



Article

Comparative Insecticidal Efficacy of Symbiotic Bacteria (*Xenorhabdus* and *Photorhabdus*) and Their Bacterial Fractions Against Fall Armyworm, *Spodoptera frugiperda*

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Abstract

Background: The fall armyworm, *S. frugiperda*, is a globally threatening insect pest of agricultural crops, including in Thailand. Symbiotic bacteria in the genera *Xenorhabdus* and *Photorhabdus*, which are derived from entomopathogenic nematodes, can produce diverse bioactive compounds with insecticidal activities. However, no study on Thai isolates of these symbiotic bacteria against *S. frugiperda* has been reported, leading to a significant gap in local biopesticide development. **Methods:** Two symbiotic bacterial isolates, *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH), were isolated from Thai entomopathogenic nematodes and cultured in LB broth at 10⁸ CFU/mL. Three bacterial fractions, i.e., whole-cell suspension, cell supernatant, and cell pellet, were prepared from each isolate. Insecticidal bioassays were conducted by topical application on second- and fifth-instar larvae of *S. frugiperda* (10 larvae × 5 replicates per treatment, CRD). Larval mortality was recorded daily for seven days, and median lethal time (LT₅₀) was calculated. Data were analyzed by two-way ANOVA with Duncan's Multiple Range Test ($p < 0.05$). **Results:** The results indicated that both bacterial isolates caused significantly higher larval mortality than controls across all treatments ($p < 0.05$). *X. miraniensis* (bMH16.1_TH) showed higher virulence, achieving ~80% mortality of second-instar larvae within 24 h and 100% by day three. Whole-cell suspensions consistently produced the fastest and highest mortality with the lowest LT₅₀ values in both larval stages. Second-instar larvae were significantly more susceptible than fifth-instar larvae. The shortest LT₅₀ was recorded in second-instar larvae treated with *X. miraniensis* supernatant at 0.616 days, while fifth-instar larvae generally showed higher LT₅₀ values across all treatments. **Conclusions:** Our comparative study demonstrated that the insecticidal efficacy varied between the two symbiotic bacterial isolates and among their fractions. Whole-cell suspensions of *X. miraniensis* (bMH16.1_TH) produced the highest larval mortality and the smallest LT₅₀ values, particularly against early-instar larvae. These findings demonstrated that local symbiotic bacteria are promising candidates to be developed as sources of biopesticides against this insect pest. In addition, characterizing active insecticidal compounds, evaluating field efficacy, and assessing safety to non-target organisms need to be further studied to support integration of these biopesticides in sustainable pest management programs.



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1. Introduction

Fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), is one of the most destructive lepidopteran pests, affecting a wide range of agricultural crops worldwide. This insect is polyphagous; it can infest more than 350 plant species, including corn, rice, sorghum, sugarcane, cotton, soybean, wheat, and vegetables [1]. Damage on economic crops in many regions has threatened agricultural production and food security. Due to its high reproductive ability, capability to migrate, broad host range, and potential to develop resistance to chemical insecticides, *S. frugiperda* has become an invasive insect species throughout major agricultural regions worldwide [2,3]. This pest can cause substantial economic losses in corn production. Yield losses of up to 77% have been reported when crops are attacked by late-instar larvae, while damage to leaves, silks, and tassels ranging from 25% to 50% can reduce grain yield by as much as 58% [4,5]. Since its first detection in Thailand in 2018, infestations have rapidly spread across the country and it has become a major insect pest [6–8]. Chemical insecticide applications are the primary control strategy, but they have negative impacts on the environment and non-target organisms, and there is a growing problem of insecticide resistance. There have been reports of resistance in this insect species to organophosphate (OP) and pyrethroid (PYR) insecticides in several areas of America and Africa [9]. Hence, alternative approaches, such as the use of biological control agents, have received considerable attention as they are promising eco-friendly tools for insect management.

