Parasites & Vectors

REVIEW

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The great potential of entomopathogenic bacteria *Xenorhabdus* and *Photorhabdus* for mosquito control: a review

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Abstract

The control of insects of medical importance, such as *Aedes aegypti* and *Aedes albopictus* are still the only effective way to prevent the transmission of diseases, such as dengue, chikungunya and Zika. Their control is performed mainly using chemical products; however, they often have low specificity to non-target organisms, including humans. Also, studies have reported resistance to the most commonly used insecticides, such as the organophosphate and pyrethroids. Biological control is an ecological and sustainable method since it has a slow rate of insect resistance development. Bacterial species of the genera *Xenorhabdus* and *Photorhabdus* have been the target of several research groups worldwide, aiming at their use in agricultural, pharmaceutical and industrial products. This review highlights articles referring to the use of *Xenorhabdus* and *Photorhabdus* for insects and especially for mosquito control proposing future ways for their biotechnological applicability. Approximately 24 species of *Xenorhabdus* and five species of *Photorhabdus* have been described to have insecticidal properties. These studies have shown genes that are capable of encoding low molecular weight proteins, secondary toxin complexes and metabolites with insecticide activities, as well as antibiotic, fungicidal and antiparasitic molecules. In addition, several species of *Xenorhabdus* and *Photorhabdus* showed insecticidal properties against mosquitoes. Therefore, these biological agents can be used in new control methods, and must be, urgently considered in short term, in studies and applications, especially in mosquito control.

Keywords: Entomopathogenic bacteria, *Aedes aegypti*, Mosquito-borne arboviruses, *Xenorhabdus nematophila*, *Photorhabdus luminescens*, Biological control

Background

It is widely known that various species of mosquitoes can transmit pathogens that cause debilitating injuries in world populations, often endangering the lives of millions of people. Among the mosquito-borne viruses are dengue, chikungunya [1], West Nile virus, yellow fever [2] and Zika [3].

The presence of chikungunya virus has been notified in more than 45 countries, highlighting the epidemic that occurred in India in 2005 with 1015 confirmed cases [4]. In Brazil, the first autochthonous cases were reported in September 2014, with subsequent emergence of cases in several regions, in a short period of time [5]. In 2016, 63,810 cases of chikungunya were confirmed in this country [6].

West Nile fever virus was first isolated in 1937 from an infected person in Uganda, Africa [7]. After that, sporadic transmission was observed in more temperate parts of Europe and endemic in tropical areas of Africa, northern Australia and South Asia. The virus was introduced in North America in 1999, which spread and became a public health problem [8]. After its introduction in America, in

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2010 approximately 1.8 million people have been infected, resulting in 1308 deaths [9].

Since 2016 there has been the re-emergence of the Zika virus, with outbreaks of transmission by mosquitoes, causing a threat to public health worldwide [3, 10–14]. Regarding dengue virus, it is expanding globally, being present in more than 100 countries [15], with about 2.5 billion people living in an infection risk area [16–18]. Currently this virus is found on all continents [13, 18]. Because of this global viral spread, dengue has become an important public health problem [15, 19], being a threat to approximately 390 million people [3, 16].

According to Laughlin et al. [19], considering complications of dengue such as hemorrhagic fever and shock syndrome, is among the most important re-emerging infectious diseases in the world. This arbovirus is the one with the greatest impact on human morbidity and mortality compared to other arboviruses [20] due to the high virulence of the etiological agent [21]. Their four viral serotypes can be transmitted by females of *Aedes aegypti* mosquitoes (Diptera: Culicidae), and, to a lesser extent, *Aedes albopictus* [3, 12, 14, 22].

Aedes aegypti has a daylight hematophagous behavior and is extremely anthropophilic and endophilic, often being found in urban and suburban environments, where the life-cycle occurs with great proximity to humans [23]. Female oviposition occurs preferably in clean water present in artificial containers [24]. *Aedes albopictus*, popularly known as the Asian tiger, is also a daylight hematophagous mosquito, and can be found competing with *Ae. aegypti* in natural and artificial containers outside of houses. Also, it has expanded its habitat to temperate areas in urbanized regions due to intense climate change [18, 25].

These often-devastating arboviruses seem to continue to affect millions of people worldwide [26]. Therefore, vector control, whether in immature or adult stages, is a crucial strategy for preventing the expansion of these diseases [26, 27].

For the production of this narrative review, keywords were chosen: *Xenorhabdus*; *Photorhabdus*; insect and mosquito control; arbovirus and *Aedes*. All articles that contained information relevant to the purpose of the review were selected and used for their construction. As we propose that these bacterial species can be used to control mosquitoes, no temporal delimitation was made for the inclusion of the articles, aiming at a greater range of results that could be used.

Current vector control and their resistance to some compounds

Vector control is a method of extreme relevance to minimize the transmission of disease agents by mosquitoes [28, 29]. Therefore, limiting the impact of mosquito-borne diseases is an important goal for global public health agencies [26]. For the control of culicids several methods have been used, which result in reduction of population density, reduction of life span, or impediment of contact with the use of repellent compounds [30].

The methods for genetic control are in the study phase for *Ae. albopictus* and *Ae. aegypti*, also requiring considerations on the possibility of its implementation [30–32]. Thus, chemical control is generally considered the first method of choice [27].

In addition, several strategies for combating *Aedes* spp. have been used, such as the elimination of potential breeding sites, biological and chemical control with the use of repellents (contact precaution) and application of synthetic insecticides [14, 29, 30].

Among the most used compounds, organophosphates (temephos and fenthion) and growth inhibitors (diflubenzuron and methoprene) for larval control [14, 33–35]. However, due to the high frequency of use of these compounds, several populations of *Aedes* spp. have become resistant over the years [14, 26, 29, 36–38].

Chemical control may have disadvantages, such as effects on non-target organisms, environmental pollution, in addition to the development of insecticide resistance [27, 39–43]. Furthermore, repeated doses and high doses of chemical insecticides can cause an imbalance between the culicid population and their natural enemies, and also cause toxic effects on the environment and small mammals that co-inhabit the surrounding area [40]. Thus, it is necessary to reduce the use of chemicals and develop ecological products for the control of vector mosquitoes [44].

