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DEVELOPMENT OF INNOVATIVE BIOPESITICIDES FOR THE CONTROL OF *AEDES ALBOPICTUS* MOSQUITOES

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DOCTORAL THESIS

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Mustapha TOURAY

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LIST OF SYMBOLS AND ABBREVIATIONS

°C **:** Degree Celsius

µl **:** microliter

ACP **:** acyl carrier protein

AT **:** acyltransferase

ATCC **:** American Type Culture Collection

BGC **:** Biosynthesis gene clusters

Bti*:* *: Bacillus thuringiensis* subsp. *israelensis*

Cm **:** centimeter

Cfu **:** colony forming units

DNA **:** Deoxyribonucleic Acid

DH **:** dehydratase

easyPACId **:** easy Promoter Activated Compound Identification

EPN **:** Entomopathogenic Nematode

ER **:** enoylreductase

FAS : fatty acid synthases

IJ **:** Infective Juvenile

ITN : Insecticide treated nets

KR **:** ketoreductase

KS **:** ketosynthase

ml **:** milliliter

nAChRs : Nicotinic Acetylcholine Receptors

NBTA **:** Nutrient Bromothymol Blue Agar

NRPS **:** Non Ribosomal Peptide Synthetase

NP : Natural product

OD **:** optical density

PKS **:** Polyketide synthetase

PirAB proteins : *Photorhabdus* insect-related proteins A and B

PPTase **:** Phosphopantetheinyl Transferase

RNA **:** Ribonucleic acid

Rpm **:** rotations per minute

Tca **:** toxin complex A

LB **:** Luria Bertani broth

WHO **:** World Health Organization

xcn **:** Xenocoumacine

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ÖZET

AEDES ALBOPICTUS SİVRİSİNEK TÜRÜYLE MÜCADELEDE YENİ VE ETKİLİ BİYOPESTİSİTLERİN GELİŞTİRİLMESİ

Touray M. Aydın Adnan Menderes Üniversitesi, Fen Bilimleri Enstitüsü, Biyoloji Programı, Doktora Tezi, Aydın, 2023.

Amaç: Küresel halk sağlığı açısından büyük önem taşıyan ve 20'den fazla viral patojene vektörlük yapabilen *Aedes albopictus* sivrisinek türüyle mücadelede *Xenorhabdus* ve *Photorhabdus* cinslerine ait bakteri sekonder metabolitlerden yeni ve etkili biyopestisitlerin geliştirilmesi.

Materyal ve Yöntem: Bu tez çalışması kapsamında farklı *Xenorhabdus* ve *Photorhabdus* türlerine ait hücreleri uzaklaştırılmış supernatant, bakteri kültürü ve hücre süspansiyonlarının *Aedes albopictus* türünün 3-4. dönem larvalarına karşı aktivitesi belirlenmiştir. Çalışmalar 24 kuyucuklu kültür plaklarında yürütülmüştür ve deney sonuçları 24 ve 48 saat sonunda kontrol edilmiştir. Bunun yanısıra bakteri supernatantlarının, sivrisinek yumurtaları üzerindeki ovisidal etkinliği de test edilmiştir. Daha sonra laboratuvar ortamında en yüksek aktivite gösteren türlere ait Δ*hfq* promotor bölgesi değiştirilmiş mutant bakteriler elde edilip etken biyoaktif madde/lerin tespit edilmesi amacıyla çalışmalar yapılmıştır.

Bulgular: Çalışmalar sonucunda kullanılan supernatantlardan, 3 *Photorhabdus* ve 13 *Xenorhabdus* türünün larvisidal bileşikler ürettiği tespit edilmiştir. Bununla birlikte neredeyse test edilen tüm türlerin bakteri kültürü larvisidal aktivite göstermiştir. Bakteri hücreleri uygulandıkları larvalarda oral toksisiteye (%59-91) sebep olmuşlardır. Genel olarak bakteri supernatantlarında sivrisinek yumurtalarına karşı ovisidal bir etki gözlenmemiştir ancak yumurtadan çıkan 1. dönem larvalarda >%75 oranında ölüm meydana gelmiştir. Mutant bakterilerle yürütülen deneyler sonucunda biyoaktiviteden sorumlu etken maddeler Fabclavin ve Xencoumacin olarak tespit edilmiştir. Fabclavinler, ticari *Bacillus thuringiensis* subsp. *israelensis* ve spinosad ürünleri ile birlikte temiz suda ve sahadan

toplanan suda 5dpa içinde aynı performansı göstermiştir.

Sonuç: Tez çalışması sonucunda elde edilen veriler, birçok *Xenorhabdus* ve *Photorhabdus* bakteri türünün hücre haliyle veya ürettiği sekonder metabolitler aracılığıyla larvisidal aktivite gösterdiğini ortaya koymuştur. Biyoaktif maddeler olan Fabclavine ve Xenocoumacin yeni biyolarvisitler olarak geliştirilebilme veya farklı etken maddelerin sentezlenmesinde model olarak kullanılabilme potansiyeline sahiptir.

Anahtar kelimeler: *Aedes albopictus*, Biyopestisit, Fabclavine, Larvisit, Xenocoumacin.

ABSTRACT

DEVELOPMENT OF INNOVATIVE BIOPESITICIDES FOR THE CONTROL OF *AEDES ALBOPICTUS* MOSQUITOES

Touray M. Aydin Adnan Menderes University, Graduate School of Natural and Applied Sciences, Biology Program, Doctorate Thesis, Aydin, 2023.

Objective: Determination of the larvicidal activity of bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus* and bioactive compound/s against *Aedes albopictus* mosquito which is capable of serving as vector of over 20 different arboviruses of major global public health concern.

Material and Method: The efficacy of cell-free supernatant (CSF), growth culture and re-suspended bacterial pellet against late 3rd-4th stage *Ae. albopictus* larvae was evaluated in wells of a 24-well plates. Larval mortality was assessed after 24 and 48 h. The ovicidal effects of these bacteria CFS on mosquito eggs were also evaluated. Supernatants of the most effective *Xenorhabdus* spp. Δ*hfq* promoter exchange mutants were used to identify the bioactive compound/s.

Results: Three *Photorhabdus* spp. and 13 *Xenorhabdus* spp. release larvicidal compounds in CSF. Cell growth culture of nearly all tested species exhibited larvicidal activity. Bacterial cells exhibited oral toxicity (59-91%) against exposed larvae. Considering ovicidal activity, overall, bacterial CSF were ineffective against mosquito eggs but after hatching, >75% of emerged 1st instar larvae died in treatments. Using the easyPACId approach, the larvicidal compounds were identified as Fabclavine and Xencoumacin. Fabclavines were as effective as commercial *Bacillus thuringiensis* subsp. *israelensis* and spinosad products within 5dpa in distilled and field collected water.

Discussion: An extensive number of *Xenorhabdus* and *Photorhabdus* display larvicidal activity as cells or by producing secondary metabolites with larvicidal activity. The bioactive compounds, Fabclavine and Xenocoumacin, can be developed in novel biolarvicides or can be used as a model to design and synthesize other compounds.

Key words: *Aedes albopictus*, Biopesticide, Fabclavine, Larvicidal, Xenocoumacin.

1. INTRODUCTION

In the past century, there has been a drastic decline in the toll of death from infectious diseases, particularly in industrialized nations, as a result of improved therapeutics as well as better living conditions and standards. In resource-limited areas of the world like Africa, South Asia and South America, however, insect-vectored infectious diseases like malaria, yellow fever, dengue, zika, leishmaniasis, bluetongue disease, etc. continue to cause epidemics affecting thousands to millions of people and animals. These diseases account for ~17% of the estimated worldwide burden of communicable diseases and are transmitted during feeding by hematophagous dipterous insects like mosquitoes, sandflies, midges, fleas, tsetse flies, black flies, lice, reduviid bugs (Roberts, 2002; Hamer and Bhutta, 2015).

Mosquitoes are a group of small flies within the Culicidae family found all over the world from the arctic to the tropics. They have a long hairy, slender and segmented body with long legs, and most of their body is covered with scales. They are grouped into three subfamilies: Culicinae, Anophelinae, and Toxorhynchitinae. Adult females of Anophelinae and Culicinae have an elongated proboscis designed for piercing and sucking blood from vertebrate animals; male proboscis is adapted for sucking plant nectar and juices. Toxorhynchitinae lack piercing-sucking mouthparts and larvae are predacious on mosquito larvae and other aquatic microorganisms (Eldridge, 2005; Service, 2012; Harbach and Besansky, 2014). Currently there are over 3500 described mosquito species in the world. Species in the genera *Aedes, Anopheles, Culex, Mansonia, Psorophora, Haemagogus,* and *Sabethes* are prominent and efficient vectors of bacterial, viral and nematode pathogens of life-threatening diseases which may directly or indirectly impact public health, restrict rural and urban development as well as compromise food security (Eldridge, 2005; Service, 2012; WHO 2017).

Aedes (*Stegomyia*) *albopictus* Skuse (the Asian tiger mosquito) vectors over 20 different arboviruses of major global public health concern (Figure 1, Table 1). They include yellow fever, which leads to kidney and liver failure, and jaundice; Dengue Fever, which can give patients a characteristic skin rash; Zika virus, which can cause birth defects

like microcephaly during pregnancy; and Chikungunya virus, which can leave victims with debilitating joint pain (Wilder-Smith et al., 2017; Becker et al., 2021). The epidemic potential of these arboviral diseases has increased globally since the 1950s. Foremost among these diseases, Dengue has the greatest impact on human morbidity and mortality in more than 100 countries. In the last 30 years, dengue incidence has increased 4-fold with over 100 million infections, half a million cases of dengue hemorrhagic fever, and at least 40.000 deaths reported annually. Most cases occur in children aged 15 and under in resource-poor countries (Gubler, 2002; Hamer and Bhutta, 2015; Zheng et al., 2021). Similarly, yellow fever is endemic in 13 Central and South American countries and 34 African countries and every year 200.000 cases and 29.000-60.000 fatalities are reported even though there is an effective vaccine (YF vaccine) (WHO 2014; Gianchecchi et al., 2022). Approximately, 600.000 Chikungunya and Zika cases annually are seen in the Americas. Zika has been prevalent in mainly South America. Zika virus transmission persists at low levels in several countries in the Americas and in other endemic regions and the number of Zika cases continue to decline globally since 2017.

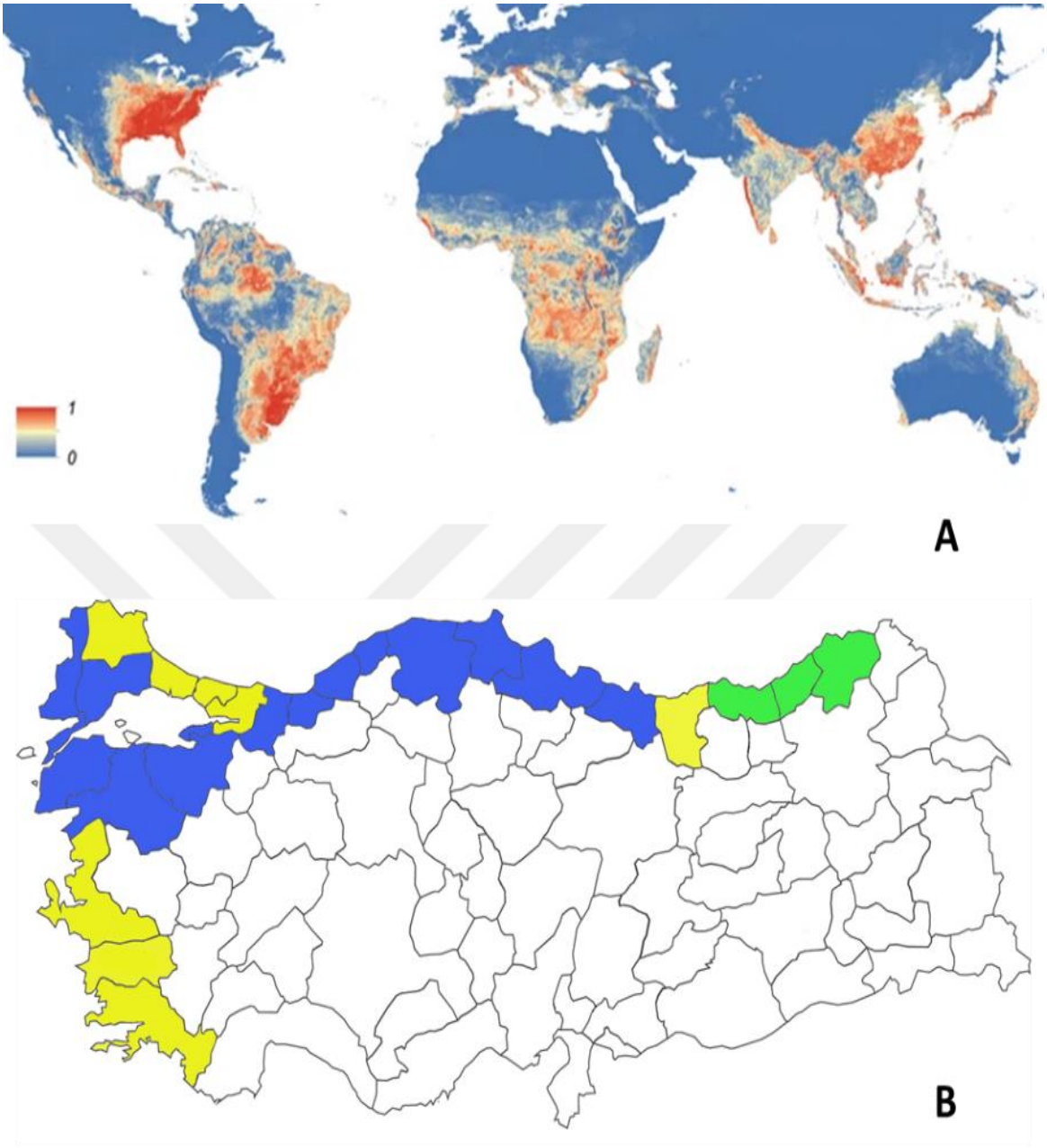


Figure 1.1. Global map depicting the probability of occurrence of *Aedes albopictus* around the world (Kraemer et al., 2015) (A). Distribution of *Aedes aegypti* and *Ae. albopictus* in Turkiye (B). Yellow and blue (M. Akiner, personal communication) indicate the presence of *Aedes albopictus* and green color indicates presence of *Aedes aegypti* and *Ae. albopictus*

Table 1.1. Some arboviral diseases transmitted by *Aedes albopictus*

Disease	**Agent**	**Family**	**Hosts**	**Mosquito vectors**	**Vaccine**	**Treatment**
Dengue	Dengue virus 1-4	Flaviviridae	Human	*Ae. aegypti*, *Ae. albopictus*	CYD-TDV vaccine*	Palliative
Yellow fever	Flavivirus	Flaviviridae	Human	*Ae. aegypti*, *Ae. albopictus*, *Haemagogus spp.*	YF vaccine	Palliative
Zika	Zika virus	Flaviviridae	Human	*Ae. aegypti*, *Ae. albopictus*	–	Palliative
Chikungunya	Chikungunya virus	Togaviridae	Human	*Ae. aegypti*, *Ae. albopictus*	–	Palliative
West Nile virus	West Nile virus	Flaviviridae	Human	*Culex* spp, *Ae. albopictus*	–	Palliative
La Crosse encephalitis	La Crosse virus	Peribunyaviridae	Human	*Ae. triseriatus*, *Ae. albopictus*, *Ae. japonicus*	–	Palliative
Venezuelan equine encephalitis	Venezuelan equine encephalitis virus	Togaviridae	Equine species, Human^{+}	*Aedes* spp.	TC-83, C-84	Palliative
Eastern equine encephalitis	Eastern equine encephalitis virus	Togaviridae	Equine, Birds, Human	*Culiseta melanura*, *Aedes* spp.	–	Palliative
Japanese encephalitis	Japanese encephalitis virus	Flavividae	Equine, pigs^{+}, human^{+}	*Culex* spp., *Ae. albopictus*	–	Palliative
Dirofilariasis (Heartworm disease)	*Dilofilaria immitis*; *D. repens* nematodes	Onchocercidae	Dogs, wolfs, foxes, cats, Human^{+}	*Ae. albopictus*, *Ae caspius* and *Cx. pipiens sl*	–	Antifilarial medication

*Live attenuated virus licensed in 2015 and approved for use in children aged 9–16 years. $^{+}$ Dead-end hosts

Transovarial transmission of certain arboviruses can occur in this mosquito (Ferreira-de-Lima and Lima-Camara, 2018). *Aedes albopictus* can also transmit filarial nematodes in the genera *Dirofilaria* and *Seratia* which infect domestic animals such as dogs (Paupy et al., 2009; Medlock et al., 2012).

Aedes albopictus is a synanthropic mosquito originally native to tropical and sub-tropical regions of Asia. It is an invasive and a highly adaptive species currently undergoing a dramatic global expansion. The persistence and dissemination of this mosquito toward higher latitudes has been greatly facilitated by the transport of its drought-resistant and dispausing eggs in bamboo plants, used tires, and artificial containers during global trade and shipping activities to temperate regions in northern latitudes (Kraemer et al., 2015).