Entomopathogenic nematodes (EPNs) are among the effective biological control agents. The pathogenicity of EPNs is primarily determined by their symbiotic bacteria, principally *Xenorhabdus* and *Photorhabdus* spp. Once the EPNs enter the host body, these bacteria are released into the insect hemocoel, where they reproduce and release diverse bioactive compounds, including toxins, enzymes, antimicrobial metabolites, and immunosuppressive compounds, leading to host death within a short period [10,11]. Practical applications of whole EPNs in field conditions can reduce nematode survival and infectivity due to constraints by environmental factors such as temperature fluctuations, UV radiation, and soil moisture availability [12]. These limitations, combined with other challenges, such as maintaining viable nematode cultures and stability during storage and transportation, have led to interest in exploiting bacterially derived metabolites and cell preparations as standalone biopesticides [13]. Therefore, the utilization of symbiotic bacteria has been considered an alternative potential control strategy. These symbiotic bacteria are responsible for suppressing the host's immune system, inducing digestive organ malfunctions, and causing host death [8]. Various species of *Xenorhabdus* and *Photorhabdus* have been explored from several regions, including Thailand. Refs. [14,15] reported several Thai EPN-associated symbiotic bacteria that have demonstrated insecticidal potential against *Aedes aegypti* larvae. The results suggested that native bacterial isolates may harbor distinct virulence traits effective against economically important insects adapted to tropical conditions.

The deployment of symbiotic bacteria depends on suspension preparations. Several investigations have demonstrated that the insecticidal activity of bacteria varies among fractions. The whole-cell suspension is a fraction that contains viable cells, which can multiply and continue to produce toxins after infection in the host body. Cell-free supernatant is a suspension without living cells and contains extracellular materials, secreted toxins, and other bioactive metabolites, whereas cell pellet suspension retains intracellular substances

and membrane-associated virulence factors [16–18]. Hence, each fraction can perform different activities according to its composition. To use them as biological control pesticides, the effectiveness of each fraction should be examined to optimize bacterial preparations. Considering the suitable applications, the developmental stage of the insect host has a significant influence on susceptibility to microbial infection, particularly in early stages that are considerably susceptible to pathogen infections. Even though, at these stages, they consume less plant tissue, they are in the phase of dispersing to colonize host plants. In contrast, later instars cause significant damage due to higher food consumption capacity and can increase resistance to microbial infection as they approach full development [19,20]. Identifying the most susceptible larval stages is therefore critical for designing targeted biocontrol strategies that maximize pest mortality while minimizing the quantities of biopesticide required.

Despite the demonstrated potential of EPN-associated symbiotic bacteria as biocontrol agents against important insect pests, no study has evaluated Thai isolates against *S. frugiperda*. This gap limits our understanding of the insecticidal efficacy of native bacterial strains against one of the major agricultural pests in Thailand and other tropical regions. To clarify this gap, we evaluated the insecticidal efficacy of *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH), derived from Thai EPN populations, against *S. frugiperda*. We compared the pathogenicity of different bacterial fractions, including whole-cell, cell supernatant, and cell pellet fractions, against second- and fifth-instar larvae. The findings of this study may contribute to the development of locally sourced bacterial biopesticides as effective and sustainable alternatives for fall armyworm management in Thailand and other tropical agricultural regions.

2. Materials and Methods

2.1. Collection and Rearing of Fall Armyworm (*S. frugiperda*)

Larvae of fall armyworm, *S. frugiperda*, were collected from infested maize fields in northern Thailand. The species were verified according to the identification procedures of [21]. The larvae were maintained individually in 20 mL plastic containers and fed fresh pesticide-free maize leaves under laboratory conditions (25 ± 2 °C, $60 \pm 10\%$ RH, and a 14:10 h light:dark photoperiod). Pupae were placed in a plastic container and transferred into an adult emergence cage ($30 \times 30 \times 30$ cm), with young maize plants provided as an oviposition substrate. Upon emergence, the adults were provided with a 10% sugar solution as food. The insects were reared under laboratory conditions for at least two generations prior to the tests. This was to eliminate potential effects of previous environmental exposure, especially pesticide residues and/or infections by other natural enemies. Only healthy larvae and those with no visible signs of injury or deformity were selected for the experiments to ensure the homogeneity of experimental outcomes.