Biological control of vectors

The nutrition source of *Aedes* larvae comes from decaying organic matter, rich in bacteria, fungi and protozoa present in natural or unusual containers [45]. Some bacterial species may produce secondary toxins and metabolites capable of inducing larval death. Thus, symbiotic bacteria possibly cause pathogenicity after being ingested by mosquitoes [14].

Biological vector control is an ecological and sustainable method since it has a slow rate of insect resistance development [27]. Insecticide activities have been investigated in several microorganisms, including bacteria [46, 47], protozoa [48] and fungi [49]. The Gram-positive bacterium *Bacillus thuringiensis israelensis* (*Bti*) has been widely used as a biolarvicide in aquatic environments for mosquito control [50] and some species of the family Simuliidae [51–53]. In addition, *Bti* was an excellent candidate for fly control due to its entomopathogenic activities [50, 54], being widely used in recent years as

researchers developing studies to improve its effectiveness [44].

The World Health Organization recommends the use of biolarvicides derived from *Bti* and *Bacillus sphaericus* (syn. *Lysinibacillus sphaericus*) to control mosquito larvae, because they are alternative products that do not cause harm to the environment. Nevertheless, there are few options for bacterial larvicides available [55, 56]. The use of *Bti* also presented an impact on the prevalence of malaria [57]. Due to its mechanism of action, with release of toxins in the midgut of the larvae, the development of insect resistance can be hampered [26]. Although some authors have already recorded the occurrence of mosquitoes resistant to *Bti* [58, 59].

Other biological agents have been described for the control of *Aedes* species, such as: (i) fungi *Metarhizium anisopliae* and *Beauveria bassiana* [49, 60, 61]; (ii) protozoan *Acanthamoeba polyphaga* [48]; (iii) the copepod *Macrocyclops albidus* [62]; (iv) as well as bacteria of the genera *Xenorhabdus* and *Photorhabdus* [13, 29, 44, 50, 63–65].

Symbiotic nematoid bacteria and insect control

The study of bacterial species of the genera *Xenorhabdus* and *Photorhabdus* has been the target of several research groups, aiming at their use in agricultural, pharmaceutical and industrial products [43, 66–68]. The interest in studying these bacteria is justified by some evidence available in the literature, such as: (i) having genes that are capable of encoding low molecular weight secondary toxins and metabolites with insecticide activities [43, 69–71], antibiotic [43, 69, 72–74], antifungals [43, 69] and antiparasitic [69, 75–78]; (ii) laboratory research points to the success of these bacteria in pest control [27, 79]; (iii) *Photorhabdus luminescens* releases toxins with activities in the insect intestinal epithelium [59, 80]; (iv) *P. luminescens* in conjunction with *B. thuringiensis kurstaki* inhibits the growth of *Spodoptera littoralis* [81]; (v) *Xenorhabdus ehlersii* protein (XeGroEL) is effective against *Galleria mellonella* [82, 83]; (vi) acaricide and antibacterial activities have been reported for *Xenorhabdus stockiae* PB09 [84, 85]; (vii) *Xenorhabdus stockiae* PB09 showed miticidal activity against *Luciaphorus perniciosus* [86]; and (viii) the supernatants of the culture of *Xenorhabdus nematophila* and *P. luminescens* prevented the feeding of ants, crickets and wasps [87, 88], among others.

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are Gram-negative, optional anaerobic, belonging to the family *Enterobacteriaceae* [89–91], which stand out for their entomopathogenic potential [29, 92]. Approximately 24 species of *Xenorhabdus* and five species of *Photorhabdus* have been described worldwide to have insecticidal properties [14, 75, 93–96].

In nature, some species such as *X. nematophila* and *P. luminescens* developed a symbiotic relationship with helminths of the class Nematoda, Steinernematidae for *Xenorhabdus* and Heterorhabditidae for *Photorhabdus* [13, 14, 27, 44, 59, 97–101]. In the nematode host, the bacteria reside in the receptacle located in the intestine [102, 103].

Although these two bacterial species (*X. nematophila* and *P. luminescens*) have different evolutionary origins, the life-cycle is similar [104] and both are highly pathogenic for various insect species [13]. The cycle occurs as follows: the larvae of entomopathogenic nematodes (EPNs) live in the soil of several ecological systems searching for insect larvae as prey [105]. When found, it penetrates the insect's body through natural openings, such as the mouth, anus or spiracles [69], or they directly reach the hemocoel by boring a hole into the insect's skin, where symbiotic bacteria will be released by regurgitation (e.g. Heterorhabditidae) and defecation (e.g. Steinernematidae) [106, 107]. Once inside the hemocoel, the bacteria actively replicate, and release compounds that have the potential to suppress the immune response of the host insect, this being a protection strategy for symbiosis with the nematode [108, 109]. Taking into account the immunosuppressed state of the host, bacteria multiply in the hemocoel, initiating a fatal septicemia for the insect [99], causing its death in about 24 to 48 hours. Soon after, the carcass is bio-converted by bacteria, forming a rich food source for the nematodes as well as for themselves. Nematoid larvae grow and reproduce, giving rise to new youth stages. Furthermore, reproduction and development of the nematodes is actively supported by the bacteria by a yet unknown mechanism [14, 29, 44]. With food depletion, symbiotic association occurs again and the new helminth larvae adopt a free life phase (soil), where they actively transport their endosymbiotic bacteria and searching for new insect hosts [13, 99, 101, 110] (Fig. 1).

The symbiotic relationship, for example, between *Steinernema* and *Xenorhabdus* infecting insects, is mutually beneficial for the helminth-bacterium dyad, because carcasses become a nutritional source and breeding site for both the helminths and the bacteria. Furthermore, it is important to highlight that endosymbiotic bacteria are essential for the death of the next insect that will be parasitized, playing a crucial role in the survival of these nematodes [111].

In terms of specificity, *X. nematophila*, *X. hominickii* and *Photorhabdus temperata temperata* were isolated from *Steinernema carpocapsae*, *Steinernema monticolum* and *Heterorhabditis megidis*, respectively [103, 112, 113].

