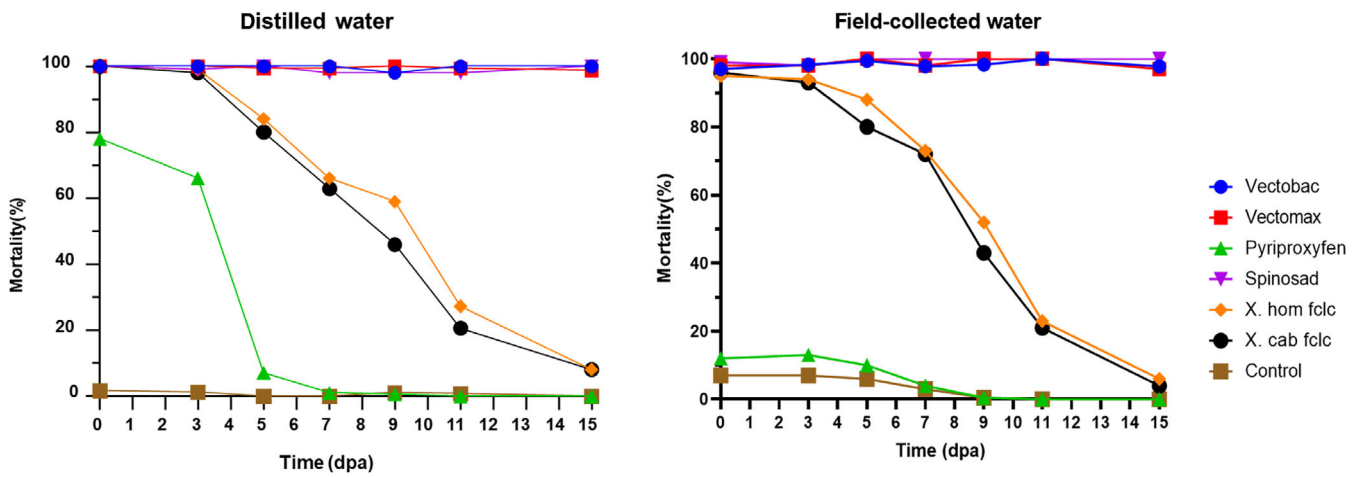

Figure 6. Residual effects of fabclavine and commercial biolarvicides in clean (distilled) water and field collected water.

fabclavines from *X. cabanillasii* and *X. hominickii* increased to 98 and 96%, respectively. *Lysinibacilus sphaericus* and Serbate were ineffective in field-collected water. Statistical difference occurred between the negative control, *L. sphaericus* and Serbate with the other treatments after 24 h ($F = 229.034$; $df = 7,80$; $P < 0.001$) and 48 h ($F = 242.315$; $df = 7, 80$; $P < 0.001$) (Fig. 5(B)).

As for the residual/longevity effects, we observed that Spinosad, Vectomax and Vectobac maintained their efficacy at 100%

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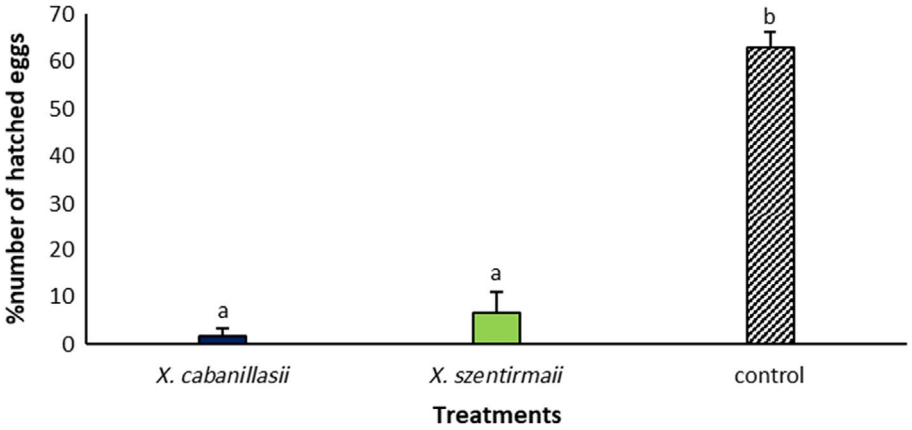

Figure 7. Mean effects of supernatants obtained from *Xenorhabdus szentirmaii*, *X. cabanillasii* bacteria against Aedes albopictus egg hatching. Lowercase show the comparisons of ovicidal results, respectively ($P < 0.05$).

mortality for 15 days whereas, there was a downward trend in effects of fabclavine from *X. hominickii* and *X. cabanillasii*. These fabclavines caused 100% mortality on 1 and 3dpa; then there was a gradual decrease in efficacy from 5 dpa until 15 dpa. *Lysinibacillus sphearicus* was ineffective in both treatments, whereas Serbate was only effective in distilled water (Fig. 6).

3.4 Effects of cell-free supernatants of Xenorhabdus and Photorhabdus on mosquito egg hatching

There was a statistical difference in the percentage of *Ae. albopictus* eggs that hatched after exposure to *X. cabanillasii* and *X. szentirmaii* CFS. (Fig. 7). Eggs in wells with bacterial supernatants did not hatch 5 dpa compared to control ($F = 67.035$; $df = 2,35$; $P < 0.001$). However, after 5 days when we replaced the supernatant with clean water, the eggs were observed to hatch.