2.2. Isolation and Multiplication of Symbiotic Bacteria

The two symbiotic bacteria were isolated from their entomopathogenic nematode hosts according to [14,15]. *Xenorhabdus miraniensis* (bMH16.1_TH) (GenBank: KY404031) is associated with the entomopathogenic nematode *Steinernema websteri*, and *Photorhabdus akhurstii* (bSBR11.1_TH) (GenBank: MK478079) is associated with *Heterorhabditis indica*. Each nematode–bacterium complex suspension (50 µL) was cultured on Petri dishes with NBTA (Nutrient Agar (NA), Bromothymol blue, and 2,3,5-Triphenyl tetrazolium chloride, supplemented with Ampicillin (50 mg/mL)) [22]. They were incubated for 48 h at 25 ± 2 °C in the dark, before bacterial colonies were selected. After three to four days of room-temperature incubation, bacterial colonies were selected based on their characteristics. *Xenorhabdus* species were verified as rounded colonies with smooth margins and the ability

to absorb colorants [23], whereas *Photorhabdus* was identified based on a light or dark green colony color with a convex or umbonated surface on NTBA [24].

2.3. Preparations of Bacterial Fractions (Whole-Cell, Cell Supernatant, and Cell Pellet Suspensions)

Each symbiotic bacterial isolate mentioned above was transferred from NBTA medium stock culture to Luria–Bertani (LB) medium and incubated at 25 °C in the dark and shaken at 150 rpm for 24 h. Subsequently, 100 mL of the bacterial suspension was transferred into 250 mL of fresh LB broth and further incubated under the same conditions for 48 h, following the methods described by [15].

The bacterial suspension was then serially diluted using a ten-fold dilution method, and the bacterial density was determined by the spread-plate technique, which resulted in an approximate concentration of 10^8 CFU/mL. The same stock bacterial suspension was divided into two equal volumes. The first volume was used as the whole-cell suspension and applied directly to this concentration. The second volume was transferred to prepare the cell supernatant and cell pellet suspensions. To separate these fractions, the suspension was centrifuged at $10,000 \times g$ for ten minutes. After centrifugation, the cell supernatant was collected and stored in 15 mL centrifuge tubes and used without filtration. The cell pellet fraction was prepared by adding the same volume of sterile distilled water to the remaining fraction, containing bacterial cells. Each fraction was tested using five technical replicates within a single experimental run.

The whole-cell suspension consisted of the bacterial culture in LB broth. After centrifugation, the supernatant was collected and used directly without further filtration. The bacterial pellet was resuspended in sterile distilled water to the original culture volume. All three bacterial preparations were kept at 4 °C until the bioassays.

2.4. Insecticidal Bioassay

Three treatments consisting of 0.5 mL of each bacterial suspension (whole-cell, cell supernatant, and cell pellet suspensions) and a control treatment were examined. Second- and fifth-instar larvae were tested separately. Each treatment consisted of five replicates with ten larvae per replicate in a completely randomized design (CRD).

Each larva was individually placed in a Petri dish (5.5 cm in diameter) containing a detached maize leaf as food for the fall armyworm. For each treatment, 0.5 mL of the bacterial suspension was applied topically to larvae, to the leaf surface, and on the bottom of the Petri dish, to ensure that the tested larvae received an adequate volume of bacterial suspensions (Figure 1). A nominal bacterial dose of approximately 5×10^7 CFU was used in each treatment. Sterile distilled water was used for control treatment. Treated larvae were maintained under laboratory conditions, and mortality was recorded daily for seven days.

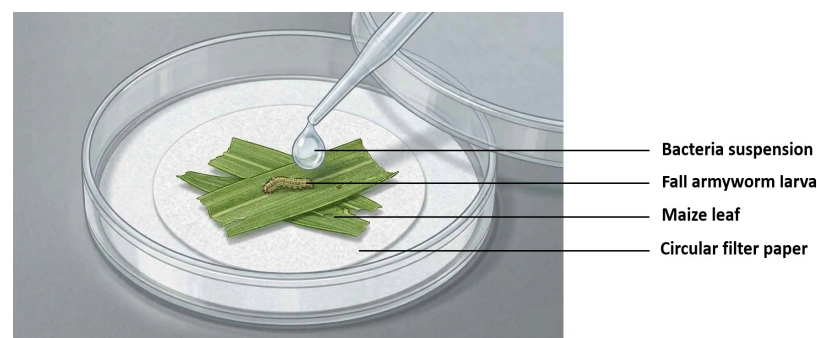


Figure 1. Illustration of the bacterial suspension application procedure used for the insect bioassays.