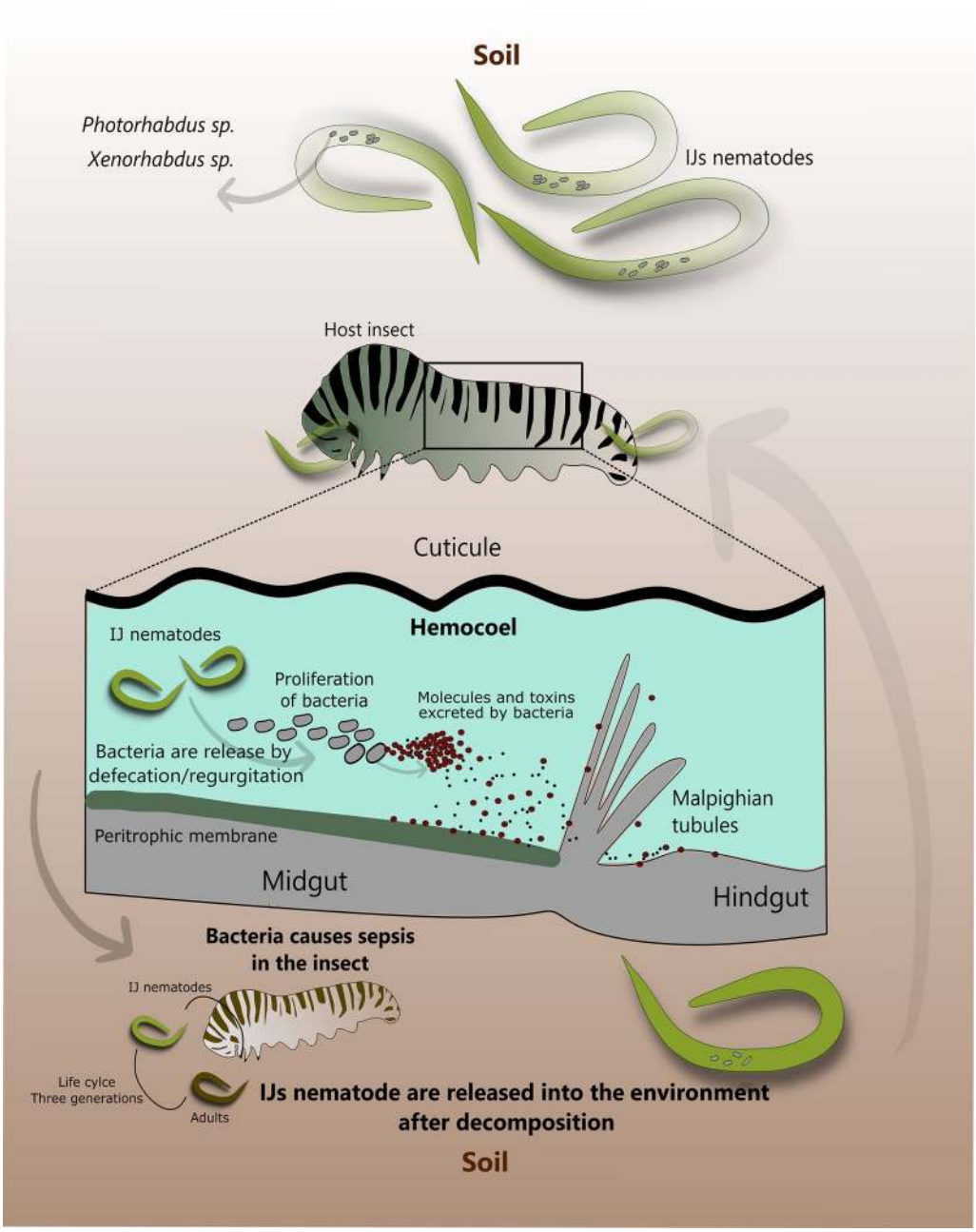

Fig. 1 Schematic drawing of the entomopathogenic nematode cycle, with the *Photorhabdus* and *Xenorhabdus* bacteria, demonstrating their symbiosis. The nematodes roam freely in the soil until they find a host insect, in the scheme represented by a caterpillar. The nematodes, when entering the host and settling in the hemocoel, release the bacteria through defecation or regurgitation. The bacteria proliferate in the hemocoel and become infectious, when they release toxic molecules to the host, leading to their death. Nematodes use the host's carcass to reproduce and return to the habitat carrying the bacteria, restarting the cycle until the nematodes find a new host insect

On the other hand, *X. nematophila* is not able to colonize *Steinernema scapterisci* [114]. Yooyanget et al. [14] described the importance of studying the species-specific identification in the mutualism between nematodes and entomopathogenic bacteria to obtain information about their diversity, as well as distribution in space, to additional studies of bioactive compounds that can be used in mosquito control.

Other differences in nematode-bacterial interactions have been described. For example, *Xenorhabdus innexi*

usually associates with *S. carpocapsae*, but with only one to five cells in the intestine of the nematode. *Xenorhabdus nematophila* colonizes the entire intestinal receptacle [115]. *Photorhabdus asymbiotica* is a species pathogenic to humans. However, *Photorhabdus asymbiotica australis* maintains an entomopathogenic symbiosis with nematodes of the genus *Heterorhabditis* [59, 100, 116].

Regarding the interaction of the bacteria with insects, in the moth *Manduca sexta*, infected with *Photorhabdus* and *Xenorhabdus*, colonization occurs primarily in the anterior portion of the midgut and then spreads to the posterior intestine [29]. *Photorhabdus* sp. secrete toxins that are able to destroy the intestinal epithelium of this insect, resulting in the interruption of the host's feeding process [29]. It is also noteworthy that in conjunction with insect feeding the virulence of the *Xenorhabdus* species is altered [29, 117].

Xenorhabdus are pathogenic to insects even in the absence of nematodes, as they are able to kill them after experimental injection. Thus, several studies are being developed in order to use *Xenorhabdus* for pest control [118]. Plants expressing certain genes of *X. nematophila* can become resistant to some insect species [119, 120]. For example, oral ingestion of transgenic *Arabidopsis thaliana* (expressing a gene from the *P. luminescens* toxin complex) was highly toxic to *M. sexta*, conferring plant resistance to insects and their oral mortality [121].