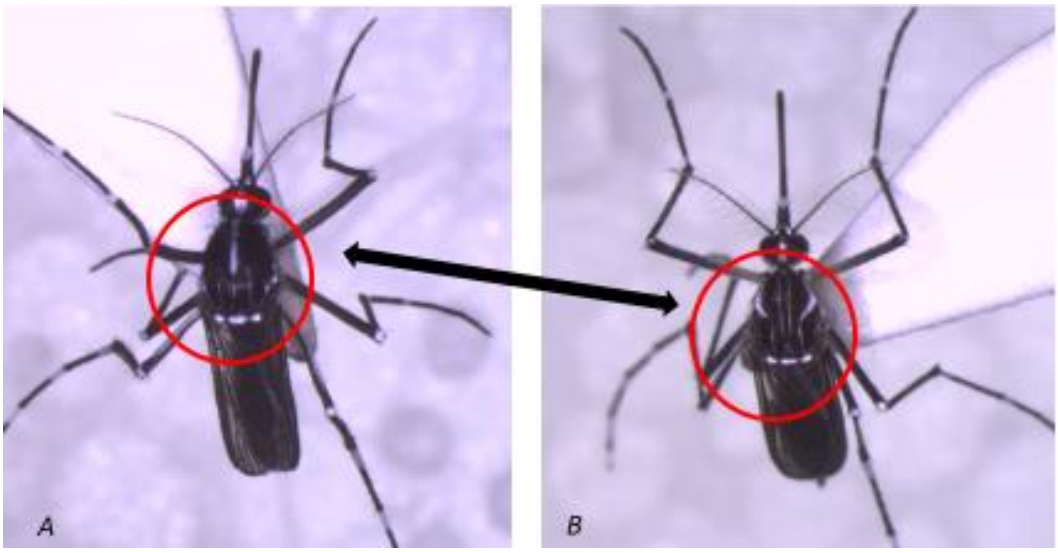
Eggs of temperate strains are capable of withstanding cold conditions up to -10 °C. Drastic deviations in global climate caused by natural or anthropogenic activities that increase greenhouse gas emissions has also made its establishment in new habitats easier (Thomas et al., 2012; Faraji and Unlu, 2016). It is now widely distributed in at least 30 countries throughout the tropics, subtropics and temperate regions of the world outside Asia (Benedict et al., 2007; Fonseca et al., 2013). In Europe, *Ae. albopictus* has been detected in Austria, Albania, Bosnia and Herzegovina, Belgium, Bulgaria, Croatia, France, Czech Republic, Greece, Romania, Russia, San Marino, Germany, Malta, Italy, Montenegro, Monaco, the Netherlands, Slovakia, Slovenia, Serbia, Switzerland, Spain, Turkiye, United Kingdom and the Vatican City (Vaux and Medlock, 2015; Medlock et al., 2015; 2017).

In Turkiye, *Ae. albopictus* is found in Thrace and Istanbul in Thrace Region, in the provinces of Artvin, Rize, and Trabzon in the Black Sea Region and most recently in İzmir and Aydın in Aegean regions (Figure 1) (Akiner et al., 2016, 2018; Demirci et al., 2021; Şakacı, 2021; Yavasoglu et al., 2021). It has been detected in various other provinces especially around ports (M. Akiner, personal communication). This species is primarily found in discarded tires, which are stored and can stay undisturbed for a long time or in cemeteries. In cemeteries there are numerous water-filled ceramic and plastic containers, blood from people and animals, sugars from flowering plants, and shelter (Vezzani, 2007; Snr et al. 2011; Susong et al. 2022). Due to its tolerance of cold temperatures and opportunistic feeding behavior on a wider host range including man, domestic and wild animals, *Ae. albopictus* has been found farer north in temperate regions than *Ae. aegypti,* which is another medically important *Aedes* species (Paupy et al., 2009; Medlock et al., 2012, 2015). International travel is an underlying factor that contributes to the dispersal and persistence of this mosquito from established areas to areas where they have invaded and become established. Once established in a new area, they can be vectors for disease transmission or increase the risk of disease emergence in their newly established home. Thus monitoring of these vector species at maritime, aerial, and terrestrial ports of entry is essential to prevent introduction to new areas (Touray et al., 2023).

1.1. Morphology and bionomics of *Aedes albopictus* mosquitoes

Aedes albopictus adults are moderately small (2-10 mm long) with black and white markings and a peculiar white band stripe located on the dorsal part of their thorax (scutum) (Picture 1 and 2). This marking pattern is due to the presence of white scale patches. This species can be confused with *Ae. aegypti* as both have similar appearances and behavior. They can be separated by the characteristic difference of stripes on their thorax: *Ae. aegypti* has two white stripes in the shape of a lyre/violin, whereas *Ae. albopictus* has one white longitudinal stripe (Picture 3) (Rueda, 2004). Both these *Aedes* mosquitoes are daytime-biting species that breed in similar sites such as in natural and artificial water-filled containers found in and around human dwellings house like tree holes, plastic containers, used tires, clogged gutters etc. (Service, 2012; Medlock et al., 2012, 2015).

Picture 1.1. *Aedes albopictus* female with characteristic black and white markings



Picture 1.2. Differences in marking pattern on thorax of *Aedes albopictus* (A) and *Aedes aegypti* (B)

Mosquitoes are holometabolous insects with four stages in their life cycle. As semiaquatic organisms, the egg, larva and pupa stages are found in water. *Aedes albopictus* females take blood meals from a variety of host including humans, domestic and wild animals, birds, amphibians, and reptiles (Medlock et al., 2015). After digesting a blood meal, gravid *Ae. albopictus* females lay eggs on the inner walls of containers with water and just above the waterline. These eggs are desiccation resistant and can remain viable for up to 8 months but under favorable conditions larva will emerge from these eggs within two to three days and will start feeding on the microorganisms and organic matter found in the environment (Berker et al., 2010). Larvae molt their exoskeleton thrice before proceeding to the pupal stage which does not feed and the tissues of the adult stage is form (Figure 2). *Aedes albopictus* are multivoltine species with 5-17 generations per year (Berker et al., 2010, 2020).

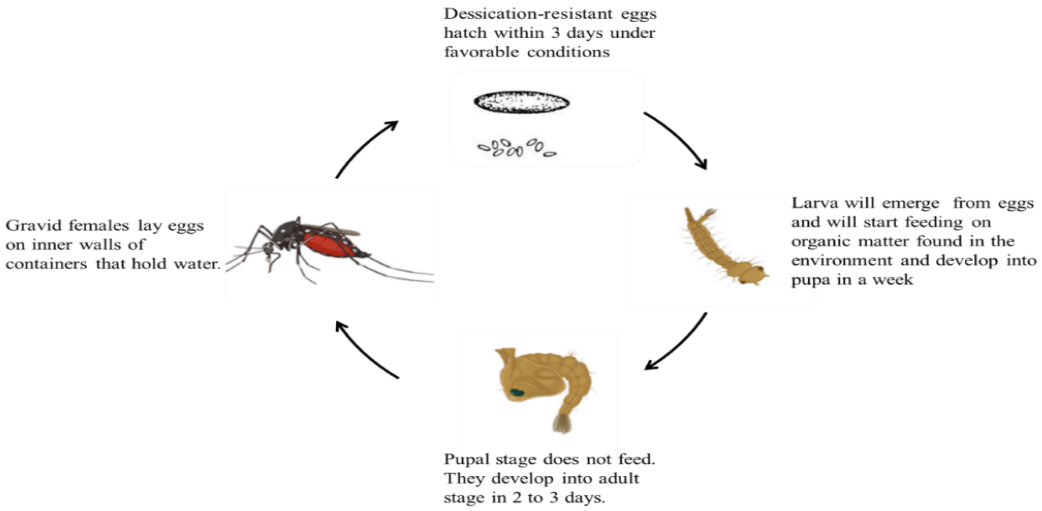


Figure 1.2. Life cycle of *Aedes albopictus* mosquitoes

1.2. Control of mosquitoes

The incidence of mosquito-borne diseases in several places are essentially spurred by climate and land-use change, ineffective control efforts, drug resistance, and various anthropogenic factor that favor the presence of mosquito vectors and thus pathogens (Patz and Olson, 2008). Since 2014, major dengue, chikungunya, zika and yellow fever epidemics have occurred in several countries, overwhelming their health systems and claiming the lives of thousands of people (WHO 2017). Annually, huge amounts of money are spent on the prevention and control of mosquito-borne disease; however, there has been a critical deficiency of vaccines or therapeutics for most of these transmitted diseases. Besides yellow fever, *Aedes*-transmitted diseases have no vaccine or effective therapeutics (MacKenzie et al., 2004; Monath, 2005; Guzman, 2005).

The best alternative option is the management of the mosquito populations to thresholds that impede transmission. But first, the most important part in the mosquito control activities is the need for a well-organized monitoring program to assess the occurrence, dispersal, and abundance of the target populations. This includes inspections of potential breeding sites, employing ovitraps and adult traps, and human-landing-collections. Breeding sites need to be inspected for mosquito developing stages. This can be done before the control stage.

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 mosquito breeding sites since most of their lifespan is in aquatic habitats (WHO, 2018). Mostly, trained personnel from the municipalities, health ministry, or tourism agencies in touristic areas around the world use pyrethroids (cypermethrin, permethrin, prallethrin, resmethrin, bifenthrin, deltamethrin, cyfluthrin, vs.) against adult mosquitoes in open areas using ultralow-volume aerial sprays. These chemicals are applied in open areas during warm summer months whereas hibernating areas such as barns are targeted in the winter. People can use indoor residual spraying or insecticide impregnated nets (pyrroles-pyrethroids nets) (Giunti et al., 2023; Touray et al., 2023).

The effects of chemical-based control, however, are generally short-termed and some have detrimental effects on human health, other non-target organisms and the environment. In biological control, predators, parasites, pathogens, competitors of mosquitoes or their toxins are used to control mosquito populations (Debach and Rosen 1991; Moazami 2011; van Lenteren et al., 2018). Mosquitoes have a variety of natural enemies ranging from viruses, bacteria, fungi, nematode, other insects to vertebrate predators. 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, *Lysinibacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) bacteria and spinosad toxin obtained from *Saccharopolyspora spinosa* bacteria are the only bacterial larvicidal products available to control mosquito larvae. These larvicides are applied to mosquito breeding sites to kill larva before they develop into adults (Lacey, 2007; Benelli, 2016). *Bti* and *L. sphaericus* are soil-inhabiting bacteria that during sporulation produce parasporal crystalline inclusions/bodies containing multiple gut disrupting toxins, which are toxic when ingested by mosquito larvae as well as blackfly and fungus gnat larvae. Spores produced can only be activated by larva's gut pH and enzymes (Gupta and Dikshit, 2010; Lacey and Georgis, 2012; Damalas and Koutroubas, 2018). Spinosad, composed of spinosyn A and spinosyn D factors, is active on contact and by ingestion; it affects insect nervous system by targeting nicotinic acetylcholine receptors (nAChRs) (Hertlein et al., 2011; Bunch et al., 2014).

Besides these larvicidal agents insect growth regulators such as methoprene, pyriproxyfen and diflubenzuron are used to control larval stages. The chemicals mimic insect hormone. They disrupt the development of insects by causing physiological alterations that prevent or impede reproduction, egg-hatch, and molting and they present low acute mammalian toxicology and are relatively safety to non-target organisms (Bellini et al., 2020; Giunti et al. 2023).

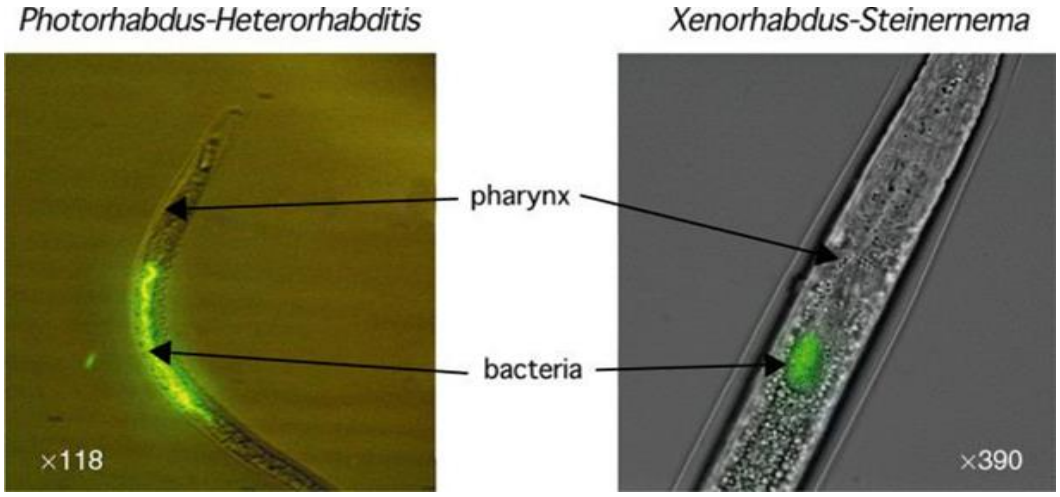
These aforementioned control strategies have had successes in implemented countries (Becker, 2006; Hiwat et al., 2012); but, recently there has been a dearth in the armamentarium of vector control mainly due to increased resistance to available pesticides, lack of new approaches, and/or new pesticides and formulations with novel modes of action to augment and supplant current ones. Also, chemical pesticides should only be a last resort because of the major health and environmental risk linked with their use (WHO, 2018). In the case of *Bti/L. sphaericus,* these biolarvicides sediment quickly and adsorb to organic matter found in mosquito breeding sites; this reduces their efficacy. Despite 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. There are reports of resistance to *L. sphaericus;* its toxin targets a single receptor in larval midgut which increases risks of resistance (Lacey 2007; Wirth, 2010; Silva-Filha et al., 2021). Besides, *Ae. albopictus*'s inclination to breed in small pockets of water in natural and artificial containers around urban and suburban settings makes it difficult to manage, unlike other species which lay their eggs in larger more accessible and locatable bodies of water like marshes or flooded backwaters. This makes it quite hard for mosquito-control managers to detect and treat these sites with pesticides or other control measures (Medlock et al., 2015; Faraji and Unlu, 2016).

For decades, there has been a significant and increasing demand for the development of biopesticides which are less harmful to human health and the environment to supplant or complement current mosquito control products (Lacey and Georgis, 2012; WHO 2017). 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 (Damico, 2017). Several potential new substances are being investigated and have been reported in the literature as promising biopesticides from fungus, bacteria and plants (Dahmana et al., 2020; Spinozzi et

al., 2021). Entomopathogenic bacteria in the genera *Photorhabdus* and *Xenorhabdus* are up-and-coming novel bioactive compound sources.

1.3. Entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*

Entomopathogenic bacteria in the genera *Photorhabdus* and *Xenorhabdus* are Gram negative, motile, facultative anaerobic and non-spore-forming organisms assigned to the family Morganellaceae and class γ-Proteobacteria (Adeolu et al., 2016). These enteric bacteria are symbionts found in the gastrointestinal tracks of *Steinernema* (Fam: Steinernematidae) and *Heterorhabditis* (Fam: Heterorhabditidae) nematodes, respectively (Hazir et al., 2022) (Picture 3). 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 (Brivio and Mastore, 2018; Shapiro-Ilan et al., 2017, 2020). These nematodes are easily mass-produced biopesticides that do not require registration for use in several countries. They are easily applied with standard spray equipment to control insect pests in soils or against those in cryptic habitats (e.g., tree trunks, rhizomes). They can even be applied with certain chemical pesticides (Gulcu et al., 2017; Nalinci et al., 2021; Touray et al. 2021)



Picture 1.3. Location of *Xenorhabdus* and *Photorhabdus* bacteria in respective nematode host (Goodrich-Blair and Clarke, 2007)

Xenorhabdus and *Photorhabdus* have mainly found in association with entomopathogenic nematodes (EPNs). Only *P. asymbiotica* has been isolated from human wound infections and soil. Twenty-seven *Xenorhabdus* and 23 *Photorhabdus* species have been isolated from 102 *Steinernema* and 17 *Heterorhabditis* species (Hazir et al., 2022). They reside in the alimentary canal of infective juvenile (IJ) or 3rd dauer stage of the nematodes. *Xenorhabdus* are found sequestered in a special intestinal vesicle of *Steinernema* IJs, whereas *Photorhabdus* are found in the anterior part of *Heterorhabditis* IJ guts (Boemare, 2002). In these associations, IJs are responsible for infecting the circulatory system of arthropod host via natural openings of host or by penetrating through thin cuticle, and housing and protecting bacteria from unfavorable environmental conditions. They cruise or ambush insect hosts in soil in response to physical and chemical cues from host. Vectored *Photorhabdus* and *Xenorhabdus* bacteria grow prolifically in insect hemoceol and produce a starling array of secondary metabolites enzymes, and toxins that suppress insect immunity, perform defense functions against competitors and deters natural enemies (Shapiro-Ilan et al., 2012, 2017; Gulcu et al., 2017). In insect host, IJs inside this cadaver continues its life cycle by proceeding into the other larva stages and finally in to an adult stage. The adult stages mate and produce new juvenile stages whilst feeding on digest insect remains and proliferating bacteria symbiont. Just before deserting depleted host in search of a new victim, nematodes retrieve and store bacteria (Kaya and Gaugler, 1993; Forst et al., 1997; Stock and Goodrich-Blair, 2008) (Figure 1.3).