4 DISCUSSION

This study showed that the different *Xenorhabdus* and *Photorhabdus* bacterial species had larvicidal effects on *Ae. albopictus* larvae. Treatment type (i.e., cell-free supernatant, bacterial growth culture, bacterial cell suspension) had a significant effect on mosquito larval mortality. Overall, mortality caused by effective species ranged between 52 and 100%, and higher mortalities occurred when bacteria are applied as growth culture, which contains both metabolites and bacterial cells. Shah *et al.*[51] highlighted that certain bacteria can release toxic metabolic compounds with larvicidal activities out of their cells. Supernatant composition varies widely between *Xenorhabdus* and *Photorhabdus* species and even between strains of the same species.[57] Three *Photorhabdus spp.* and 13 *Xenorhabdus spp.* were found to release larvicidal compounds in CFS whereas, bacterial cell suspensions of 21 species exhibited oral toxicity. Some of these bacteria do not release larvicidal compounds but their cells can exert toxicity when ingested by larvae. Similar results have been reported by other studies using bacterial pellets or crude supernatants (broth bacterial culture) of different *Xenorhabdus* and *Photorhabdus* species/strains against important mosquito species such as *Ae. aegypti*.[46,47,49,61]

Our study, as a first, demonstrates that the bioactive mosquito larvicidal compounds were fabclavine from *X. szentirmaii* and xenocumacin from *X. nematophila*. Fabclavines and xenocoumacins are water-miscible, non-ribosomal-synthesized peptide/polyketide peptide compounds with corresponding genes found mainly in *Xenorhabdus spp.*[58,62] These compounds have been demonstrated to possess antibacterial,[52] antifungal,[43,63] and antiprotozoal[45] activity and their main function is to basically maintain a monoxenic environment within infected host by inhibiting the growth of various prokaryotic and eukaryotic organisms.[62,64–67] Supernatants from xenocoumacin-producing wildtype species (i.e., *X. nematophila*, *X. indica*, *X. miraniensis*, *X. stockiae*, and *X. doucetiae*), and all fabclavine-producing (i.e., *X. szentirmaii*, *X. budapestensis*, *X. cabanillasii*, *X. stockiae*, *X. hominckii*, *X. indica*, *P. asymbiotica*),[40,58] were highly effective against the *Ae. albopictus* larvae; despite producing these compounds, *X. kozodoi*, *X. poinarii* and *X. bovienii* were ineffective. The 32 different fabclavines reported to be produced by different species can differ greatly in structure and bioactivity e.g., *X. bovienii* produces derivatives with only the polyamine part.[58] Numerous research have described the possible application of fabclavine and xenocoumacin compounds against medical,[45,52,63] and agriculturally important pathogens.[43,58,68] Other effective strains on mosquito larvae such as *P. akhurstii*, *P. namnonensis*, *X. eapokensis*, *X. japonica*, *X. griffinae*, and *X. vietnamensis* tested in this study probably produce other compound/s with larvicidal activity. These species produce neither fabclavine nor xenocoumacine but have larvicidal activity; this needs to be investigated in the future. Thus far, other larvicidal compounds reported are toxin complex a (tca) protein,[69] PirAB proteins,[70] and anthraquinones (1,3-dimethoxy-8-hydroxy-9,10-anthraquinone and 3 methoxychrysazine)[71] from *Photorhabdus spp.* and *Xenorhabdus* lipopeptide toxin (Xlt) from *X. innexi*. Xlt is believed to be biochemically similar and homologous to fabclavines and can be found secreted from cells into growth media and retained on the cell surface.[72,73]

We compared the effects of fabclavines with current commercially available larvicidal products. Fabclavines were as effective as the products with *Bti* and Spinosad as active ingredients in clean water and in field collected water. Serbate was only effective in clean water whereas, *L. sphaericus* was ineffective in both tested environments. Reportedly, *Aedes* species are less susceptible to *L. sphaericus*.[74,75] As for the longevity, spinosad, vectomax and vectobac maintained their efficacy for 15 days whereas, there was a downward trend in effects of fabclavine from >94% to approximately 15%. The growth culture of *X. nematophila* has been observed to have a short longevity in water maintaining 100% efficacy against *Ae. aegypti* until the 4th day before a drastic decrease to 20% by the 11th day.[47]

Interestingly, we observed that the significantly few eggs treated with bacterial supernatants hatched compared to the control and when we transferred unhatched eggs into clean water, larvae emerged within 24 h. This data shows that larvae in the eggs can sense the possible toxicity of compounds in the bacterial supernatants. Touray *et al.*[76] demonstrated that the supernatants of of *Xenorhabdus spp*. and *Photorhabdus spp*. effectively deterred *Ae. albopictus* oviposition. They showed that compounds such as fabclavines could potentially be used to prevent mosquitoes from breeding around human dwellings and simultaneously killing of immature stages hereby greatly influencing mosquito species establishment, population densities, and dispersion in conducive areas.

In conclusion, our study demonstrates that an extensive number of *Xenorhabdus* and *Photorhabdus* display larvicidal activity as cells or by producing secondary metabolites with larvicidal activity against *Ae. albopictus*. Using the easyPACId technique we identified that the bioactive compounds are fabclavine and xenocoumacin. These compounds can be developed in novel biolarvicides or can be used as a model to design and synthesize other compounds.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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