When released into the hemolymph of various insects, *Photorhabdus* bacteria are highly pathogenic [122]. It is important to emphasize that so far, no resistance to these bacteria has been reported in insect populations [58, 59, 80]. Some toxins of *P. luminescens* have a mode of action that differ from the toxins of *B. thuringiensis* and, these toxins for insect control can serve as potential alternatives [110, 123].

Even at low doses, *X. nematophila* demonstrates high toxicity to larvae of *Galleria mellonella*. After inoculation of bacteria (independent dose), the colony reached more than one million colony-forming units (CFU's) in a short period of time ($\leq$ 24 hours). A trial conducted with adults of *Drosophila melanogaster* inoculated with *X. nematophila* showed similar results, with rapid death of the insects, but the colony reached one million CFU's in a shorter time ($\leq$ 18 hours). The same adults of *D. melanogaster* seemed to be highly resistant to *X. innexi* [114], which is also not effective in the death of larvae of *M. sexta*, while *X. nematophila* is highly toxic to both insects [43].

The insecticide activity of *Xenorhabdus* and *Photorhabdus* species is related to protein production [66, 110, 124, 125] and secondary metabolites [13, 69, 75, 126–128]. The secretion of toxins of high molecular weight by *P. luminescens* and *X. nematophila* plays an important role in insect mortality [29, 66, 125]. As described, pathogenicity is related to cell replication and production of toxins in the hemocoel causing histological injury, and septicemia [117].

Samples of *Xenorhabdus* produced toxins (Tcs) that induce immunosuppression in insects by inhibiting eicosanoid synthesis [108, 129]. *Xenorhabdus nematophila* produces about eight suppressor metabolites of insect immunity [109]. *Xenorhabdus budapestensis* produces hybrid compounds called fabclavins, which exhibit antibiotic and insecticide activities [69, 73, 130, 131]. Some species of *Photorhabdus* produce a variety of toxins including Tcs (toxin complexes), Mcf (make caterpillars floppy), Pvc (*Photorhabdus* virulence cassettes) and Pir (insect-related protein) [132]. The Tcs destroy epithelial cells from the middle intestine of insects, similar to δ-endotoxin of *B. thuringiensis* and acting on the actin cytoskeleton by the ADP-ribosyltransferases TccC3 and TccC5 in *P. luminescens* [132, 133]. On the other hand, Mcf promotes hemocytes apoptosis in the hemocoel [134]. It was also observed that *M. sexta* and *G. mellonella* are susceptible to Pvc [135] (Fig. 2).

Predictive genes of toxins, proteases and haemolysins are abundant in the TT01 strain of *P. luminescens laumondi*, which may be involved in pathogenicity [136]. In this strain, Pir proteins are related to insect death. The proteins are encoded by genes *plu4093-plu4092* for PirA and *plu4437-plu4436* for PirB, respectively. The corresponding proteins have similarity with δ endotoxins of *B. thuringiensis* and with a growth regulatory protein of *Leptinotarsa decemlineata*. *Photorhabdus luminescens* and *P. asymbiotica* Pir proteins heterologously produced in *Escherichia coli* have the ability to cause the death of larvae of *G. mellonella*, with high toxicity [137].

Previously, Bode [69] suggested further studies on bacterial secondary metabolites due to the possibility of their use as new control agents of agricultural pests and/or vector insects, such as mosquitoes. Some secondary metabolites produced by *X. nematophila* and *P. temperata temperata* are supposed to be responsible for the suppression of the enzyme phospholipase A_2, causing impairment in the eicosanoid biosynthesis [138] and consequently in the immune response of insects [139]. Two bacterial metabolites that can inhibit phospholipase A_2 are oxindole and benzylideneacetone in insects [138, 140]. Such metabolites have also been reported as potentiators of toxicity of *B. thuringiensis* against lepidopterans and coleopterans, acting in suppressing the immune response of these insects. [109, 141]. In cultures of *X. nematophila* and *P. temperata temperata*, seven metabolites with the function of inhibiting phospholipase A_2 were identified [138].