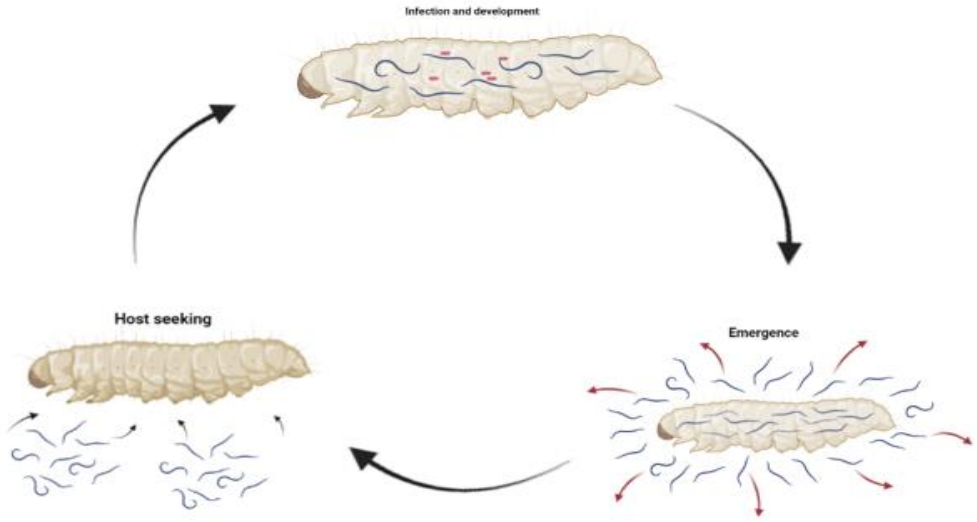


Figure 1.3. Life cycle of entomopathogenic nematode and symbiotic bacteria

Since this nematode-bacteria pairing are obligate insect parasites effective against a wide range of insect host, the nematodes are mass-reared and applied to soil as biological control agents to control pestiferous arthropods, which are a menace to agriculture and public health. Thus, they serve as important alternatives to chemical insecticides, nearly all of which have harmful side effects on the environment and on unfortunate non-target organisms (Hominick et al., 1996; Glazer, 2002; Poinar and Grewal, 2012).

Bacteria in both genera are Gram negative motile rods with peritrichous flagella. They have the ability to absorb dye on Nutrient Bromothymol Blue Agar (NBTA) and MacConkey agar; *Xenorhabdus* forms dark blue convex, umbonated and mucoid colonies on NBTA that swarm and red colonies on MacConkey whereas, *Photorhabdus* forms dark green, convex, umbonated, mucoid and gummy colonies on NBTA, red colonies on MacConkey agar, and hemolysis zones around colonies streaked on sheep or horse blood agar. They also can ferment various carbohydrates (glucose, fructose, maltose, etc.) without gas production. Distinct difference between these bacteria is that *Xenorhabdus* bacteria are catalase negative while *Photorhabdus* are bioluminescent and catalase positive (Boemare, 2002; Boemare and Akhurst, 2006).

1.4. Secondary metabolites of *Photorhabdus* and *Xenorhabdus*

All bacteria produce antimicrobial compounds as defense/survival strategies in competitive environment. *Photorhabdus* and *Xenorhabdus* bacteria produce a plethora of biologically active compounds that 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 (Webster et al., 2002; Tobias et al., 2017; Raja et al. 2021). These compounds are mostly non-ribosomal peptides (NRP), polyketides (PK) or hybrids thereof biosynthesized by fatty acid synthases (FAS), polyketide synthases (PKS), or non-ribosomal peptide synthetases (NRPS) enzymes and modified post-translationally by phosphopantetheinyl transferase (pptase) (Bode, 2009; Beld et al., 2014).

Antibacterial compounds are nematophin, xenorhabdin, xenortide, xenocoumacin, benzylidenacetone, PAX peptides, fabclavine, darobactin; antifungal compounds are nematophin, xenocoumacin, trans-cinnamic acid, PAX peptides, cabanillasin, stilbene derivatives; antiprotozoal compounds are fabclavines, xenortides, xenoumacin, szentiamide; and insecticidal compounds are dithiolopyrrolone derivatives, rhabduscin etc (Stock et al., 2017; Dreyer et al., 2018; Imai et al., 2019; Booysen and Dicks, 2020; Gulsen et al., 2022; Cimen et al., 2022) (Figure 4 and 5).

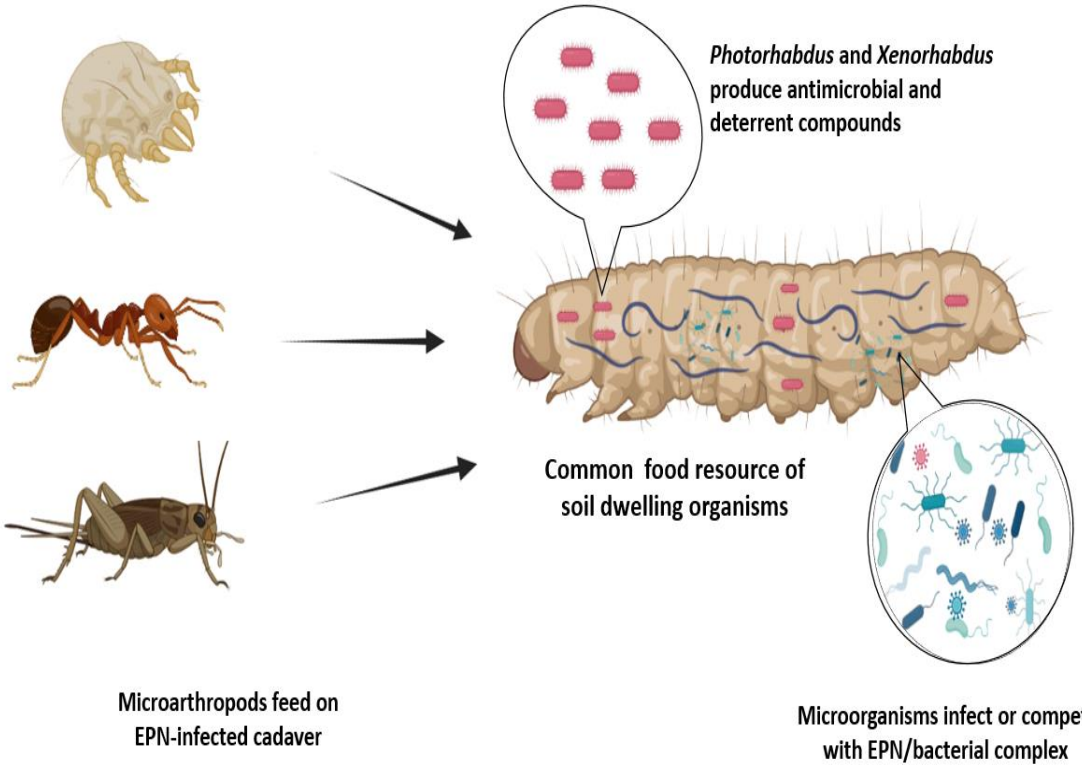


Figure 1.4. *Photorhabdus* and *Xenorhabdus* bacteria produce metabolites to defend against natural enemies

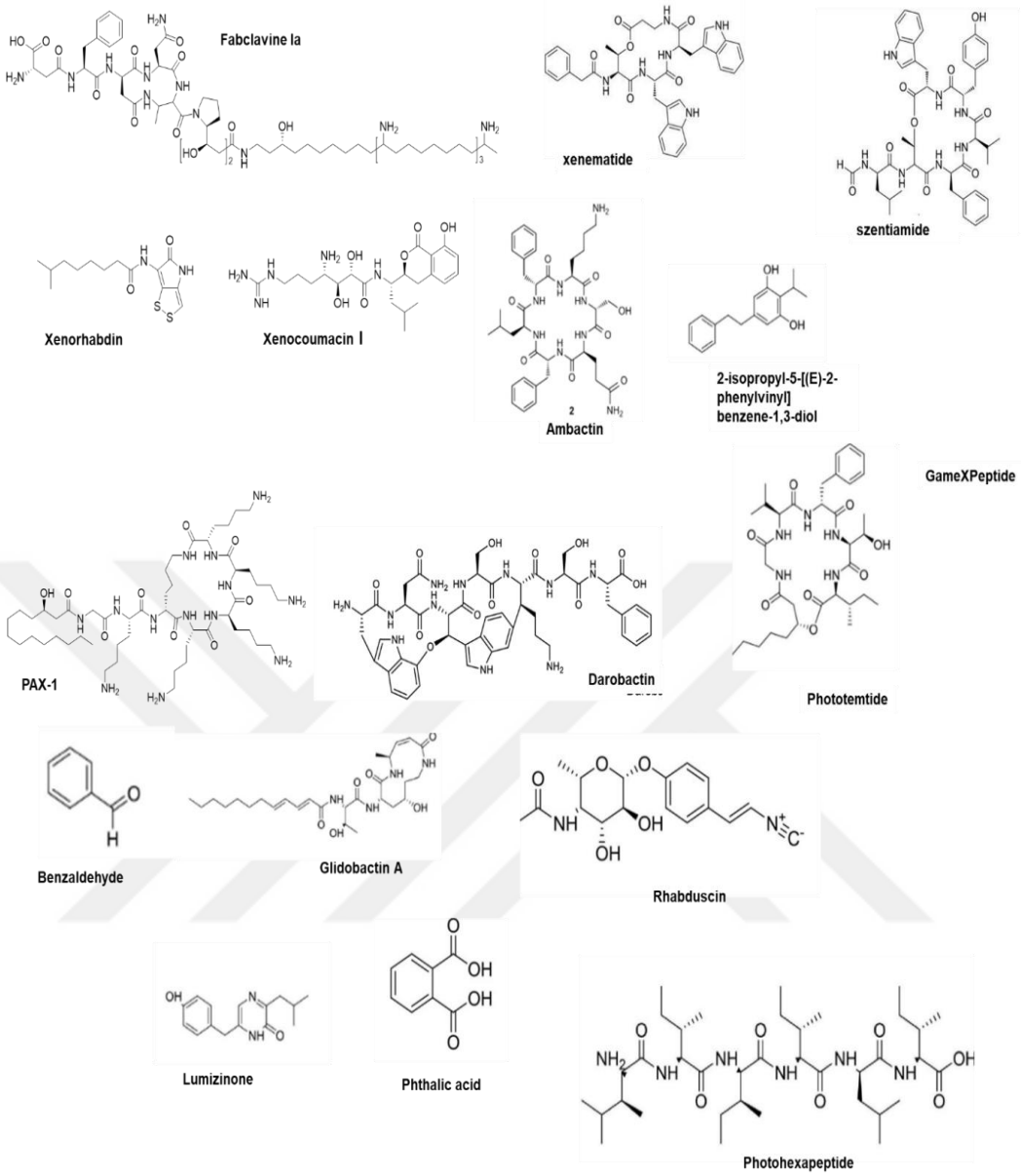


Figure 1.5. Structures of selected natural products from *Photorhabdus* spp. and *Xenorhabdus* spp.

Photorhabdus and *Xenorhabdus* have a substantial portion of their genomes (>6%) encoding 21-41 different biosynthesis gene clusters (BGCs) of these enzymes. Most of these are yet to be characterized (Tobias et al. 2017, 2018) and there is a substantial variation among different species or strains of a specific species (Reimer et al., 2014; Dreyer et al., 2018). The size of these gene clusters can be >100 kb and are located in more than 20 different genetic loci. In comparison, *Streptomyces* species, another prolific NP source, have <5% of their genomes allocated to NP production (Bode, 2009; Joyce et al., 2011; Beld et al., 2014; Schimming et al., 2014).

Non-ribosomal peptide synthetases, as multi-modular enzymes that do not require a mRNA template, sequentially condense amino acid monomers using a thiotemplate mechanism to obtain the primary structures of NRPs. The sequences of NRPs are encoded in NRPS modules made of adenylation (A), peptidyl carrier protein (PCP) or thiolation (T), and condensation (C) domains (Figure 6). Each of these modules is enzymatically capable of selecting, activating, and transferring a single amino acid onto specialized carrier domains, and can form peptide bonds between an amino acid and the C-terminus of a previous peptide chain (Fischbach and Walsh, 2006; Hwang et al., 2020; Zhao et al., 2020). Recently, an in-depth genomic comparison of 81 different *Phtotohabdus* species/strains for NRPS showed that 44% of bacteria genomes consisted of 990 NRPS BGCs clustered into 275 gene cluster families (GCFs); only 13 of these GCFs could be annotated with known BGCs. These NRPS BGCs encoded 351 novel peptides (Du et al., 2022).

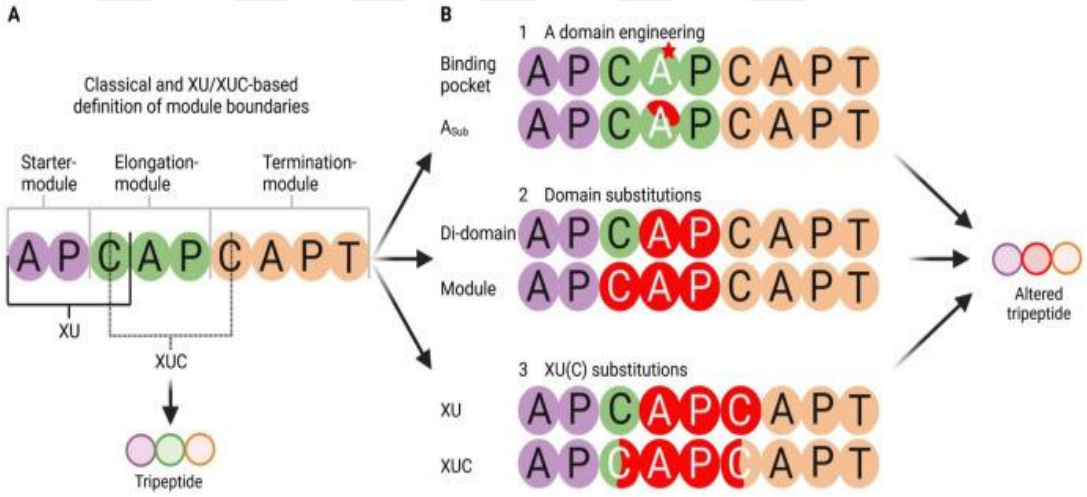


Figure 1.6. NRPS module domains. A: Adenylation; T: Thioesterase; P: Peptidyl carrier protein; C: Condensation (Wenski et al., 2022)

In contrast, polyketide biosynthesis involves a stepwise addition of two carbon ketide units, which can be derivatives of acetate thioesters or other short carboxylic acids (Ansari et al., 2004) (Figure 7). Different enzyme domains are involved in polyketide biosynthesis: acyltransferase (AT), ketosynthase (KS), thioesterase (TE) and optional domains. In this multi-enzyme complex, acyltransferase catalyzes the attachment of substrates like acetyl or malonyl to the acyl carrier protein (ACP) and ketosynthase (KS). Then ketosynthase elongates the carbon chain by decarboxylative Claisen condensation of

substrates attached in ACP. The reaction continues by joining ketoreductase (KR). This enzyme reduces keto ester, dehydratase (DH) dehydrates the compound, whereas enoylreductase (ER) reduces the carbon-carbon double bond in the molecule (Risdan et al., 2019; Wang et al., 2020). There are three PKS types that share similar synthetic processes: type I which are non-iterative multimodular megasynthases; type II composed of monofunctional proteins with an iterative action and type III which are simple homodimers of ketosynthases. PKS type I and II predominantly synthesize aromatic polyketides (Risdan et al., 2019; Hwang et al., 2020; Wang et al., 2020).

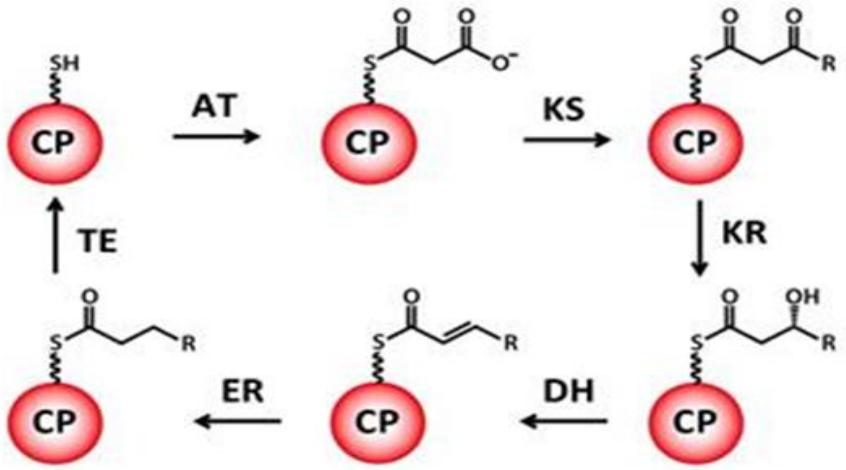


Figure 1.7. reaction occurring in polyketide synthases. ACP: acyl carrier protein; DH: dehydratase; KS: ketosynthase; AT: acyltransferase; ER, enoylreductase; KR: ketoreductase. (Vance et al., 2016)

1.4.1. Identification of natural products produced by *Xenorhabdus* and *Photorhabdus*

Natural products (NP) produced by plants, fungi, and bacteria in their respective ecological niches have proven to be important starting points for the development of novel medical drugs and agricultural agents. Research into the identification of these NPs in microbes including *Xenorhabdus* and *Photorhabdus* have tended to use a traditional bioassay-guided approach: microorganism cultivation, chemical extraction of metabolites produced, and final structure elucidation. However, the recent advent of high throughput sequencing technology has revealed that this approach is inadequate in identifying NPs,

especially those considered silent under laboratory conditions (Schneider et al., 2008; Reen et al., 2015; Katz and Baltz, 2016; Imai et al., 2019). Fortunately, unlike plants and fungi, bacteria genomes have relatively small size with high coding densities and NP genes and machinery involved in their biosynthesis is mostly compact and clustered with no introns present: this reduces sequence cost, enables the fast identification of diverse bacterial gene clusters and research using expression methods can begin directly from chromosomal DNA (Bode and Muller, 2005). With such advantages, scientist can mine microbial genomes and target biosynthetic gene clusters of NP with techniques such as knocking down, introducing or heterologous expression of microbial genes, regulating promoters, inducing mutations, or changing cultivation conditions. This is to stimulate SM gene expression so as to more objectively exploit the potential of bacteria

Natural products can be identified via the manipulation of regulatory proteins associated with NP production. Several regulatory mechanisms and transcription factors such as global regulators LeuO, Hfq, HexA and Lrp have been described (Joyce et al., 2003, 2006; Goodrich-Blair and Clarke, 2007; Richards and Goodrich-Blair, 2009; Clarke, 2016; Eckstein and Heermann, 2019). Although the molecular details of secondary metabolite production are not fully understood, work on the importance of these proteins after the deletion of individual encoding genes have demonstrated that they affect NP production (Brinkman et al., 2003; Cowles et al., 2007; Clarke, 2016; Bozhüyük et al., 2016; Engel et al., 2017). Tobias et al. (2016) reported that deletion of the hfq gene resulted in the cessation of all known natural product production in *P. luminencens*. Hfq is a RNA chaperone ubiquitous in bacteria, archaea and eukaryotes. It plays important roles in gene expression at the post-transcriptional level via binding to small regulatory RNA (sRNAs), small noncoding RNAs, and mRNAs; this facilitates and accelerates mRNA translation or initiates mRNA degradation and prevents translation. It also mediates the interaction between these small regulatory RNAs and mRNA involved in the regulation of virulence, quorum sensing etc. (Guisbert et al., 2007; Vogel and Luisi, 2011). Another important protein, phosphopantetheinyl transferases (ppt) is a holo-acyl carrier protein synthase found in all life forms. This enzyme activates natural product biosynthetic clusters by converting inactive linked and iterative apo-synthases to active holo-synthases post translationally (Beld et al., 2014).