2.5. Mortality Assessment

Larvae were considered dead when they showed no response to gentle stimulation with forceps. The observed mortality was calculated as

$$\text{Observed mortality} = (\text{Total number of dead larvae} \times 100) \div \text{Total number of tested larvae} \quad (1)$$

2.6. Median Lethal Time (LT₅₀)

The median lethal time (LT₅₀) of each treatment was calculated based on days after infection, with the objective of comparing the period (days) that each bacterial isolate required to induce 50% death in the examined larvae. This value compared the pathogenicity among bacterial isolates and fractions.

2.7. Statistical Analysis

Mortality data from the control and treatment groups (each bacterial isolate) were analyzed using SPSS 17.0 at a significance level of $p < 0.05$. Two-way analysis of variance (ANOVA) was performed to evaluate the effect of bacterial treatment and infection status (infected vs. control) on larval mortality. When significant differences were detected, post hoc comparisons were conducted using Duncan's Multiple Range Test (DMRT) to determine differences among treatments and observation times after infection. The lethal time (LT₅₀) values were estimated using SPSS software following the method described by [25].

3. Results

3.1. Insecticidal Activity of Symbiotic Bacteria Against *S. frugiperda*

Both *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH) exhibited significant insecticidal activity against second- and fifth-instar larvae of *S. frugiperda* compared to the control ($p < 0.05$) (Figures 2 and 3). No mortality was observed in the control treatments throughout the experimental period.

Among the tested bacterial isolates, *X. miraniensis* (bMH16.1_TH) consistently induced higher and more rapid larval mortality than *P. akhurstii* (bSBR11.1_TH). In second-instar larvae, *X. miraniensis* (bMH16.1_TH) caused considerable mortality within 24 h after infection and reached 100% on day three (Figure 2A). Mortality caused by *P. akhurstii* (bSBR11.1_TH) increased more gradually over time, with mortality generally occurring later, with around 30% on the first day after infection. The mortality increased at a slower rate and reached 100% later on day five or six (Figure 2B).

In both larval stages, whole-cell suspensions exhibited considerably greater and more rapid insecticidal activity than supernatant and cell pellet fractions among the examined bacterial fractions ($p < 0.05$) (Figures 2 and 3). The whole-cell fraction of *X. miraniensis* (bMH16.1_TH) killed approximately 80% of the second-instar larvae in the first 24 h and 100% of the larvae in three days. While the cell pellet required up to six days to reach 100% mortality, the supernatant fraction treatment reached full mortality by day five (Figure 2A).

Infection by *P. akhurstii* (bSBR11.1_TH) resulted in a lower mortality rate (Figure 2B). The whole-cell fraction caused higher and faster mortality, with total mortality observed on day five and day six in the second and fifth instars, respectively. Day five of the second-instar treatment showed a 100% mortality rate, despite the supernatant fraction causing slower mortality. On the seventh day following infection, both fractions resulted in 100% death in the older stage (Figure 3A,B).

3.2. Efficacy of Different Bacterial Fractions on Larval Mortality

In both larval stages, whole-cell suspensions exhibited comparable higher insecticidal activity than supernatant and cell pellet fractions among the examined bacterial fractions

($p < 0.05$), particularly during the early period after infection (Figures 2 and 3). The whole-cell fraction of *X. miraniensis* (bMH16.1_TH) killed approximately 80% of the second-instar larvae in the first 24 h and 100% of the larvae in three days. While the cell pellet and the supernatant fractions required up to five days to reach 100% mortality. Infection by *P. akhurstii* (bSBR11.1_TH) resulted in a lower mortality rate. The whole-cell fraction caused higher and faster mortality, with total 100% mortality observed on day five and day six in the second and fifth instars, respectively. Day five of the second-instar treatment showed a 100% mortality rate, despite the supernatant fraction causing slower mortality. On the seventh day following infection, both fractions resulted in 100% death in the older stage (Figure 3A,B).