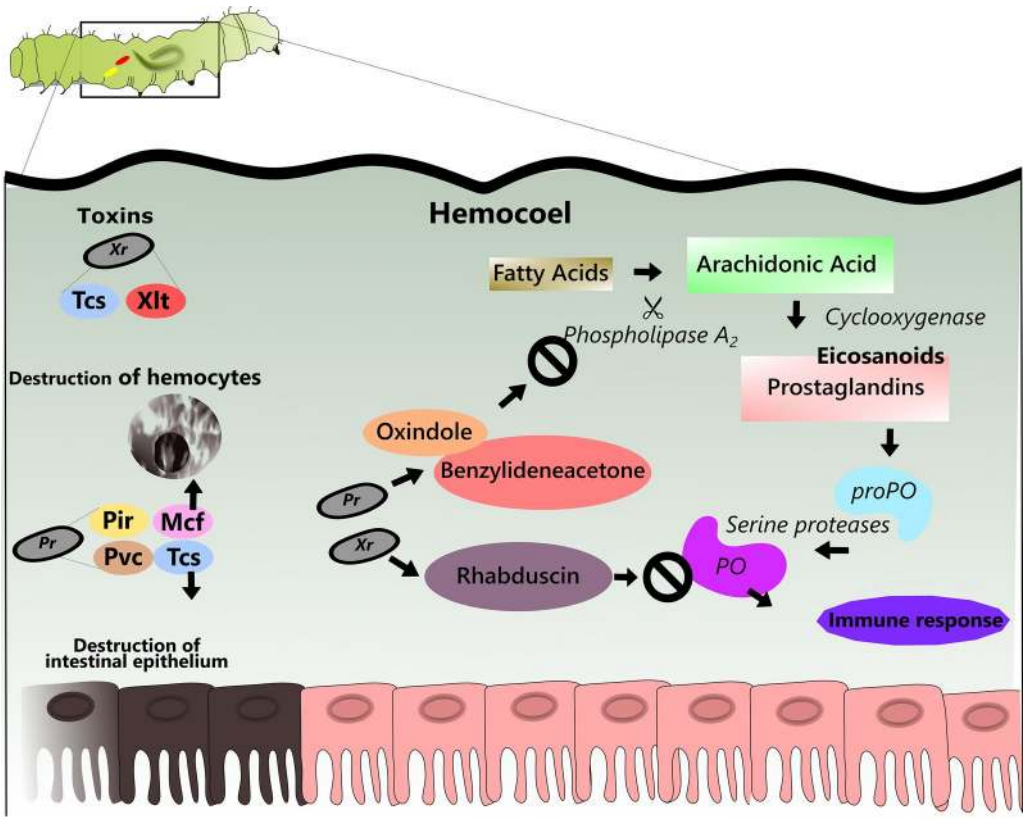

Fig. 2 Schematic drawing of toxins and mode of action of some compounds produced by the bacteria *Xenorhabdus* and *Photorhabdus*. *Xenorhabdus* can produce toxin complexes that induce immunosuppression in insects by inhibiting eicosanoid synthesis. The *Xenorhabdus* lipoprotein toxin produced by *X. innexi* has toxic properties against culicids. *Photorhabdus* also produces toxin complexes, which have activity directly in the intestinal epithelium of insects, leading to their destruction. Make caterpillars floppy causes apoptosis in hemocytes in the hemocoel. *Photorhabdus* virulence cassettes, encode genes that are toxic action against some species of lepidopterous. Insect-related protein is highly toxic and is similar to δ endotoxins of *Bacillus thuringiensis*. *Photorhabdus* can produce toxins that directly affect Phospholipase A_2, while *Xenorhabdus* produces toxins that inhibit phenoloxidase produced through prophenoloxidase, directly affecting the insect's immune system. *Abbreviations*: Xr, *Xenorhabdus*; Tcs, toxin complexes; Xlt, *Xenorhabdus* lipoprotein toxin; Pr, *Photorhabdus*; Mcf, make caterpillars floppy; Pvc, *Photorhabdus* virulence cassettes; Pir, insect-related protein; PO, phenoloxidase; proPO, prophenoloxidase

The phospholipase A_2 enzyme has a function of catalyzing fatty acids (mainly acynic acid) that will later be oxygenated by cyclooxygenase and lipooxygenase enzymes for the production of prostaglandins and leukotrienes, respectively, which are mediators of the immune response in insects [44, 138, 139, 142, 143]. Prostaglandins induce the release of profenoloxidase (proPO) of oenocytoids in plasma, for the formation of active phenoloxidase (PO) [144] which, in insects, is indispensable for the execution of humoral and cellular immune responses [44, 145].

Another common mechanism of *Xenorhabdus* in insect immunosuppression is direct suppression of the PO enzyme that is present in hemolymph in the inactive proPO form. PO is activated by proPO cleavage by protease serines [146]. Secretion of rebduscin by *X. nematophila* inhibits the activation of PO [138, 147] (Fig. 2).

Xenorhabdus innexi in association with *Steinernema scapterisci* is effective in killing some insects [114, 148, 149], mainly crickets [43]. In order to verify the immunosuppression capacity of insects by this bacterial species, Kim et al. [43] evaluated the inhibition capacity of PO activation in *M. sexta*. However, there was no secretion of immunosuppressive metabolites that could be detected in the trial performed with cell cultures. The same authors identified that the genome of *X. innexi* has a reduction in gene complements predicted to encode virulence determinants compared to other species of the same genus. However, *X. innexi* secret Xlt (*Xenorhabdus* lipoprotein toxin), which is a lipopeptide with toxic properties for culicids [118, 150].

The impact of *Photorhabdus* and *Xenorhabdus* on mosquito control

Previously, several authors described that *X. nematophila* secretes proteins and secondary metabolites that are effective in the control of culicids [69, 104], such as benzylideneacetone, [151] iodine, [72] phenethylamides and indol derivatives, [126, 128] xenorhabdins and xenooxides [128], and xenocoumacins [127] (Fig. 3).

Consequently, Gill et al. [152] described the benefits of using biological control agents with different mechanisms of action, because the synergistic effect could increase larvicidal potential and decrease the selection of resistant populations.

Ahantarig et al. [59] evaluated the larvicidal potential of *P. asymbiotica* against *Ae. aegypti*. The PirAB protein of this bacterial species were heterologously produced in *E. coli* for oral administration in larvae of the first stage of *Ae. aegypti* and *Ae. albopictus*. Mortality rates of up to 100% for the two mosquito species were observed. The concentration of 0.33×10^6 cells/ml of PirAB produced by *E. coli* was sufficient for killing all *Ae. aegypti* larvae within 24 hours. The bioassay was also performed with the copepod *Mesocyclops thermocyclopoides*, a species