Another approach is the “promoter exchange” approach called easyPACId approach (easy Promoter Activated Compound Identification) (Figure 8). This is a biotechnological approach that allows scientist to identify genomic regions coding natural products and use these fragments for gene inactivation studies (Bode et al., 2015, 2019). With this approach we can evaluate the respective biological activity of a compound by comparing a wild-type strain with an induced (usually production of the NP derived from BGC overexpression) and the non-induced promoter exchange mutant (usually a non-producer due to the missing expression) (Bode et al., 2015, 2019; Bozhüyük et al., 2016). First a Δ*hfq* mutant strain is generated; this mutant there is a complete loss of NPs production. Next, in mutants with a Δ*hfq* background natural promoters, which might have unknown methods of regulation through specific transcription factors or other unidentified mechanisms, are replaced with strong constitutive or inducible promoters such as the arabinose inducible P_{BAD}/AraC system. With this method sometimes a higher production titer is observed compared to the wild-type level. This approach permits direct bioactivity analysis of the corresponding supernatant instead of the onerous isolation of single compound/s (Bode et al., 2015, 2019; Bozhüyük et al., 2016).

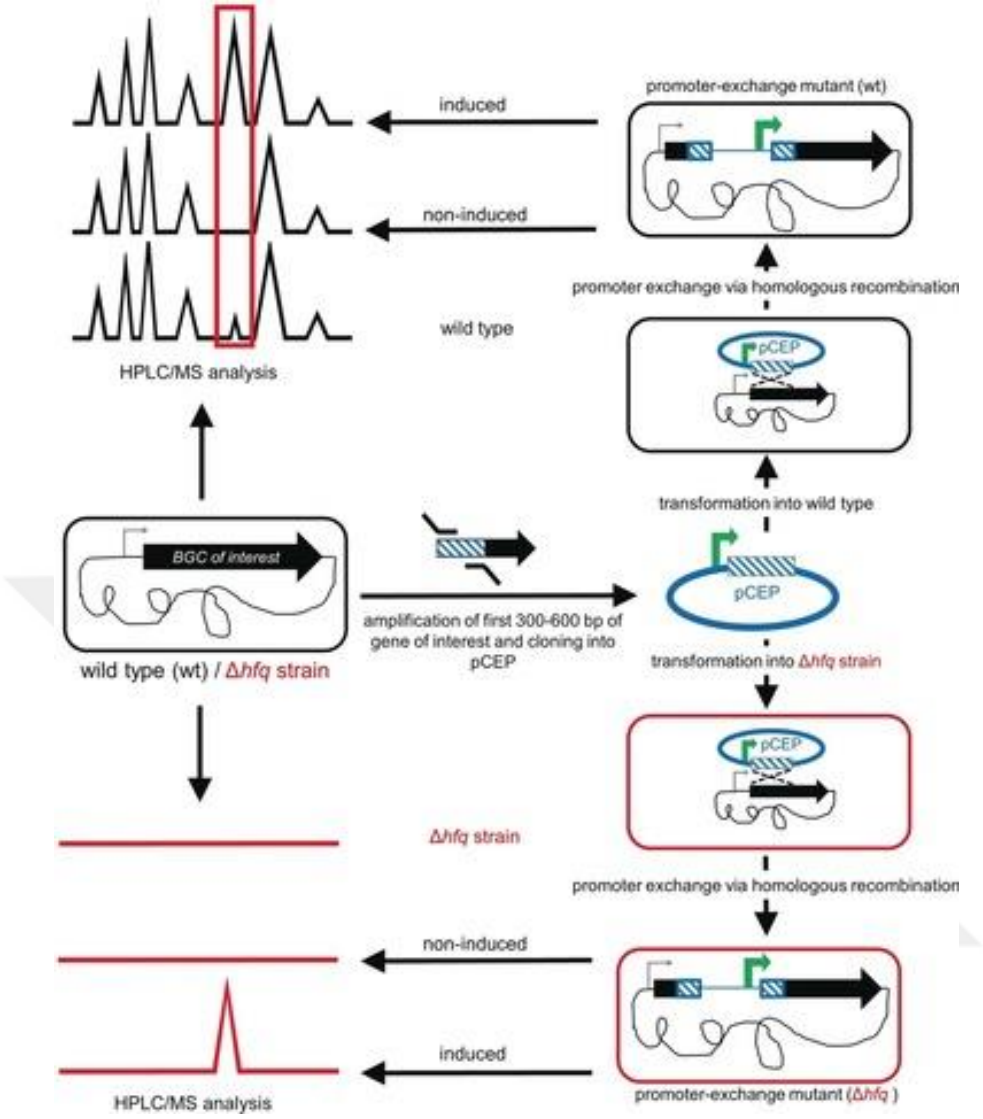


Figure 1.8. Schematic diagram illustrating the easyPACId approach (easy Promoter Activated Compound Identification) used to identify the biological properties of a desired biosynthetic gene cluster (BGC) in wild type (Bode et al., 2019)

1.5. Objectives of this thesis

Secondary metabolites of *Xenorhabdus* and *Photorhabdus* are an important source of a wide range of novel antimicrobial compounds of increasing agricultural, medical and pharmaceutical importance.

Research carried out in this thesis;

1. Investigates the larvicidal efficacy of cell growth cultures, cell free supernatants (CFS) and bacterial cell suspensions of an extensive list of entomopathogenic bacteria in the genera *Xenorhabdus* and *Photorhabdus* against *Aedes albopictus* larvae (3^{rd} -4^{th} stages).

2. Assesses the ovicidal effects of bacterial supernatants on the eggs of *Ae. albopictus* and subsequent effects on the emerged larvae (1^{st} stage).

3. Aims to identify the novel larvicidal compound/s in the supernatants of *X. szentirmaii* and *X. nematophila* using mutants generated using the easyPACid biotechnological approach.

2. LITERATURE REVIEW

Morgan et al. (1997) evaluated the survival of *Xenorhabdus* and *Photorhabdus* bacteria in water. They demonstrated that *X. nematophilus* (pLV1013) and *P. luminescens* (pLV1012) cells release into river water began to decline and after 6 days were later undetectable whereas in sterile river water cells detectable for only 2 days.

Ahantarig et al. (2009) isolated PirAB proteins (*Photorhabdus* insect-related proteins A and B) from *P. luminescens* TT01. They reported that these proteins are encoded between the plu4093 and plu4436 loci, are orally toxic to 1st stage larvae of *Ae. aegypti* and *Ae. albopictus* and are similar to *B. thuringiensis* endotoxins. These proteins exhibited no activity against *Mesocyclops thermocyclopoides* – a crustacean used as a biological control agent against mosquito larvae.

O.S. Silva et al. (2013) evaluated the oral pathogenicity of cell suspensions (10^7-10^8 cfu/ml) of different *Xenorhabdus* and *Photorhabdus* bacteria against mosquito larvae. They observed that *P. luminescens* killed 73 % and 83 whereas, *X. nematophila* killed 52 % and 42 % of *Ae. aegypti* larvae in fed and unfed treatment. In contrast, J.L.R. Silva et al. (2017) tested the effects of different concentrations of *P. luminescens* and *X. nematophila* supernatants (at concentrations ranging from 12 to 54%) against *Ae. aegypti* larvae. They observed that overall *X. nematophila* (LC_{50}=14.14%) was more effective than *P. luminescens* (LC_{50}=21.18%). The authors also assessed the residual effects of *X. nematophila* by adding new larvae to treated water. They observed >50 mortality up to 8 days after treatments.

Park et al. (2016) reported synergistic interactions between Bti and bacterial metabolites of *X. nematophila, X. hominckii, P. temperata* subsp. *temperata* against *Ae. albopictus* larvae. LC_{50} values of Bti were scaled down from 2.9 x 10^5 spores/ ml to 1.5 x 10^5 spores/ml, 1.7 x 10^5 spores/ml, and 1.9 x 10^5 spores/ml, respectively. Bti's pathogenicity was enhanced after the bacterial metabolites inhibited the immune system of *Ae. albopictus*. This combination had displayed similar effects on *Culex pipiens pallens*. They also noted that heat treatment had no effects on the bacterial supernatants suggesting that the bioactive

compound is non-proteinaceous. Furthermore, “Dip-Kill”, which is a new mosquitocidal Bti formulation made up of 10% Bti (10^{10} spores/ml), 80% *X. nematophila*-cultured broth, and 10% preservative was found to be more effective (100%) at 1.000 ppm compared to Bti product.

Wagutu et al. (2017) conducted studies using cells plus metabolites (cells/ml), cells-only (cells/ml) and metabolites-only of *Photorhabdus* spp., and *Xenorhabdus* spp., isolated from *H. indica* and *S. karii,* respectively, against *Anopheles* mosquito larvae. They demonstrated that cells plus metabolites and metabolites-only of both bacteria caused 50% death of *Anopheles* mosquito larvae after 72 h and >80% mortality after 96 hours. They also observed that the cells-only treatment exhibited 50% mortality after 96 hrs.

Vitta et al. (2018) evaluated the oral pathogenicity of cell suspensions (10^7-10^8 cfu/ml) of *X. indica, X. stockiae, P. luminescens* subsp*. Akhurstii* and *P. lum.* subsp. *hainanensis* against *Ae. aegypti* and *Ae. albopictus* larvae. They found that mortality of *Ae. aegypti* ranged between 68-80%, whereas that of *Ae. albopictus* was between 66-81% at 72 h after exposure.

Bode et al. (2015, 2019) developed a biotechnological technique called the easy Promoter Activated Compound Identification approach (easyPACId) for the identification of natural products. This technique allows researchers to investigate and compare the effects of a natural products (NPs) encoded in a specific gene and synthesized by non-ribosomal peptide synthetases (NRPS) or polyketide synthetases (PKS) enzymes using mutants with regulable gene. Promoter sequences of these genes are replaced with inducible promotors. Some studies have used this approach to identify some bioactive compounds.

Shah et al. (2021) compared the larvicidal effects of the CFS and bacterial cell suspension of *P. laumondii* and *X. nematophila* against *Ae. aegypti*. They showed that while both the supernatant and bacterial cell suspensions of *X. nematophila* caused >91% larval mortality after 48 h, whereas only the bacterial cell suspension of *P. laumondii* was effective against the mosquito larvae, indicating that some EPN-symbiotic bacteria release toxic metabolic compounds with larvicidal activities out of their cells.

Incedayi et al. (2021) used the easyPACId approach to identify the novel acaricidal compound in *X. szentirmaii* and *X. nematophila* against *Tetranychus urticae* and its side effects on the predatory mites, *Phytoseiulus persimilis* and *Neoseiulus californicus*. Their

results showed that CFS of *X. nematophila* (pCEP_kan_XNC1_1711) which contains xenocoumacin caused >97% mortality on immature and adult stages of *T. urticae* and <36% mortality on the predatory mites at 7dpa. The acaricidal compound in *X. szentirmaii* could not be determined. Likewise, Cimen et al. (2021) used easyPACId technique and determined that fabclavines, a heat stable natural products synthesized by a PKS and NRPS hybrid system, was responsible for the antifungal activity of *X. szentirmaii* against the chestnut blight, *Cryphonectria parasitica*. In another study, Gulsen et al. (2022) showed that fabclavines, xenocoumacins, xenorhabdins and PAX peptides possessed antiporotozoal activities against *Acanthamoeba castellanii, Leishmania tropica, Trichomonas vaginalis,* and *Trypanosoma cruzi* using Δhfq *X. szentirmaii, X. nematophila* and *X. doucetiae* mutants.

3. MATERIAL AND METHOD

3.1. Bacteria Species

The different wild-type *Xenorhabdus* and *Photorhabdus* bacterial species as well as the promoter exchange mutants provided by Prof. Dr. Helge B. Bode from Geothe University, Germany, used in this study are given in Table 2, 3 and 4.

Table 3.1. List of *Xenorhabdus* wildtype bacterial species.

Bacterial species			
1	*Xenorhabdus bedingii* DSMZ 4764	13	*X. japonica* DSMZ 16522
2	*X. bovienii* SS-2004	14	*X. kozodoi* DSMZ 17907
3	*X. budapestensis* DSMZ 16342	15	*X. miraniensis* DSMZ 17902
4	*X. cabanillasii* JM26-1	16	*X. stockiae* DSMZ 17904
5	*X. doucetiae* DSMZ 17909	17	*X. szentirmaii* DSMZ 16338
6	*X. eapokensis* DL20	18	*X. thuongxuanensis* 30 TX1
7	*X. ehlersii* DSMZ 16337	19	*X. vietnamensis* DSMZ 22392
8	*X. griffinae* DSMZ 17911	20	*X. koppenhoeferii* DSMZ 18168
9	*X. hominickii* DSMZ 167903	21	*X. nematophila* ATCC 19061
10	*X. indica* DSMZ 17382	22	*X. poinarii*
11	*X. innexi* DSMZ 16336	23	*Xenorhabdus* sp. TS4
12	*X. ishibashii* DSMZ 22670		

Table 3.2. List of *Photorhabdus* wildtype bacterial species.

1	*Photorhabdus akhurstii* DSMZ 15138
2	*P. asymbiotica* ATCC 43949
3	*P. laumondii* TT01
4	*P. thracensis* DSMZ 15199
5	*P. namnaoensis* PB 45.5
6	*P. kayaii* DSMZ 15194

Table 3.3. *Xenorhabdus spp.* Δ*hfq* pCEP-KM-xy *mutants* used in this thesis.

Bacteria species	Mutant name	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
	Δhfq 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

3.2. Mosquito species

Aedes albopictus mosquito larvae were collected from a graveyard in Güzelçamlı, Aydın, Turkiye. Larvae, which can survive in small collections of water, were found in clay vases and plastic bottles used to water plants on graves (Picture 4). Collected larvae were brought to the Vector Control laboratory of the Biology Department at Aydın Adnan Menderes University and reared in 45 × 45 × 45 cm insect cages (Bugdoms) placed in an insectarium with 27±1°C temperature, 70% relative humidity and under a 14D:10L

photoperiod. Adults were used to confirm species using morphological identification keys (Schaffner et al., 2001). Cotton pads soaked in 10% sugary water was available ad libitum to adult mosquitoes. Female mosquitoes were regularly fed blood from live quails every three to five days; cylindrical containers with water and filter paper on the sides were provided for egg laying. Hatched larvae were fed daily with ground fish food flakes (Kauffman et al., 2017).

Picture 3.1. Site of *Aedes albopictus* mosquito collection.

3.3. Preparation of cell-free supernatant, growth culture, and bacterial suspension

From stock cultures, *Xenorhabdus* and *Photorhabdus* bacteria were streaked on Luria-Bertani agar and then a single colony was inoculated and incubated in Luria-Bertani broth (10 ml) on a rotary incubator at 28ºC 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: one served as growth culture used in the larvicidal assays and the other was centrifuged at 10.000 rpm at 4ºC for 10 min. The supernatant was transferred into another centrifuge tube and filtered through a 0.22 µm Millipore filter (Sartorius, Goettingen-Germany). The remaining bacterial pellets were re-suspended with sterile physiological saline and the turbidity was adjusted to OD_{600nm}= 1.0 by spectrophotometer. Hence, cell-free supernatant, growth culture and re-suspended bacterial pellet ready for use in bioassays (Hazir et al., 2016, 2022) (**Figure 3.1.**).