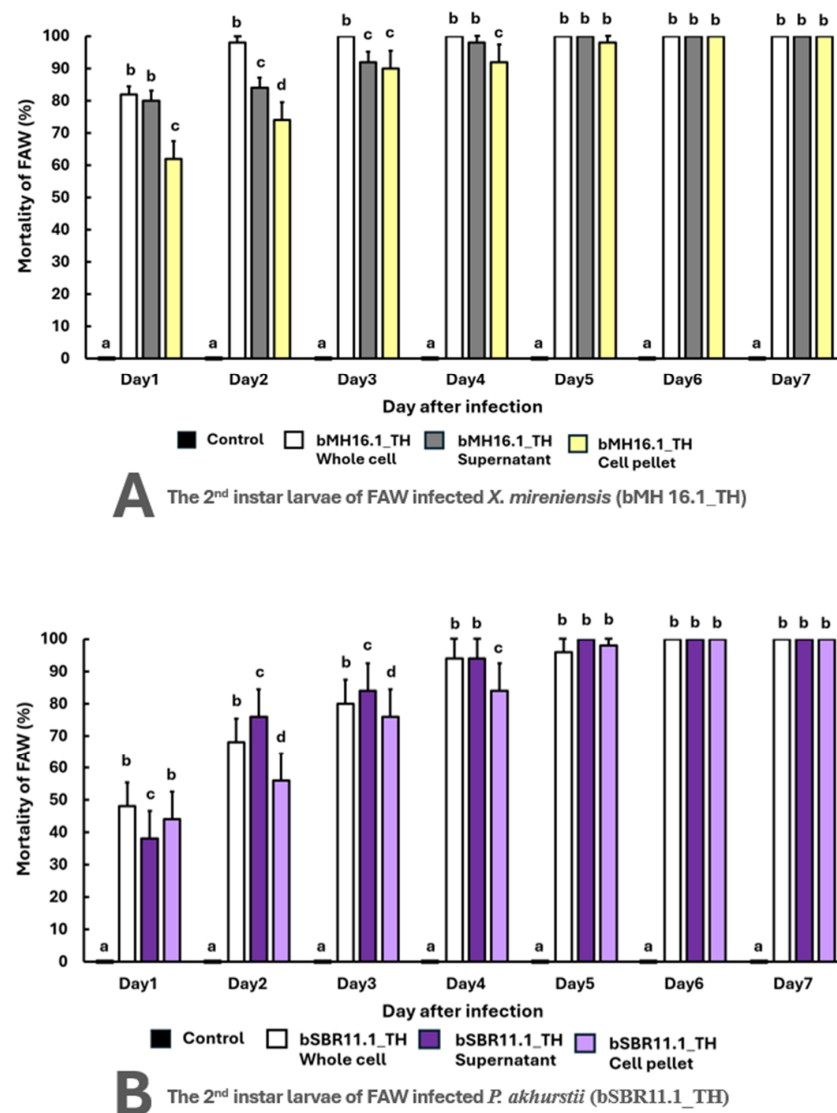


Figure 2. Mortality of the second-instar larvae of fall armyworm (*S. frugiperda*) after being infected by *X. miraniensis* (bMH16.1_TH) (A) and *P. akhurstii* (bSBR11.1_TH) (B) over seven days of observation. Error bars represent $\pm$ SD. Different lowercase letters indicate statistical significance between treatments within a bacterial isolate ($p < 0.05$).

3.3. Efficacy of Larval Instar Developmental Stages

Larval susceptibility to bacterial infection depends upon developmental stages. In all three bacterial fractions, the time required to reach complete mortality was consistently longer in older larvae. Second-instar larvae were significantly more susceptible, as evi-

denced by higher mortality and shorter time to death ($p < 0.05$) (Figures 1 and 2). The results indicated a clear reduction in susceptibility with increasing larval age.