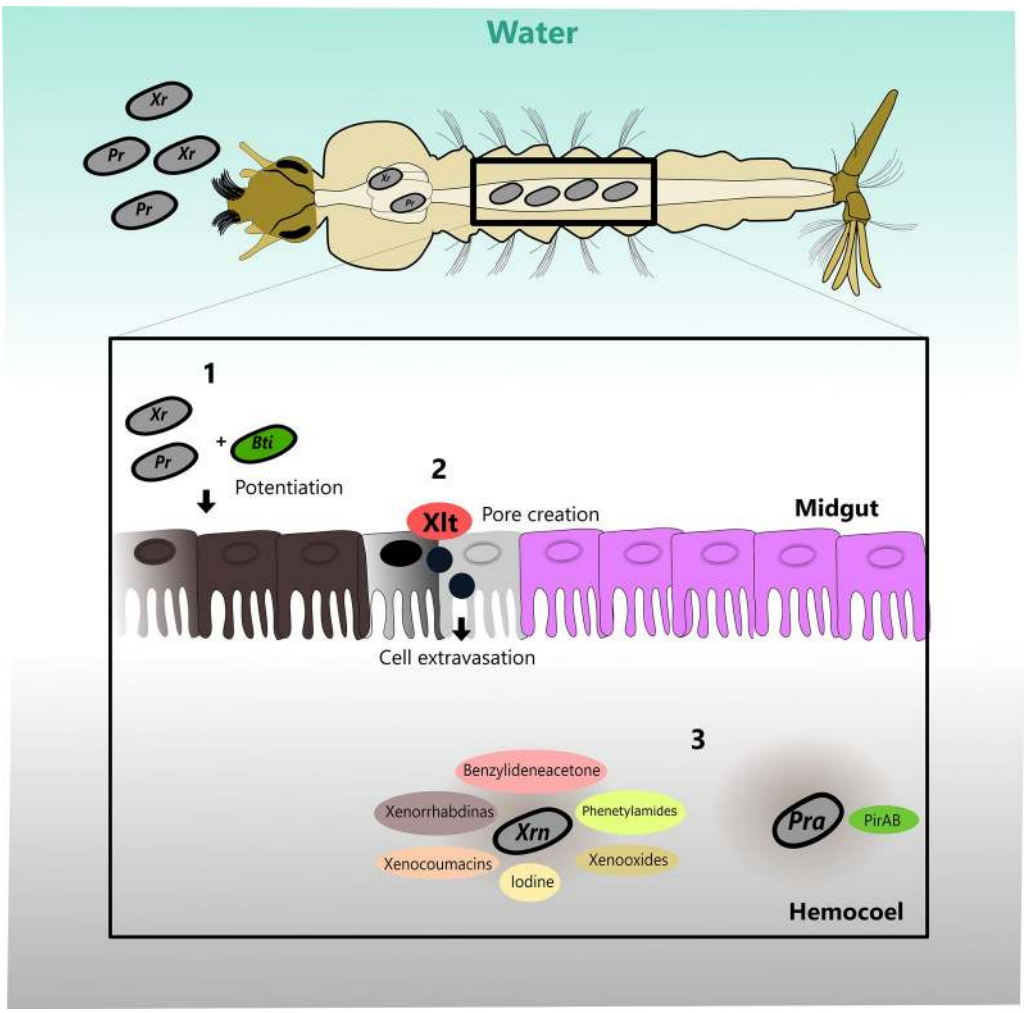

Fig. 3 Schematic drawing summarizing the mechanisms related to *Xenorhabdus* and *Photorhabdus* for the control of culicids. **1** *Xenorhabdus* and *Photorhabdus*in increase the toxic effect of Cry4Ba derived from *Bacillus thuringiensis* var. *israliensis* against *Aedes aegypti*. **2** *Xenorhabdus* liprotein toxin has the ability to create pores on the apical surface of cells in the anterior midgut of mosquitoes but in the anterior portion of the middle intestine, causing cell death. **3** *Xenorhabdus nematophila* (*Xrn*) secretes proteins and secondary metabolites that are effective in the control of culicids, while *Photorhabdus asymbiotica* (*Pra*) produce PirAB proteins, which have already been tested on *Aedes albopictus*, *Aedes aegypti* and are toxic even by oral administration. *Abbreviations*: Xr, *Xenorhabdus*; Pr, *Photorhabdus*; *Bti*, *Bacillus thuringiensis* var. *israliensis*; Xlt, *Xenorhabdus* lipoprotein toxin; *Xrn*, *Xenorhabdus nematophila*; *Pra*, *Photorhabdus asymbiotica*

used for larvae control (L1) of *Aedes*, whose result was negative for toxicity, as no mortality was observed.

The set of proteins that form PirAB have a greater toxic effect for *Ae. aegypti* larvae when compared with other Pir proteins in the oral bioassay [59]. It is noteworthy that the path of exposure of the larvae can interfere with the results. For example, Waterfield et al. [137] observed greater insecticide activity when injecting PirA + PirB proteins into the hemocoel of *G. mellonella*.

Shrestha et al. [153] evaluated culture fluids of five different isolates of *Photorhabdus* sp. on pathogenicity. Three days after oral ingestion of bacteria, the mortality of *Culex pipiens pallens* larvae was greater than 90%. However, of the insects tested, *P. luminescens laumondii* (TT01) could not cause mortality.

Subsequently, Silva et al. [29] evaluated the toxicity of *P. luminescens* and *X. nematophila* against *Ae. aegypti* larvae when they were feeding with these bacteria. After ingestion of bacteria, both species were toxic to the mosquito larvae within 96 hours. The authors also observed cannibalism among the larvae in all bioassays, after exposure to both bacterial species. This factor had previously been discussed by Koenraadt & Takken [154], who described several biotic and abiotic factors that can cause larval stress in aquatic environment. In this case, the presence of bacteria is discussed as a biotic factor that triggers cannibal behavior. Thus, Silva et al. [29] demonstrated that even in the absence of nematodes, the bacteria have a larvicidal effect in *Ae. aegypti* after being ingested. However, the molecular mechanism how the bacteria kill the larvae is still unknown.

In the context of mosquito control, several analyses were made aiming the establishment of new effective agents such as: (i) mixture of the culture broth of *P. temperata temperata* with *B. thuringiensis tenebrionis*, called "Col-Kill", demonstrated efficacy in the control of the coleopteran *Phaedon brassicae* [141]; (ii) for the control of lepidopteran *Plutella xylostella* and *Spodoptera exigua*, the mixture called "Dual Bt-Plus" by *B. thuringiensis kurstaki* and *B. thuringiensis aizawai* with culture broth *X. nematophila* [155]; and (iii) some authors described the ability of *Xenorhabdus* and *Photorhabdus* bacteria to increase the toxic effect of Cry4Ba derived from *Bti* against *Ae. aegypti* [29, 64]. In addition, mixtures of *X. nematophila* or *P. temperata temperata* were tested to verify the suppression effect of immune responses on insects and consequently increased toxicity of *B. thuringiensis* [44]. These authors also hypothesized that some metabolites of *Xenorhabdus* and/or *Photorhabdus* could be related to inhibition of eicosanoid synthesis and increased *Bti* toxicity against mosquitoes. Thus, they used a mixture of *Bti* spores with *X. nematophila* culture broth containing metabolites. This solution showed greater efficacy in the control of *Ae. albopictus* mosquitoes and *Cx. pipiens pallens*, with an increase in *Bti* toxicity against these insect species. Based on these results, they developed an insecticide called "Dip-Kill" [44]. The culture broths of *Xenorhabdus hominickii* and *P. temperata temperata* were also able to increase the toxicity of *Bti* against culicides [44].