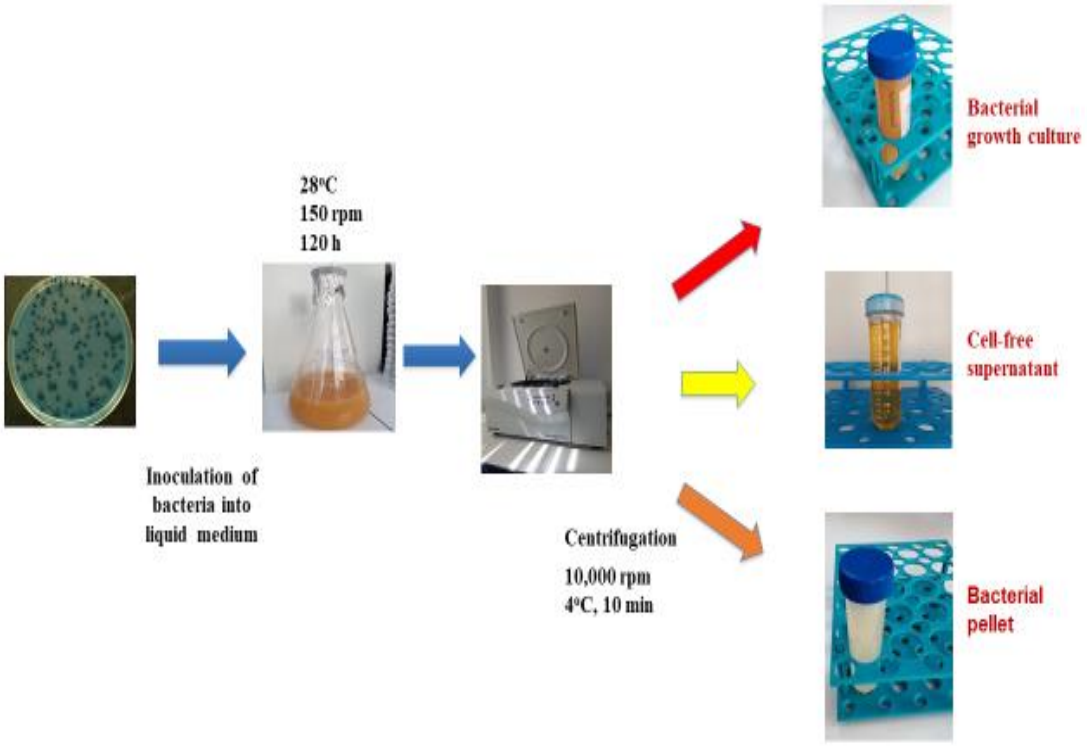


Figure 3.1. Preparation of cell-free supernatant, growth culture and bacterial suspension of *Photorhabdus* and *Xenorhabdus*

3.4. The larvicidal efficacy of cell-free supernatants, growth cultures, and cell suspensions of entomopathogenic bacteria in the genera *Xenorhabdus* and *Photorhabdus*

The efficacy of cell-free supernatant, growth culture and re-suspended bacterial pellet against late third to fourth early instar larvae of *Ae. albopictus* was evaluated according to Shah et al., (2021) in wells of a 24-well plates (**Figure 3.2.**). Each well had 10 mosquito larvae in one ml of water with 50% of prepared cell-free supernatant, growth culture and re-suspended bacterial pellet. Distilled water was used as the negative control. Larval mortality was assessed after 24 and 48 h. Dead larvae were touched with a fine tipped brush to confirm death.

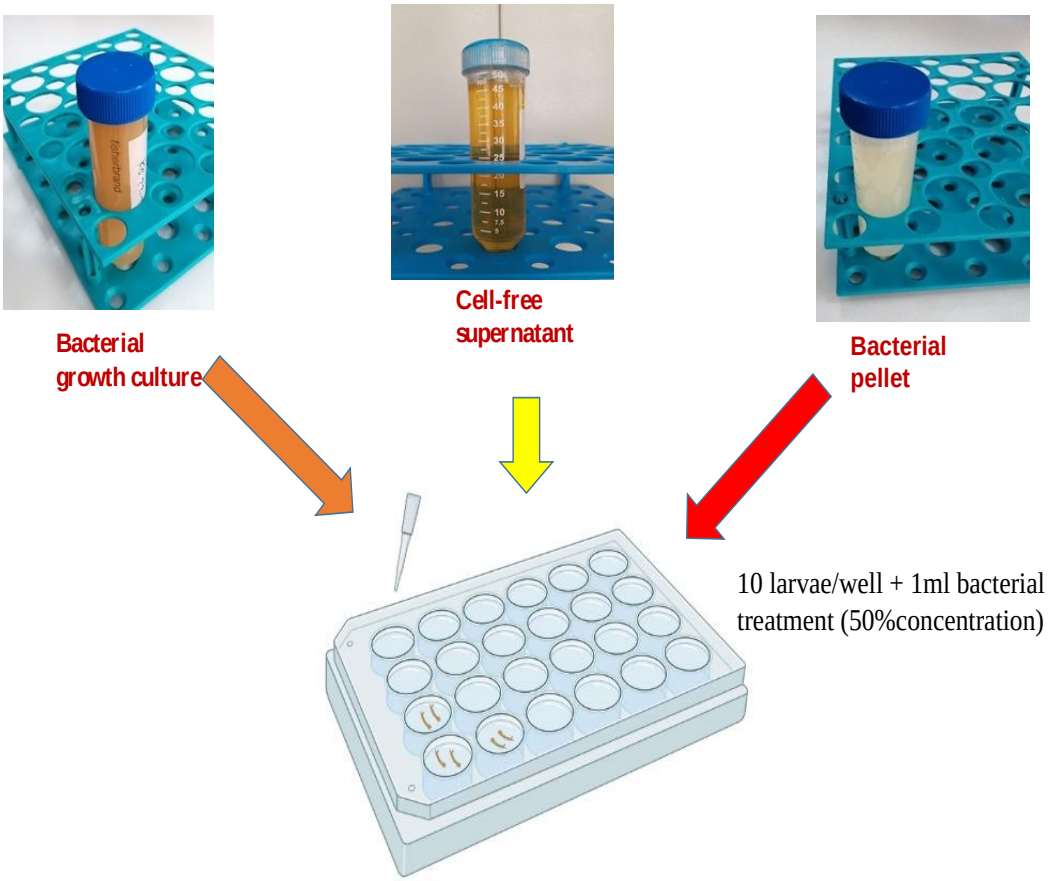


Figure 3.2. Establishing the larvicidal efficacy of *Xenorhabdus* and *Photorhabdus* bacteria

3.5. Ovicidal effects of cell-free supernatants of *Xenorhabdus* and *Photorhabdus*

The ovicidal effects of *Xenorhabdus* and *Photorhabdus* bacteria cell-free supernatant on *Ae. albopictus* eggs was evaluated in wells of a 24-well plates. Briefly, 10 mosquito eggs deposited on filter papers were transferred in to wells using a fine brush. Then one mL of water with 50% of cell-free supernatant was added. Plates were incubated at 27±1ºC for 5 days after which the number of hatched eggs was counted. The ratio of alive and dead larva was also recorded.

3.6. Identification of the bioactive larvicidal compounds

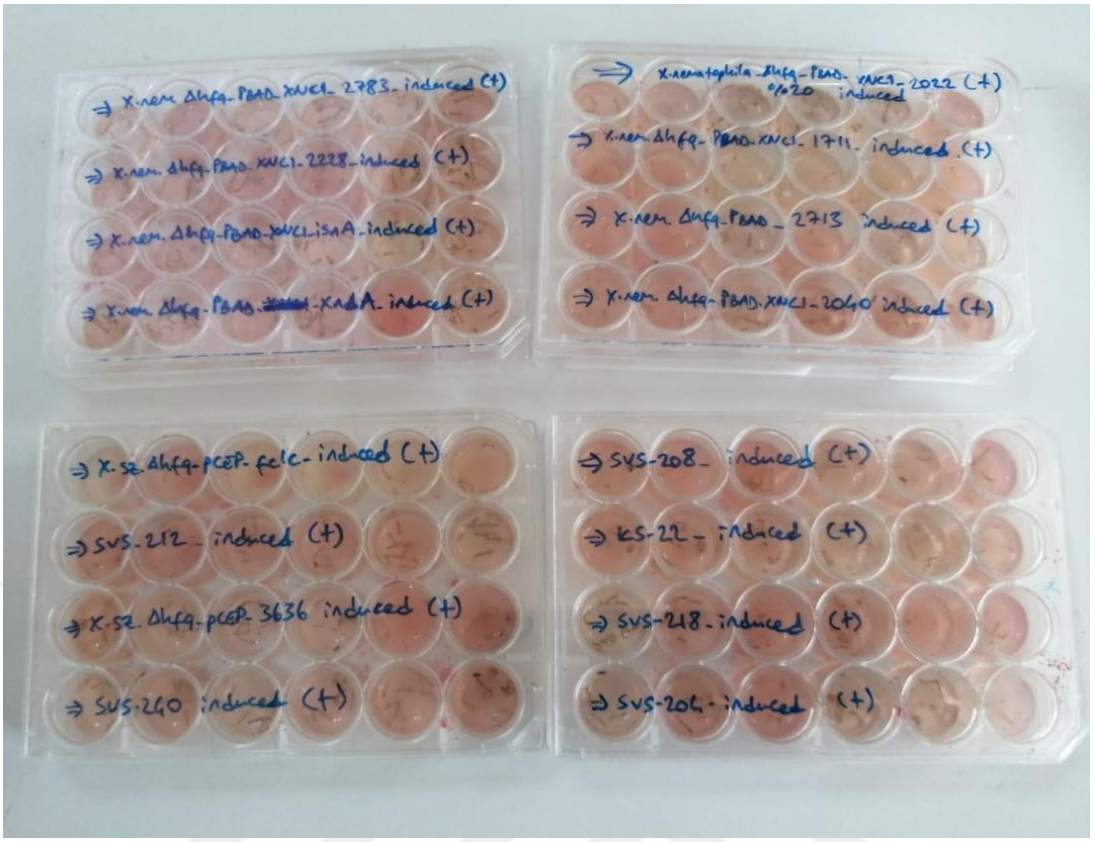
3.6.1. Creating promoter exchange mutant strains

The bioactive larvicidal compound(s) was identified using the easyPACId approach developed by Bode et al. (2015; 2019). First, Δhfq mutant of *X. budapestensis*, *X. doucetiae*, *X. cabanillasii*, *X. hominickii*, *X. stockiae*, *X. nematophila*, and *X. szentirmaii* species were first created. Then the native promoter regions of selected natural product biosynthetic gene clusters of these bacteria were exchanged with L-arabinose inducible promoter P_{BAD} by the integration of the pCEP-KM plasmid (Bode et al., 2015, 2019). 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 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 as described in detail by Tobias et al. (2017b) and Bode et al. (2019).

3.6.2. Obtaining cell-free supernatants from different *Xenorhabdus* spp. Δhfq promoter exchange mutants

The cell-free supernatants of the different *Xenorhabdus* spp. Δhfq promoter exchange mutants were obtained as described before in section 3.3. Briefly a single mutant colony of the mutants was streaked on LB agar supplemented with a 50 μg/mL 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 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_{600}) 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 (Bode et al., 2019; Wenski et al., 2020). Cultures of non-induced mutants had no L-arabinose. The cell-free supernatants 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 (Hazir et al., 2016). The cell-free supernatants were stored at -20°C and used within 2 weeks (Muangpat et al. 2017).

The same experimental design described above with 24-well plates was used to identify the bioactive larvicidal compound (**Picture 3.2.**).



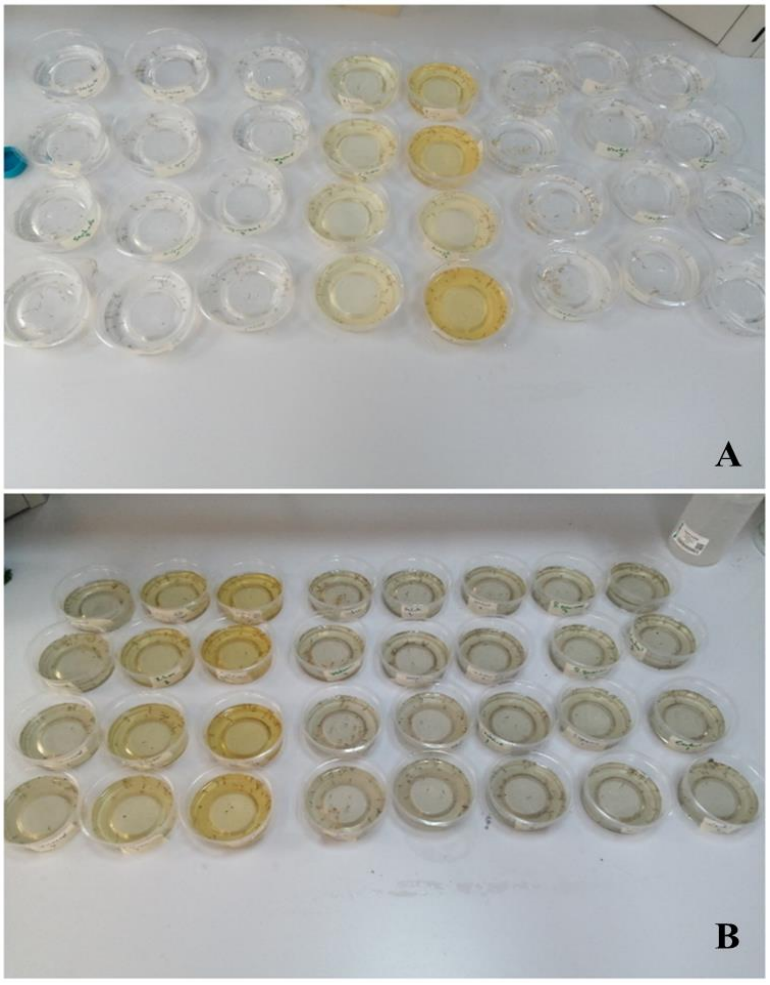
Picture 3.2. Identification of the bioactive larvicidal compounds using cell-free supernatants from different *Xenorhabdus* spp. Δhfq promoter exchange mutants

3.7. Comparing the effects of Fabclavine with other commercial products

This study was conducted to compare the efficacy of supernatants the bioactive mutants (fabclavine producing *X. cabanillasii* and *X. hominickii* mutants) with commercial larvicidal compounds of bacterial origin viz., Vectobac® (granule formulation of *B. thuringiensis* subsp. *israelensis*), *Lysinibacillus (Bacillus) sphaericus*, Vectomax® (mixture of *Bti* & *L. sphaericus*), Serbate (Pyriproxyfen- juvenile hormone), and Spinosad (**Table 3.4.**). Thirty 3rd-4th stage larvae were transferred into 150 ml plastic containers with 50 ml water which was treated with the recommended concentration of the commercial products and 50% supernatant was used. Setup was incubated at 27±1ºC and larval mortality was assessed and recorded after 4, 24 and 48 h post application (**Picture 3.3.).**

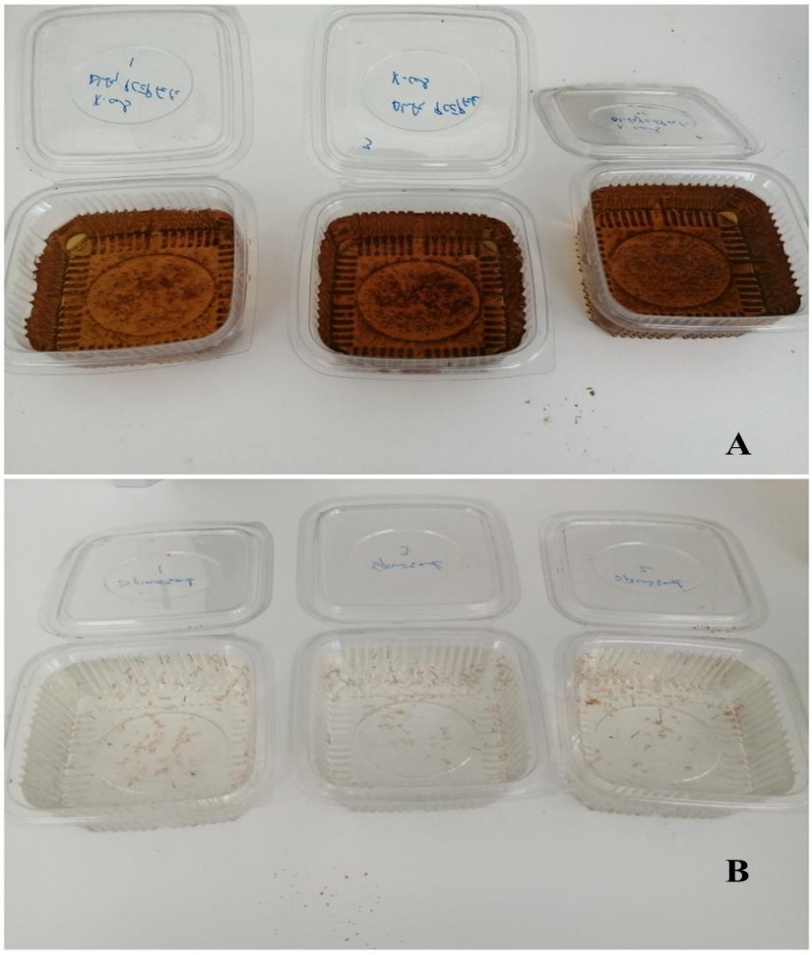
Table 3.4. Biopesticides used in this study.