The toxic effect of a bacterial cultures of *X. nematophila* and *P. luminescens*, was also tested in *Ae. aegypti* by Silva et al. [13]. Both culture broths of *X. nematophila* and *P. luminescens*, caused larvae mortality, and interfered the development of pupae and adults. These authors observed greater larvicidal stability of *X. nematophila* culture fluids exposed to high temperatures (100 °C) in contrast to *P. luminescens* culture fluids tested. The temperature labile pathogenicity of *Photorhabdus* bacteria may be related to both proteins and secondary metabolites that are relatively unstable [13]. However, bioactive compounds produced by *Xenorhabdus* are stable and therefore potential agents for a putative application in mosquito control [14].

The bacterium *X. innexi*, when injected into several species of insects, does not show insect pathogenicity, but when using cultures fluids of cells, some isolates presented larvicide activities against *Aedes*, *Culex* and *Anopheles*. The Xlt compound, derived from this bacterial species, has been described as a low molecular weight lipopeptide that has toxic activity against mosquito larvae. The protein composition has a high content of amino acids such as histidine, glycine, asparagine/aspartate, diaminobutyric acid and serine. The lipid portion has at least one oxo-fatty acid (C8 - C20) [150]. Thus, Kim et al. [118] analyzed Xlt's specificity and mechanism of action against mosquito larvae. Different doses were used for exposure of *Ae. aegypti* larvae, *Cx. pipiens* and *An. gambiae*. The authors observed that Xlt is mainly toxic to mosquito larvae, considering that pupae and adults were not affected in pathogenicity bioassays. The effects of Xlt were also observed by Kim et al. [118] in different cell strains of insects, including *Ae. aegypti* (Aag-2), *D. melanogaster* (S2) and *M. sexta* (GV1). After treatment for six hours (CL_{50} for mosquito larvae), no alterations in the cellular morphology of lepidopteran were observed. However, *Ae. aegypti* cells (Aag2) presented aggregation followed by induced apoptosis after the same exposure time. In the cell viability analysis (using SYTOX®), it was possible to observe that only mosquito cells emit fluorescence, indicating that Xlt at low doses has no toxic effects on non-target insect cells.

The Xlt toxicity was also evaluated in fibroblasts (Hs68) and mast cells (HMC-1), compared to *Ae. aegypti* strains (Aag-2) and *Ae. albopictus* (C6/36), and in 24 hours of treatment (1 ppm), more than 80% of mosquito cells were

killed. On the other hand, human cells were not affected at the same dose. Only with the dose of 50 ppm (significantly higher) of Xlt, the Hs68 fibroblast strain showed changes in cellular viability, with a decreased number of cells, but compared to mosquito cells Aag-2 and C6/36, the Hs68 strain presented greater viability after treatment with doses of 50 and 100 ppm. However, the HMC-1 human mast cell population strain showed an increase in the cell population after exposure to 10 and 50 ppm of Xlt. According to the authors, the increase in the number of HMC-1 mast cells may have occurred due to the stimulation of peptides or lipoproteins that induce the activation of these cells that are components of the immune system [156]. Thus, demonstrating that Xlt of *X. innexi* it is more toxic to mosquito cells in comparison to human cell strains [118].

Due to the existence of Xlt toxicity after ingestion by the larvae, it is possible that the mechanism of action could be similar to *Bti* toxins that act in the midgut of insects [157]. However, Kim et al. [118] suggested that Xlt has the ability to create pores on the apical surface of cells in the anterior midgut of mosquitoes but in the anterior portion of the middle intestine, causing cell death. In *Ae. aegypti* larvae at the beginning of the fourth stage, after exposure to Xlt, the pH of the anterior midgut became more acidic. According to Boudko et al. [158], the rupture of intestinal integrity causes a decrease in the pH. Although both *Bti* and Xlt act in the intestines of the larvae, it is worth mentioning that there are probably specific cell connection sites, spatially altering the place of action of biological agents against mosquito larvae [118], as it has been demonstrated that *Bti* acts at the posterior portion of the middle intestine [159, 160] (Fig. 3).

Other bacterial isolates were also tested for toxicity to insects that did not present significant effects on mortality. Some of these are listed here: (i) *Xenorhabdus stockiae* (bLPA12.2_TH, bCR7.3_TH and bPH23.5_TH), *Xenorhabdus miraniensis* (bMH16.4_TH, bMH16.1_TH and bMH4.5_TH) and *Photorhabdus* (bPY17.4_TH, bLPO16.2_TH, bMH8.4_TH and bNA22.1_TH) were not effective in the mortality of *Ae. aegypti* larvae [27]; and (ii) *Xenorhabdus japonica* (bNN165.4_TH) and *Xenorhabdus vietnamensis* (bNN167.2_TH and bNN167.3_TH) presented low toxicity to *Ae. aegypti* and *Ae. albopictus* larvae, probably due to the absence of secretion of toxic metabolites for the species of tested culicids [14]. Other factors may be related to the mortality or survival of insects, such as the number of bacterial cells ingested, production of metabolites in water and, difference in compounds secreted by different bacterial strains [14].

Recently Kajla et al. [161] described *Xenorhabdus budapestensis* fabclavines activities with mosquito-repellent action. The authors found that compounds of *X. budapestensis* cultures are capable of inhibiting artificial hematophagy of females of *Aedes*, *Anopheles* and *Culex*, probably due to the presence of fabclavines. They argue that possibly amino acids asparagine/aspartate and histidine, perhaps 2,3-diaminobutyric acid, would be related to the repellent effect of bacteria to mosquitoes [73]. It has been shown that this repellent activity of compounds secreted by *X. budapestensis* may be superior to the repellents commonly used against *Ae. aegypti*, such as DEET or picaridin [161]. The authors suggested that these bioactive compounds of *Xenorhabdus* and *Photorhabdus* may exercise larvicidal action against mosquitoes when used in breeding sites, because they are used as a food source of the larvae.

Perspectives on applicability in mosquito control

Bacteria of the genera *Photorhabdus* and *Xenorhabdus* stand out for being very effective to control *Ae. aegypti* after oral uptake by the mosquito larvae. *Photorhabdus luminescens* and *X. nematophila* were used as a food source for *Ae. aegypti* larvae. After 24 hours 50% of larvae were dead, culminating to 100% in 96 hours [29]. However, by exposing *Ae. aegypti* larvae to a series dilution of *P. luminescens* and *X. nematophila* crude culture fluids diluted in distilled water, 100% mortality was observed after 4 hours for both bacteria species. So, it seems that crude culture fluids of both *Photorhabdus* and *Xenorhabdus* are highly effective in a short period of time after oral intake to obtain mortality against larvae of *Ae. aegypti*, compared to other pathogenic bacteria for mosquitoes. For example, vegetative cells of *B. thuringiensis* need at least 12 hours to kill *Ae. aegypti* larvae [162] or, the strains of *B. thuringiensis* (SV2) and *Serratia* sp. (SV6), which only reach mortality of 50% after six and 12 hours of exposure, respectively, in larvae of *Ae. aegypti*, *Anopheles stephensi* and *Cx. quinquefasciatus* [163].

Fukruksa et al. [27] also noted advantages of the use of *Xenorhabdus* and *Photorhabdus* due to the rapid capacity of mortality range of larvae of *Aedes* spp. *Xenorhabdus ehlersii* (bMH9.2_TH) presented greater effectiveness against both fed and non-fed *Ae. aegypti* larvae, with a range of 100% mortality in up to 96 hours. On the other hand, for larvae of the same mosquito species, the isolate of *Xenorhabdus stockiae* (bLPA18.4_TH) has a mortality rate greater than 60% in 72 and 96 hours. The authors highlight the potential of isolate *X. ehlersii* bMH9.2_TH as more pathogenic, opening possibilities for *X. ehlersii* to be a biological control agent for *Ae. aegypti*.

The isolate (bNN112.3_TH) from *X. stockiae* has been tested for exposure of *Ae. aegypti* larvae, in which the authors observed 99% mortality after 96 hours. Another bioassay, with *Ae. albopictus*, demonstrated mortality of

98% of larvae after exposure of 96 hours to *P. luminescens akhurstii* (bNN121.4_TH). The authors highlight the potential of these isolates as control agents against the two species of mosquitoes to the bacteria they were exposed to [14].

The evidence that *Xenorhabdus* and *Photorhabdus* bacteria synthetize a diversity of secondary metabolites opens possibilities for these compounds to be even more specific and potent agents of biological control of mosquitoes. For example, the chemical change in the structure of fabclavines or their use in combination with chemical or biological insecticides is already established. For the use of these metabolites in the control of culicids, toxicity to other insects, aquatic organisms and humans has to be evaluated. Field applicability and feasibility of large-scale production also has to be analyzed in the future [26].

In addition, research aimed at the isolation, identification and characterization of bioactive compounds is of vital importance for elucidating the mechanisms of action of secondary toxins/metabolites that are responsible for the death of *Ae. aegypti* larvae [13, 29]. However, the molecular mechanisms of action were not elucidated. PirAB proteins are larvicide potentials for the control of vector mosquitoes, and it is necessary to conduct studies on biosafety aspects for the use of these proteins [59]. Finally, it is necessary to understand the application of these bioactive compounds to be implanted in the biological control of mosquitoes [27]. Therefore, it is necessary to study the period of activity of these entomopathogenic bacteria and their toxic compounds, as well as the time of their residual effect on mosquito breeding sites.

Conclusions

The current methods of controlling these mosquitoes, which are indicated by World Health Organization, have shown problems as a high cost for biological and chemical control. They often have low specificity for the target organisms and are therefore also toxic to non-target organisms, including humans. In addition, recent studies reported resistance to the most commonly used insecticides, such as the organophosphate temephos and pyrethroids in several populations of *Ae. aegypti* and *Ae. albopictus* distributed worldwide. Thus, the control of these insects depends on a wide variety of chemical and biological arsenals that can contribute to the prevention of their control. Entomopathogenic bacteria such as *Photorhabdus* and *Xenorhabdus* should be considered in these arsenals, since so many researchers have demonstrated their efficiency against mosquitoes. Therefore, this observation opens possibilities for more insect specific compounds and potent agents of biological control of mosquitoes. Entomopathogenic bacteria have to be urgently considered for mosquito control in the near future.

Abbreviations

Aag-2: Cell strain of *Aedes aegypti*; *Bti*: *Bacillus thuringiensis israelensis*; C6/36: Cell strain of *Aedes albopictus*; CFU's: Colony-forming units; EPNs: Entomopathogenic nematodes; GV1: Cell strain of *Manduca sexta*; HMC-1: Human mast cell lines; Hs68: Human fibroblasts cell lines; Mcf: Make caterpillars floppy; pH: Power of hydrogen; Pir: Insect-related protein; PO: Phenoloxidase; Ppm: Parts per million; *Pr*: *Photorhabdus*; *Pra*: *Photorhabdus asymbiotica*; proPO: Prophenoloxidase; Pvc: *Photorhabdus* virulence cassettes; S2: Cell strain of *Drosophila melanogaster*; SV2: Cell strain of *Bacillus thuringiensis*; SV6: Cell strain of *Serratia* sp; TccC3 and TccC5: *Photorhabdus luminescens* toxins; Tcs: Toxin complexes; Xlt: *Xenorhabdus* lipoprotein toxin; *Xr*: *Xenorhabdus*; *Xrn*: *Xenorhabdus nematophila*.

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Authors' contributions

WJS, HPL-J, RH and OSS participated in the design of the study and drafted the manuscript. OSS participated in the study coordination and helped draft the manuscript. RH participated in the review and editing. HLP-J designed and prepared the manuscript figures. WJS wrote the manuscript. All authors read and approved the final manuscript.

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