Commercial product	Active ingredient/s	Recommended concentration	Formulation
Vectobac® 12AS	*B. thuringiensis* subsp. *israelensis*	0.19 ml/L	SC; Suspension Concentrate
Vectomax® FG	Bti & *Lysinibacillus* sphaericus	1.9 g/L	WDG; Water-dispersible granule
Serbate 15C	Pyriproxyfen (juvenile hormone)	0.66 ml/L	EC; Emulsifiable concentrate
Moskill 120C	Spinosad	3.3 ml/L	SC; Suspension Concentrate
Vectolex WDG	*Lysinibacillus sphaericus*	5 g/L	WDG; Water-dispersible granule



Picture 3.3. Efficacy of fabclavine produced by *Xenorhabdus cabanillasii* and *Xenorhabdus hominickii* mutants and commercial larvicidal compounds of bacterial origin in clean (A) and field-collected (B) water.

The residual effects of these larvicidal compounds were also analyzed according to Silva et al. (2017). In this experiments, 30 mosquito larvae where dispense into plastic container (12 cm × 12 cm × 6 cm) with 300 ml water (**Picture 3.4.**). These containers were treated with the recommended concentration of the larvicidal compounds and 50% concentration of fabclavine from *X. hominickii* and *X. cabanillasii*. Larvae were added at 0, 3, 5, 7, 9, 11, and 15 dpa after treatment and larval mortality was assessed after every 24 hours of larval addition. After each assessment, larvae in each container were removed by sieving contents of the container and new health larva was added; this was continued until no significant mortality was observed for for *X. hominickii* and *X. cabanillasii*.



Picture 3.4. Residual effects of fabclavine produced by *Xenorhabdus cabanillasii* and *Xenorhabdus hominickii* mutants and commercial larvicidal compounds of bacterial origin in clean (A) and field-collected water (B)

3.8. Statistical analysis

Data on the mortality ratios of mosquito larvae and eggs dosed with the cell-free supernatant, growth culture and bacterial suspension of different *Xenorhabdus* and *Photorhabdus* bacteria, mutants and control, were arcsine-transformed to ensure the assumption of normal distributions and analyzed using one-way Analysis of Variance (ANOVA). Three-way ANOVA was also used with bacterial species, incubation time, treatment type and their interactions as the main factors taken into consideration. Means were separated using Tukey's test ($P < 0.05$) (SPSS 2004).

4. RESULTS

4.1. The larvicidal efficacy of growth cultures, cell-free supernatants and cell suspensions of entomopathogenic bacteria in the genera *Xenorhabdus* and *Photorhabdus*

According to three-way ANOVA, there was a significant difference between the effects of bacteria species, treatment type (supernatant, bacterial growth culture, bacterial pellet), assessment time (24, 48 h) and their interactions on larval mortality (**Table 4.1.**).

Table 4.1. Statistical analyses of different parameters.

Factors	**df**	**F**	**P**	**Partial η2**
Bacteria	29	276.894	0	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
Error	3060			

Comparison of the effects of the supernatant obtained from different *Xenorhabdus* and *Photorhabdus* bacteria on *Ae. albopictus* larvae showed that there was a significant difference among the species after 24 h ($F=132.509$; $df=28, 521$; $P<0.001$) and 48 h ($F=141.146$; $df=28, 521$; $P<0.001$) (Figure 10). Supernatant obtained from *P. akhurstii, P. asymbiotica, X. cabanillasii, X. hominickii,* and *X. indica* killed 100% of *Ae. albopictus* larvae. Following this, *P. namnonensis, X. stockiae, X. budapestensis, X. miranenisis, X. nematophila, X. innexi* and *X. japonica* caused larval mortality ranging between 70 and 80%, whereas *X. szentirmaii, X. vietnamensis* and *X. bovienii* caused 55, 55, and 52% mortality, respectively. This mortality increased slightly after 48 hours. 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. thouganensis* presented less than 20% mortality at all points of assessment (**Figure 4.1.).** There was no mortality in control groups.

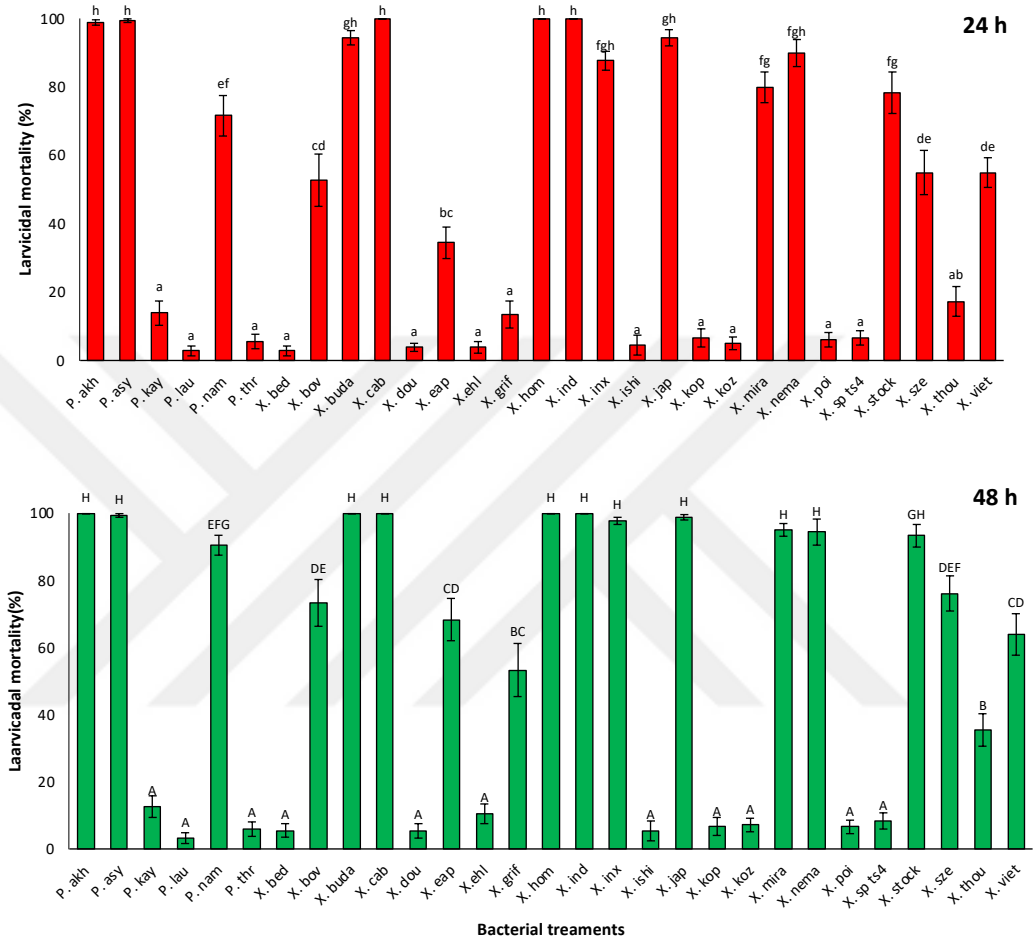


Figure 4.1. Larvicidal activity exhibited by cell-free supernatants from *Photorhabdus* and *Xenorhabdus* species. Same letter above bars indicate no statistical difference (P>0.05, Tukey test).

Growth culture from all *Xenorhabdus* and *Photorhabdus* species exhibited larvicidal activity (55-100% mortality) against mosquito larva except for *X. sp*. TS4 (**Figure 4.2**). Twenty-eight of the tested bacteria displayed efficacy with a significant difference among the treatments after 24 h exposure (F=26.146; df=28, 521; P<0.001). *Xenorhadus hominickii. X. indica, X. innexi, X. ishibashii, X. japonica, X. stockiae, X. miranensis, P. asymbiotica, P akhurstii, P. laumondii, X. bovenii, X. cabanillasii, X. doucetiae,* and *X*

vietnamensis caused > 90 larval mortality. In decreasing order of efficacy, mortality caused by *X. griffinae, X. thouganensis, X. koppenoferii P. kayaii X. budapestensis, X. nematophila*, *X, beddingii, P. thracensis, P. namnonensis, X. kozodoi* ranged between 87-60%. *Xenorhabdus eapokensis, X. poinarii* and *X. ehlersi* displayed larvicidal effects of 57%, 55%, and 43%, respectively. However, *Xenorhabdus* sp. TS4 caused only 10% mortality. 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=50.240; df=28, 521; P<0.001) (**Figure 4.2**).

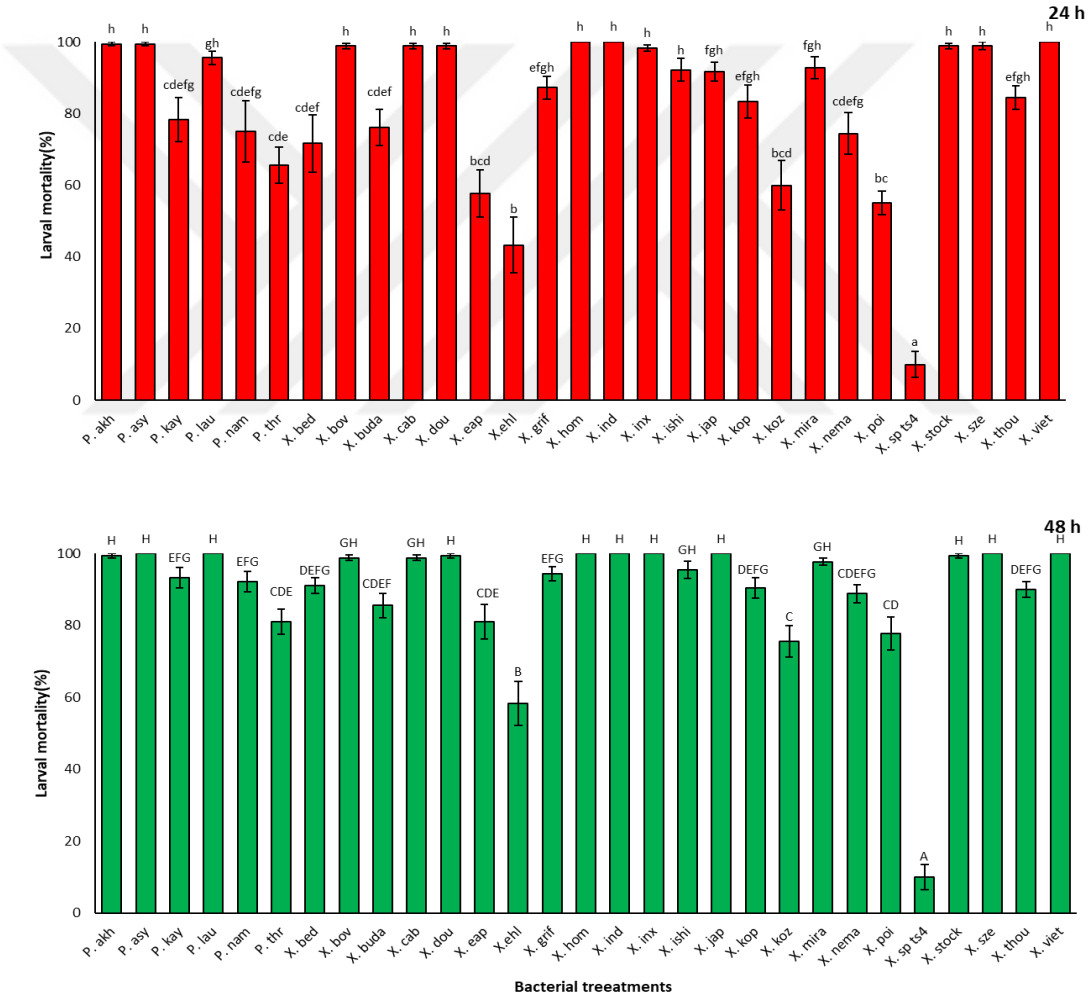


Figure 4.2. Larvicidal activity exhibited by growth cultures from *Photorhabdus* and *Xenorhabdus* species. Same letter above bars indicate no statistical difference (P>0.05, Tukey test).

In the case of treatments with bacteria pellets, *X. doucetiae* exhibited numerically highest larval mortality (91%). *Xenorhabdus budapestensis, X. cabanillasii, P. kayaii, X. ishibashii, X. ehlersii, X. indica, X. innexi, X. bovenii, X. hominickii, X. szentirmaii X. nematophila,* X. *miranensis, X. stockiae, P. asymbiotica X. thouganensis* and *X. vietnamensis* killed 86-59% of exposed larvae, whereas the least effective species were *P. laumondii, X. beddingii, P. thracensis, X. budapestensis, X. indica, X. eapokensis, P. namonensis*, and *X. ehlersii* after 24 hours (F=34.705; df=28, 521; P<0.001). There was a statistical difference in the efficacy of the bacterial pellets after 48 hours (F=79.669; df=28, 521; P<0.001) (**Figure 4.3**).

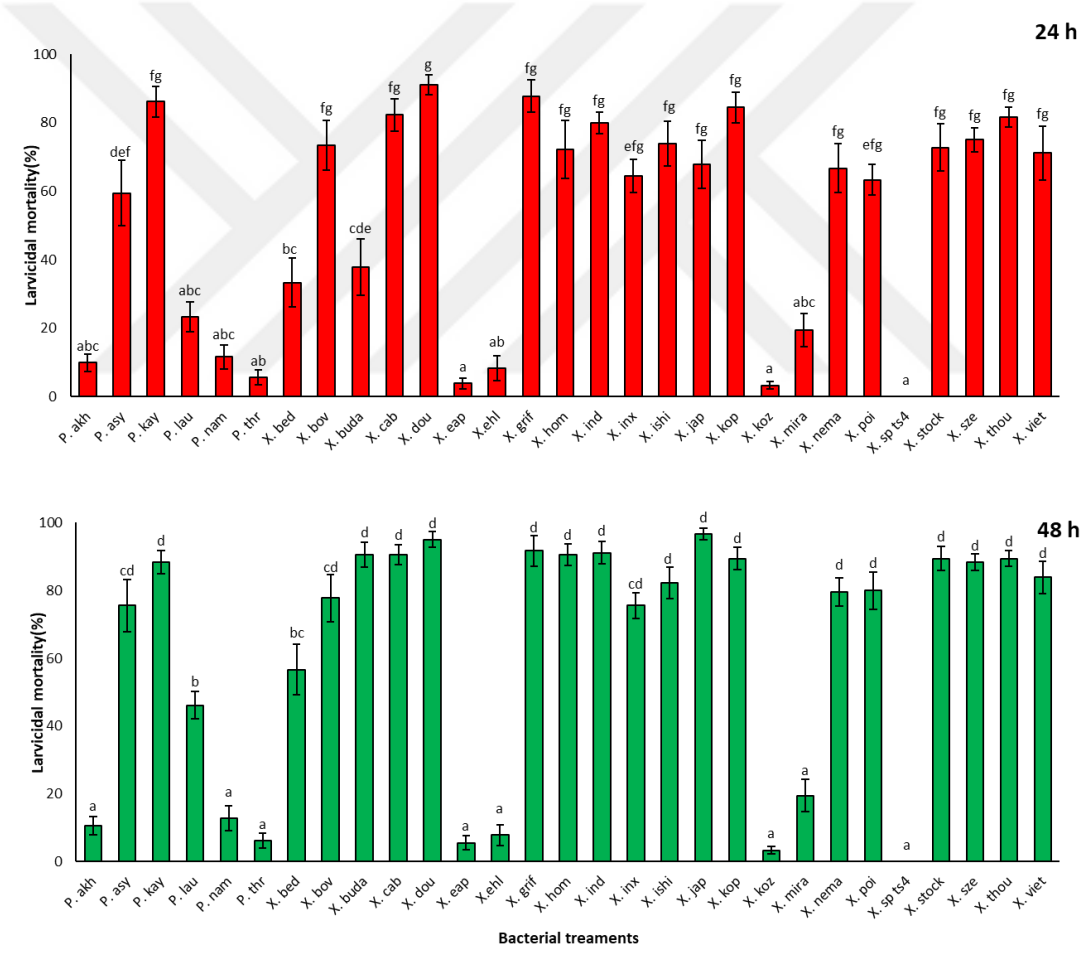


Figure 4.3. Larvicidal activity exhibited by bacterial cells of *Xenorhabdus* and *Photorhabdus* species. Same letter above bars indicates no statistical difference (P>0.05, Tukey test).

These findings show that certain bacteria can release toxic metabolic compounds with larvicidal activities out of their cells into the supernatant whereas the cells of other bacteria exert toxicity when ingested by larvae and do not release larvicidal compounds out of their cells.

4.2. Ovicidal effects of cell-free supernatants of *Xenorhabdus* and *Photorhabdus*

There was a statistical difference in the % number of *Ae. albopictus* eggs that hatched after exposure to different *Xenorhabdus* and *Photorhabdus* bacteria, Bti and spinosad (**Figure 4.4**). The lowest number of eggs hatched in *X. indica* (41%). Compared to the other treatments percentage egg hatching ranged between 48 and 85% ($F=2.753$; $df=31, 575$; $P<0.0001$). We observed that after hatching, >75% of *Ae. albopictus* 1st instar larvae that emerged died in treatments with *X. bedding, P. thracensis, P. namnonensis,* Spinosad, *X. ehlersii,* Bti, *X. japonica, X. ishibashii, P. akhurstii, X. vietnamensis, P. kayaii, P. laumondii, X. stockiae, X. innexi, X. szentirmaii, X. indica, X. bovienii, X. budapestensis, X. cabanillasii, P. asymbiotica, X. hominickii, X. griffinae, X. miranensis, X. nematophila X. thouganensis, X. koppenohoferii, Xenorhdbus* sp. TS4. This was statistically higher than the mortality observed in control, and *X. poinarii, X. doucetiae, X. kozodoi, X. eapokensis* treatments ($F=51.662$; $df=31, 566$; $P<0.0001$).

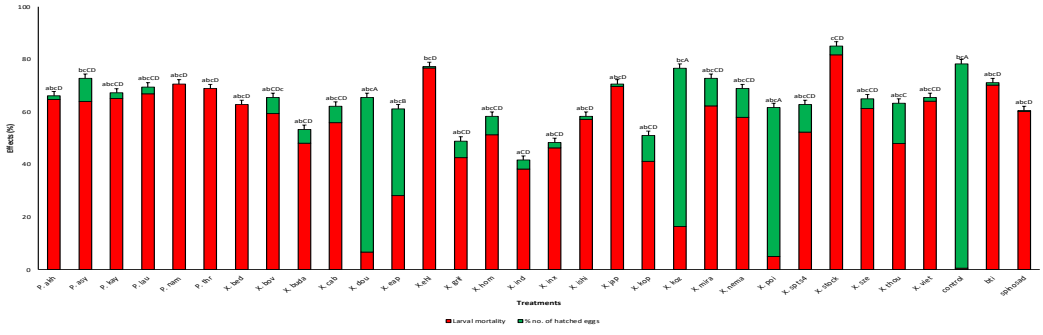


Figure 4.4. Ovicidal activity of cell-free supernatants from *Xenorhabdus* and *Photorhabdus* species and larvicidal activity against emerged larvae. Same letter above bars indicate no statistical difference ($P>0.05$, Tukey test).

4.3. Identification of the larvicidal compounds

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 wild-type cells. Results showed that the fabclavine-producing (*X. szentirmaii* Δhfq pCEP-KM-fclC) and the xenocumacin-producing strains (*X. nematophila* Δhfq_pCEP_kan_XNC1_1711) displayed larvicidal activity which increased from 76% to 95% and 40% to 70% respectively between 24 and 48 h. There was a statistically significant difference among the compounds from the tested mutant strains of *X. szentirmaii* mutants (F=178.205; df=13, 280; *P*<0.001) and *X. nematophila* mutants (F=39.179; df=11, 279; P<0.001) after 48 h (**Figure 4.5**).

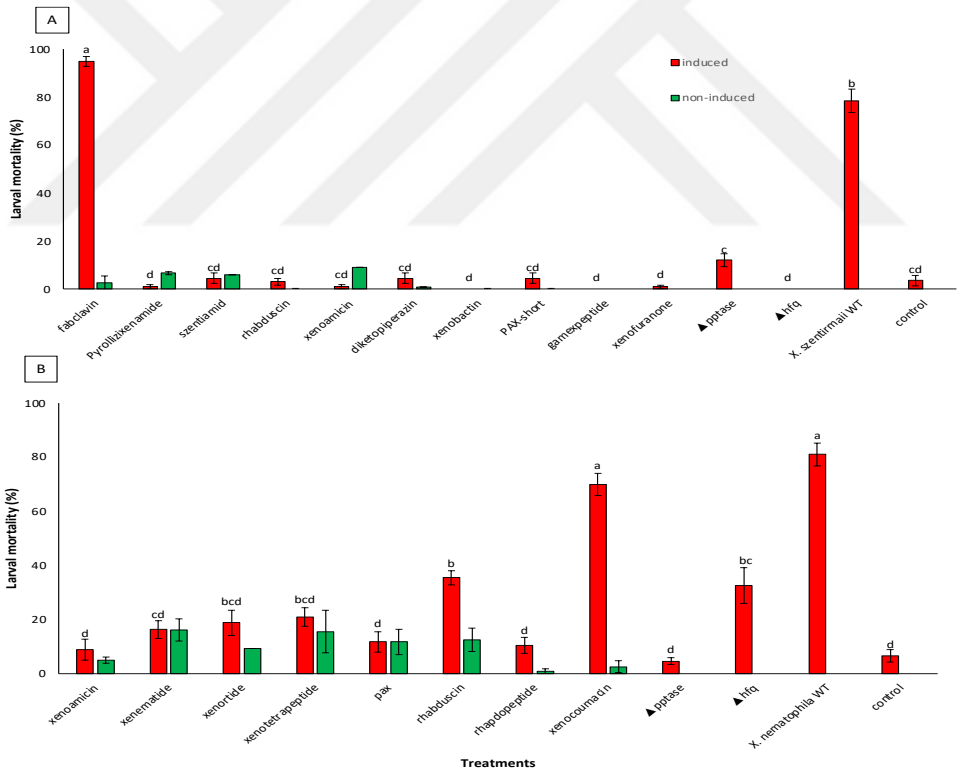


Figure 4.5. Identification of the larvicidal compound in cell-free supernatants from *Xenorhabdus szentirmaii* and *Xenorhabdus nematophila* Δhfq mutants. Same letter above bars indicate no statistical difference (P>0.05, Tukey test).

Fabclavine produced by *X. szentirmaii, X. budapestensis, X. cabanillasii, X. stockiae, X. hominickii* and *X. bovienii* differ greatly in structure and bioactivity e.g., *X. bovienii* produces derivatives with only the polyamine part (Wenski et al., 2020). These were assessed against mosquito larvae. After 24 h, *X. hominickii* and *X. cabanillasii* displayed significantly higher effects (91-96%) than *X. budapestensis, X. szenttirmaii* and *X. stockiae* which caused 71, 68, and 15% mortality, respectively ($F=111.557$; $df=6, 125$; $P<0.001$) (**Figure 4.6).** After 48 hours, a slight increment was observed in their effects ($F=171.204$; $df=6, 125$; $P<0.001$), but there was still a significant difference between the strains. Fabclavines from *X. hominickii, X. cabanillasii*, and *X. budapestensis* were effective when diluted (**Figure 4.7**).

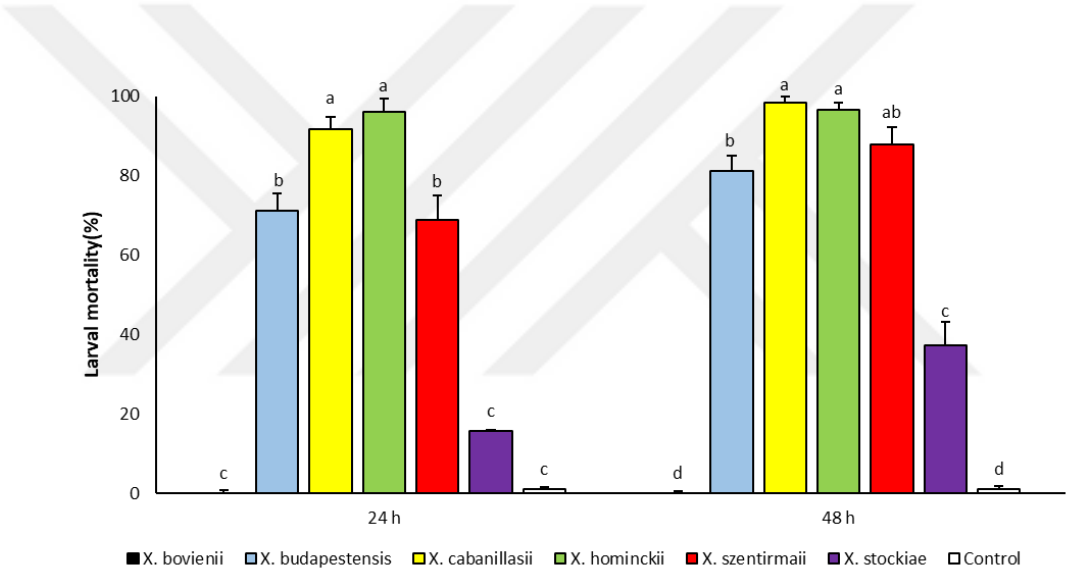


Figure 4.6. Larvicidal activity of different fabclavine types. Same letter above bars indicate no statistical difference ($P>0.05$, Tukey test).

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 ($F=15.097$; $df=3$; $P<0.001$; $\eta_p^2=0.118$), tested concentrations ($F=135.751$; $df=4$; $P<0.001$; $\eta_p^2=0.625$), and their interactions ($F=6.136$; $df=12$; $P<0.001$; $\eta_p^2=0.178$) on *Ae. albopictus* larval mortality (**Figure 4.8**).

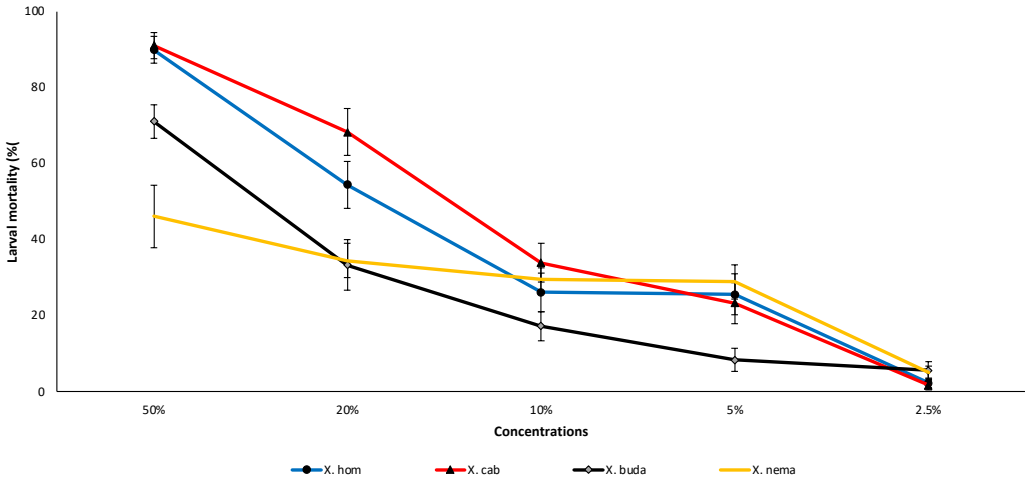


Figure 4.7. Larvicidal effects of xencoumacin from *Xenorhabdus nematophila* and fabclavines from *X. hominickii, X. budapestensis* and *X. cabanillasii* on *Aedes albopictus* after 24 h

4.4. Comparing the effects of Fabclavine with other commercial products

Results on the effects of the commercially available larvicidal products, fabclavines obtained from *X. cabanillasii* and *X. hominickii* mutants demonstrated that there were significant differences in the effects of larvicidal products, fabclavines and negative control in clean water. Four hours after treatment, Vectomax, Vectobac and spinosad caused 100%, serbate caused 77% larval whereas, fablcavines from *X. cabanillasii* and *X. hominickii* mutants exhibited 17.8 and 30.9% larval mortality, respectively ($F=348.928$; $df=7,112$; $P<0.001$) (**Figure 4.8.A**). At 24 h ($F=1101.954$; $df=7, 112$; $P<0.001$) and 48 h ($F=9649.027$; $df=7, 112$; $P<0.001$) assessments, all other larvicidal compounds, except for *L. sphaericus* which caused on 9% larval death, and fablcavines killed >94% *Ae. albopictus* larvae (**Figure 4..A**).

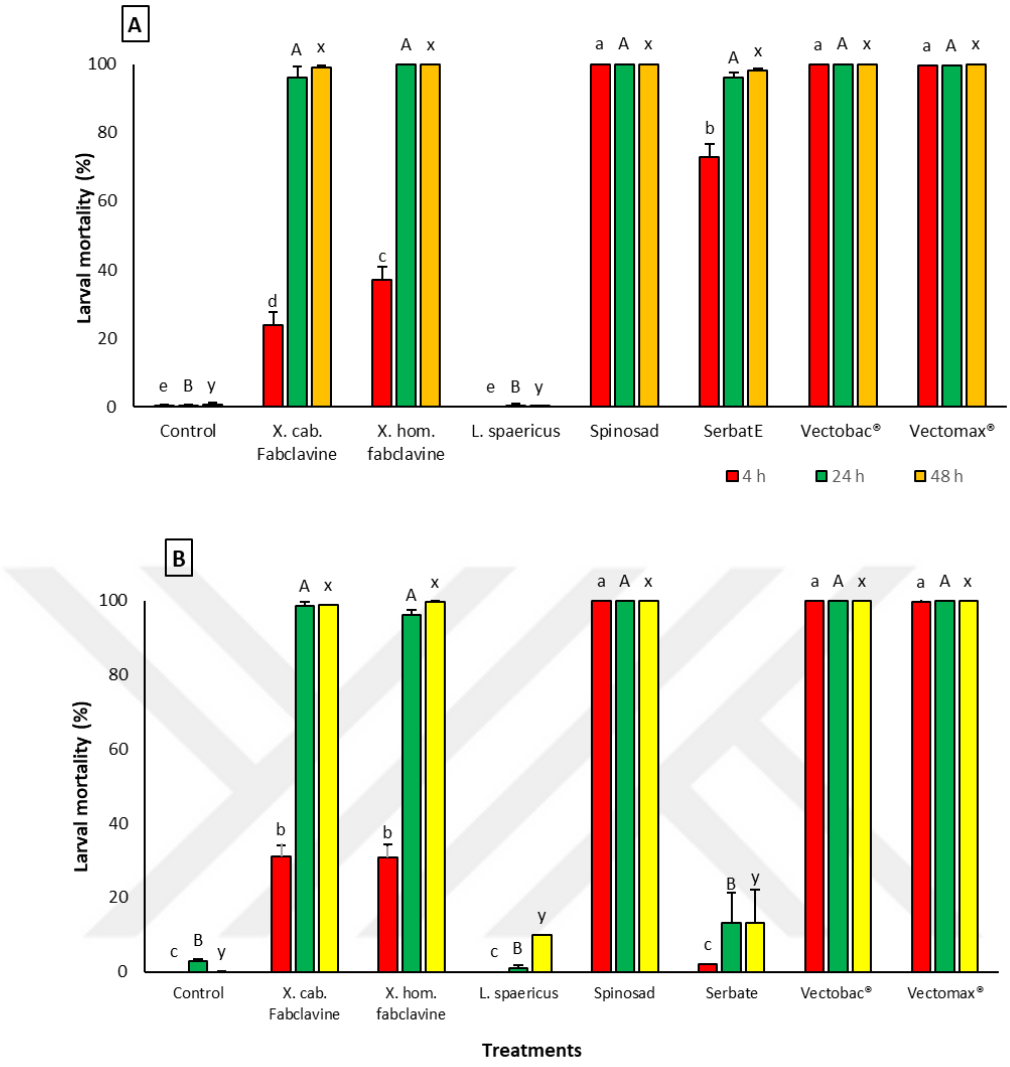


Figure 4.8. Larvicidal activity of fabclavine from *Xenorhabdus cabanillasii* and *Xenorhabdus hominickii* and commercial larvicides in clean and field-collected water. Same letter above bars indicates no statistical difference (P>0.05, Tukey test).

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 exhibited 100% mortality within 4 h ($F=735.629$; $df=7,80$; $P<0.001$) (Figure 17B). *L. sphaericus* and Serbate were ineffective in field-collected water. After 24 h, larval mortality caused by fabclavines obtained from *X. cabanillasii* and *X. hominickii* increased to 98 and

96%, respectively. Statistical difference occurred between the negative control, Spinosad 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) (**Figure 4.9.B**).

As for the residual/longevity effects, we observed that Spinosad, Vectomax and Vectobac maintained their efficacy 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 until 15 dpa. *L. sphaericus* was ineffective in both treatments, whereas Serbate was only effective in clean water (**Figure 4.9).**

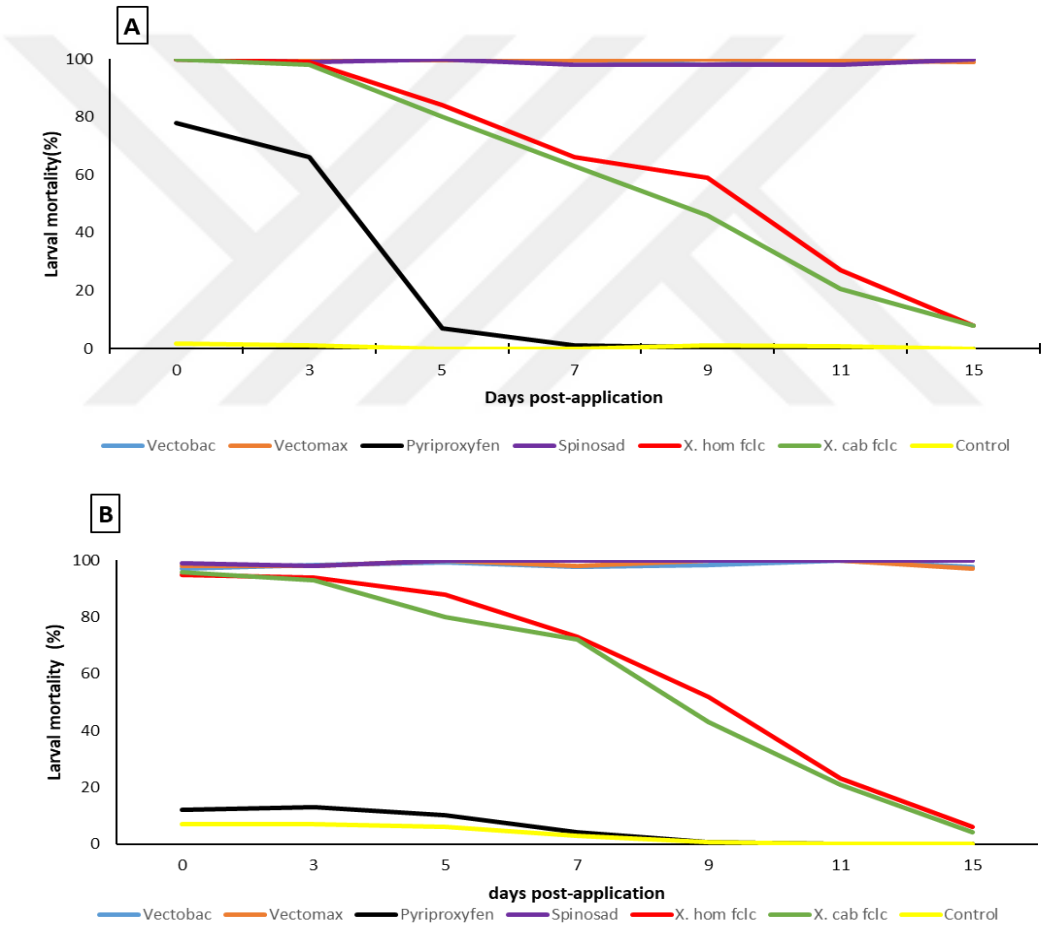


Figure 4.9. Residual effects of fabclavine from *Xenorhabdus cabanillasii and Xenorhabdus hominickii* and commercial larvicides in clean and field-collected water. Same letter above bars indicates no statistical difference (P>0.05, Tukey test).

5. DISCUSSION

This thesis performed a comprehensive screening of the larvicidal activity of the cell-free supernatant, growth culture and bacterial suspension of 22 different *Xenorhabdus* and six *Photorhabdus* species against *Ae. albopictus*. Results showed that the different bacterial species and the treatment type had significant effects on larval mortality. All treatments (i.e. supernatant, bacterial growth culture, bacterial pellet) of some bacteria caused larvicidal effects whereas, for some species either metabolites in supernatant or bacterial cells exhibited larvicidal activity.

Shah et al., (2021) 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. For example, supernatant obtained from *P. akhurstii, P. asymbiotica, X. cabanillasii, X. hominickii, X. indica, P. namnonensis, X. stockiae, X. budapestensis, X. miranenisis, X. nematophila, X. innexi, X. japonica X. szentirmaii, X. vietnamensis* and *X. bovienii* caused larval mortality ranging between 52-100%. In contrast, *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. thouganensis* presented less than 20% mortality. These bacteria whose supernatants were ineffective do not release larvicidal compounds and 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* (O.S. Silva et al., 2013; J.L.R. Silva et al., 2017; Vitta et al., 2018; Yooyanhket et al., 2018; Shah et al., 2021). O.S. Silva et al. (2013) observed that the oral pathogenicity of cell suspensions (10^7-10^8 cfu/ml) of *P. luminescens* killed 73 % and 83 whereas, *X. nematophila* killed 52 % and 42 % of *Ae. aegypti* larvae in fed and unfed treatment. In contrast, J.L.R. da Silva et al. (2017) tested the effects of different concentrations of *P. luminescens* and *X. nematophila* supernatants (at concentrations ranging from 12 to 54%) against *Ae. aegypti*. They observed that overall *X. nematophila* (LC_{50}=14.14%) was more effective than *P. luminescens* (LC_{50}=21.18%). In another study,

Wagutu et al. (2017) assessed the effects of *Photorhabdus* spp., and *Xenorhabdus* spp., from *H. indica* and *S. karii,* respectively against *Anopheles* mosquito larvae. They demonstrated that growth cultures and supernatants caused 50% larval death after 72 h and >80% after 96 hours. Vitta et al. (2018) evaluated the oral pathogenicity of cell suspensions (10^7-10^8 cfu/ml) of *X. indica, X. stockiae, P. luminescens* subsp*. akhurstii* and *P. lum.* subsp. *hainanensis* against *Ae. aegypti* and *Ae. albopictus*. They found that mortality of *Ae. aegypti* ranged between 68-80%, whereas *Ae. albopictus* was between 66-81% at 72 h after exposure. *Xenorhabdus* metabolites can even enhance the effects of other larvicidal compounds by inhibiting mosquito immune system. Park et al. (2016) reported a significant decrease in LC_{50} values of Bti from 2.9 x 10^5 spores/ml to 1.5 x 10^5 spores/ml, 1.7 x 10^5 spores/ml, and 1.9 x 10^5 spores/ml, respectively when applied with *X. nematophila, X. hominickii, P. temperata* subsp. *temperata* against *Ae. albopictus* larvae. The authors prepared a mosquitocidal formulation made up of 10% Bti (10^{10} spores/ml), 80% *X. nematophila*-cultured broth, and 10% preservative and found this formulation to be more effective (100%) at 1,000 ppm compared to Bti product alone.

Our study, as a first, demonstrates using the easyPACId method that the bioactive compounds were fabclavine from *X. szentirmaii* and xenocoumacin from *X. nematophila*. This technique allowed us to demonstrate the effects of natural products encoded in a specific genes and synthesized by NRPS or PKS enzymes using mutants with regulable gene. Promoter sequences of some genes in *X. szentirmaii* and *X. nematophila* were replaced with inducible promotors. Fabclavines and xenocoumacins are water-miscible non-ribsomally synthesised peptide/polyketide peptide compounds produced mainly by several *Xenorhabdus* spp. These compounds have been demonstrated to possess antibacterial, antifungal and antiprotozoal 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 (Mclnernery et al., 1991; Reimer et al. 2009; Fuchs et al., 2014; Shapiro-Ilan et al., 2014; Wenski et al., 2019, 2020; Gulsen et al., 2022). Correlated with data from Wenski et al. (2020) and Tobias et al. (2017b), 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*) 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 (Wenski et al., 2020). Numerous researches have described the possible application of these fabclavine and xenocoumacin compounds against medical, veterinary, and agriculturally important pathogens (Donmez-Ozkan et al., 2019; Wenski et al., 2020; Cimen et al., 2021; Gulsen et al., 2022; Abebew et al., 2022; Otoya-Martinez et al., 2023). Other compounds with larvicidal activity reported thus far are tca complex protein (Bowen et al., 1998), PirAB proteins (Ahantarig et al., 2009), and anthraquinones (1,3-dimethoxy-8-hydroxy-9,10-anthraquinone and 3 methoxychrysazine) (Ahn et al., 2013) 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 (Ensign et al., 2014; Kim et al., 2017). Ahantarig et al. (2009) reported that PirAB proteins exhibited no activity against *Mesocyclops thermocyclopoides* – a crustacean used as a biological control agent against mosquito larvae.

We compared the effects of fabclavines with current commercially available larvicidal products: Vectomax, Vectobac, spinosad, *L. sphaericus* and serbate in clean water and in field collected water. In both treaments Vectomax, Vectobac and Spinosad caused 100 % larval mortality and fablcavines from *X. cabanillasii* and *X. hominickii* killed 94% *Ae. albopictus* larvae within 24 h. Serbate was only effective in clean water. 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 ~15%. Likewise, J.L.R. da Silva et al., (2017) demonstrated that growth culture of *X. nematophila* have short longevity in water maintaining 100% efficacy against *Ae. aegypti* until the 4th day before a drastic decrease to 20% by the 11th day. Morgan et al. (1997) demonstrated that *X. nematophila* (pLV1013) and *P. luminescens* (pLV1012) cells release into river water were undetectable. Interestingly, fabclavines were reported to have oviposition deterrence effects against *Ae. albopictus* mosquitoes. The mosquitoes females sense this toxic compound through sensilla in tarsal segments of their legs before when they visit treated containers (Touray et al., to be published).

There has been a slow progress in vaccine development for most mosquito-borne infections hence mosquito control efforts on nearly all fronts i.e., surveillance, basic and

applied research, and prevention need to be ramped up. After public health officials and scientist have obtained detailed information on the species, distribution, and seasonality of mosquitoes through surveillance systems in any area, control methods are needed to reduce vector-human contact of dangerous species. Population-control efforts involve use of chemical pesticides mainly pyrethroids (permethrin, cypermethrin, prallethrin, resmethrin, deltamethrin, etofenprox, and lambda-cyhalothrin) in indoor residual spraying or impregnated into nets to control mosquitoes removal of breeding sites like standing water for mosquitoes, or use of bio-agents such as *Bacillus thuringensis israelensis* and insect growth regulators (diflubenzuron, methoprene, pyriproxyfen) (Benelli, 2015; Benelli et al., 2016). It is pertinent to develop or register new pesticides as development of resistance is widespread in most populations. Fabclavine and xenoucmacin compounds can be developed into novel biolarvicides or can be used as a model to design and synthesize other compounds.

6. CONCLUSION AND RECOMMENDATIONS

Our study demonstrates that an extensive number of *Xenorhabdus* and *Photorhabdus* bacteria, enteric bacteria associated with entomopathogenic nematodes, display larvicidal activity as cells or by producing secondary metabolites with larvicidal properties against *Ae. albopictus*. Using the easyPACId technique the bioactive compounds was identified to be fabclavine and xenocoumacin. *Aedes albopictus* is an invasive and a highly adaptive *Aedes* species currently undergoing a dramatic global expansion. It can vector viral pathogens of diseases such as yellow fever, dengue fever, West Nile fever, Zika and Japanese encephalitis in human communities. Physical, chemical and biological control approaches are used in managing mosquito populations. The emergence of strains resistant to pesticides currently used and their negative effects of these chemicals on the environment, other non-target organisms and human health has necessitated the search for safer alternatives. These natural compounds, fabclavine and xenoucoumacin, can be developed in novel biolarvicides or can be used as a model to design and synthesize other compounds.

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REPUCLIC OF TÜRKİYE

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

SCIENTIFIC ETHICS STATEMENT

I hereby declare that I composed all the information in my doctoral thesis entitled **Development of innovative biopesticides for the control of A*edes albopictus* mosquitoes** within the framework of ethical behavior and academic rules, and that due references were provided and for all kinds of statements and information that do not belong to me in this study in accordance with the guide for writing the thesis. I declare that I accept all kinds of legal consequences when the opposite of what I have stated is revealed.

Mustapha TOURAY

05 / 07 / 2023

CURRICULUM VITAE

Last name, First name **:** Touray, Mustapha

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Education

Level	Insitute	Date of graduation
Doctorate	Aydin Adnan Menderes University	2023
Master's	Aydin Adnan Menderes University	2019
Bachelor's	Adnan Menderes University	2017

Scholarhips and awards:

- Turkish Government Ibni Sina Medical Sciences Program –Full undergraduate scholarship
- Erasmus+ Placement Mobility to Romania (3 months)
- Erasmus Placement Mobility to Denmark (5 months)

WORK EXPERIENCE

Year	Place	Title
2017-2023	Laboratory for Invertebrate Animals, Aydin Adnan Menderes University,	Graduate Research Assistant
Mar-Sept 2023	Department of Plant and Environmental Sciences, University of Copenhagen	ERASMUS traineeship
Jan- Sept. 2019	College of Science, Swansea University, UK	Research Assistant–Based Overseas
Jun -Sept.	Banat`s University of Agricultural	ERASMUS traineeship

2016	Sciences and Veterinary Medicine, Romania

ACADEMIC PUBLICATIONS

1. ARTICLES

1. **Touray, M**., Bakirci, S., Ulug, D., Gulsen, S. H., Cimen, H., Yavasoglu, S. I., ... & Hazir, S. (2023). Arthropod vectors of disease agents: their role in public and veterinary health in Turkiye and their control measures. *Acta Tropica*, 106893.

2. Yavasoglu, S. İ., Wood, M. J., Alkhaibari, A. M., **Touray, M.,** & Butt, T. (2023). Potential of 3-octanone as a lure and kill agent for control of the Brown garden snail. *Journal of Invertebrate Pathology*, *198*, 107920.

3. Otoya-Martinez N., Leite LG, Harakava R, **Touray M,** Hazir S, Chacon-Orozco J, Bueno CJ. (2022). Antifungal effects of *Trichoderma* spp. and nematode-associated bacteria against the fungal pathogen *Neofusicoccum parvum* in pruning wounds of grapevine shoots. Fungal biology. (Under review)

4. Hazir, S., Kaya, H., **Touray, M.,** Çimen, H., Shapiro-Ilan, D. S. 2022. Basic laboratory and field manual for conducting research with the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, and their bacterial symbionts. *Turkish Journal of Zoology*, *46*(4), 305-350.

5. Cimen, H., **Touray, M**., Gulsen, S. H., Hazir, S. 2022. Natural products from *Photorhabdus* and *Xenorhabdus*: mechanisms and impacts. *Applied Microbiology and Biotechnology*, 1-13.

6. Gulsen, S.H., Tileklioglu, E., Bode, E., Cimen, H., Ertabaklar, H., Ulug, D., Ertug, S., Wenski, S.L., **Touray, M**., Hazir, C. and Bilecenoglu, D.K., 2022. Antiprotozoal activity of different Xenorhabdus and Photorhabdus bacterial secondary metabolites and identification of bioactive compounds using the easyPACId approach. *Scientific Reports 12*(1), 1-13.

7. Shah, F.A, Abdoarrahem, M.M., Berry, C., **Touray, M**., Hazir, S., Butt, T.M. 2021. Indiscriminate ingestion of entomopathogenic nematodes and their symbiotic bacteria by

Aedes aegypti larvae: a novel strategy to control the vector of Chikungunya, dengue, and yellow fever. Turkish Journal of Zoology, 45: 372-383.

8. Cimen, H., **Touray, M.,** Gulsen, S.H., Erincik, O., et al. 2021. Antifungal activity of different *Xenorhabdus* and *Photorhabdus* species against various fungal phytopathogens and identification of the antifungal compounds from *X. szentirmaii.* Applied Microbiology and Biotechnology.

9. Nalinci, E., Karagoz, M., Gulcu, B., Ulug, D., Gulsen, S.H., Cimen, H., **Touray, M.,** Shapiro-Ilan, D. and Hazir, S., 2021. The effect of chemical insecticides on the scavenging performance of *Steinernema carpocapsae:* direct effects and exposure to insects killed by chemical insecticides. Journal of Invertebrate Pathology, 107641.

10. Incedayi G., Cimen H., Ulug D., **Touray M**., Bode E., B., Bode H.B., E.O., Hazir S., Cakmak I. 2021. Relative potency of a novel acaricidal compound from *Xenorhabdus*, a bacterial genus mutualistically associated with entomopathogenic nematodes. Scientific Reports 11(1), 1-11.

11. **Touray M**., Cimen, H., Gulsen, S. H., Ulug, D., Erdogus, D., Shapiro-Ilan, D., Hazir, S. 2021. The impact of chemical nematicides on entomopathogenic nematode survival and infectivity. Journal of Nematology. 53, 1-17.

12. Hummadi, E.H., Dearden, A., Generalovic, T., Clunie, B., Harrott, A., Cetin, Y., Demirbek, M., Khoja, S., Eastwood, D., Dudley, E., Hazir, S., **Touray, M**., Ulug D., Gulsen, S.H., Cimen, H., Butt, T. 2021. Volatile organic compounds of *Metarhizium brunneum* influence the efficacy of entomopathogenic nematodes in insect control. Biological Control, 155, p.104527.

13. Raja, R.K., Arun, A., **Touray, M**., Gulsen, S.H., Cimen, H., Gulcu, B., Hazir, C., Aiswarya, D., Ulug, D., Cakmak, I., Kaya, H.K., Hazir, S. 2021. Antagonists and Defense Mechanisms of Entomopathogenic Nematodes and their Mutualistic Bacteria. Biological Control, p.104452.

14. Ceviz, D., Ulug, D., Cimen, H., **Touray, M.,** Hazir, S., Cakmak, I., 2020. Mode of entry of secondary metabolites of the bacteria, *Xenorhabdus szentirmaii* and *X. nematophila,* into *Tetranychus urticae.* and their effects on the predatory mites, *Phytoseiulus persimilis* and *Neoseiulus californicus.* Journal of Invertebrate Pathology, 174: 107418

15. **Touray, M.,** Gulcu, B., Uluğ, D., et al. 2020. Evaluation of the effects of different sponge types on entomopathogenic nematode. Journal of Invertebrate Pathology, 171: 107332.

2. PROJECTS

- Investigations on entomopathogenic nematodes and the Scavenger Deterrence Factor (SDF).

- Investigation of the efficacy of secondary metabolites produced by *Xenorhabdu*s and *Photorhabdus* bacteria against various human and plant pathogens.

3. POSTERS

A) Posters in International Conferences

➢ **Touray M.** & Hazır S., 2022. *Xenorhabdus* and *Potorhabdus* deter mosquito oviposition. The 8th Int`l Entomopathogens and Microbial Control Congress. Antalya Turkey

➢ Hazir, S., Ulug, D., Cimen, H., Gulsen, S. H., **Touray, M.,** et al. 2019. Do all *Xenorhabdus* and *Photorhabdus* bacteria protect nematode infected cadavers against scavengers? SIP/IOBC, Valencia.

➢ Hazir, C., Hazir, S., Ulug, D., Cimen, H., Gulsen, S. H., **Touray, M.,** et al. 2019. Does anthraquinone produced by *Photorhabdus* bacteria deter scavengers? The 7th Int`l Entomopathogens and Microbial Control Congress. Kayseri, Turkey.

➢ Hazır S., Uluğ D., Gulsen, S.H., Bode, E., Cimen, H., Wenski, S.L., Hazir, C., **Touray M.** 2018. Differences in the efficiency of scavenger deterrent factor among the different *Photorhabdus* and *Xenorhabdus* species. V. International Multidisciplinary Congress of Eurasia.