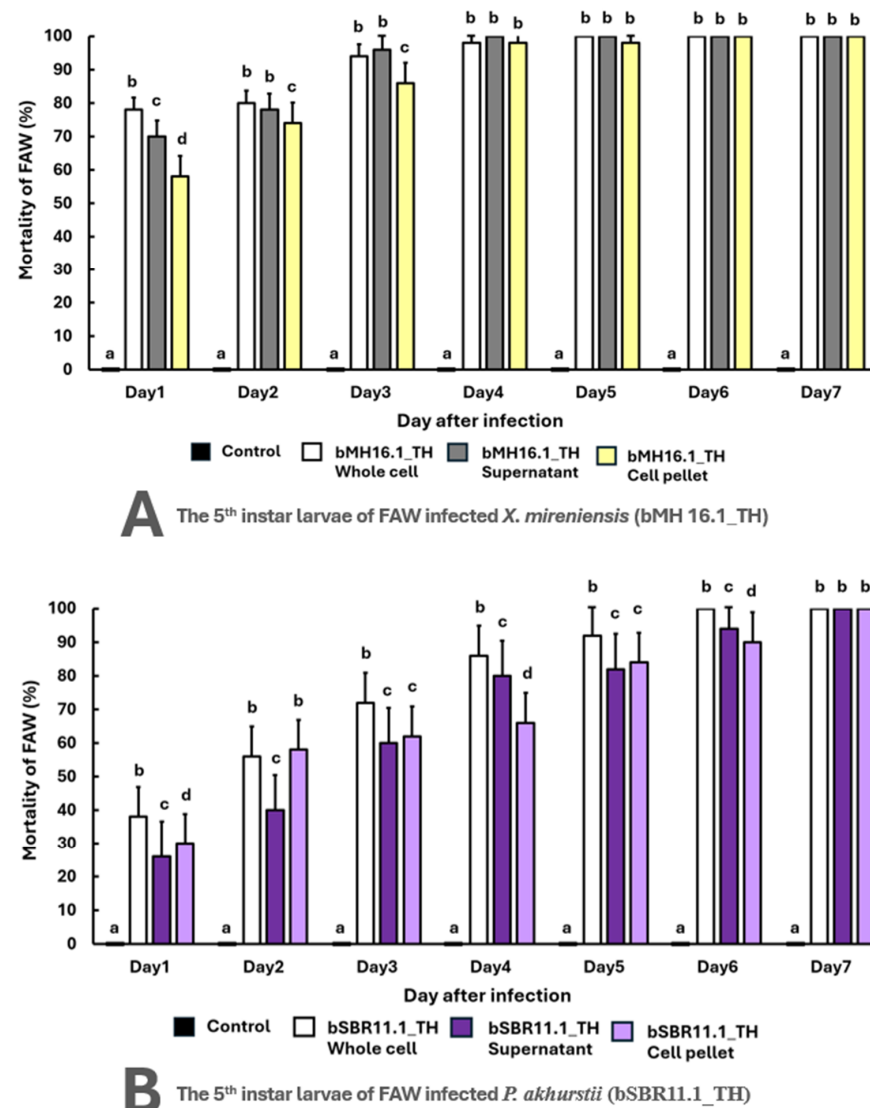


Figure 3. Mortality of the fifth-instar larvae of fall armyworm (*S. frugiperda*) after being infected by *X. miraniensis* (bMH16.1_TH) (A) and *P. akhurstii* (bSBR11.1_TH) (B) over seven days of observation. Error bars represent $\pm$ SD. Different lowercase letters indicate statistical significance between treatments within a bacterial isolate ($p < 0.05$).

3.4. Lethal Time (LT₅₀) Analysis

The median lethal time (LT₅₀) values were computed to evaluate the virulence of bacterial fractions and isolates. Higher virulence occurred with the bacteria *X. miraniensis* (bMH16.1_TH), particularly in the second-instar larvae (Table 1). The supernatant fraction exhibited the shortest LT₅₀ of 0.616 days (95% fiducial limit: 0.475–0.740 days), followed by the whole-cell suspension at 0.670 days (95% fiducial limit: 0.352–0.711 days), which was not significantly different ($p > 0.05$). The cell pellet fraction showed a substantially longer LT₅₀ of 2.197 days (95% fiducial limit: 1.178–2.560 days) and differed significantly from the other two treatments ($p < 0.05$). On the other hand, the whole-cell and supernatant fractions of *P. akhurstii* (bSBR11.1_TH) required significantly longer time to reach LT₅₀ values of 1.526 (95% fiducial limit: 1.102–1.707 days) and 1.720 days (95% fiducial limit: 1.220–2.401 days) ($p > 0.05$), respectively. The cell pellet fraction was the least effective in killing these earlier-

instar insects, with an LT_{50} value of 2.170 days (95% fiducial limit: 1.765–2.357 days) and statistically significant differences from the other two fractions ($p < 0.05$).

Table 1. LT_{50} values of second instar of fall armyworm (*S. frugiperda*) larvae exposed to two isolates of symbiotic bacteria of entomopathogenic nematodes, *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH).

Bacterial Isolates	Bacterial Fractions	T_{50} (Days)	Fiducial Limit (95%), Lower–Upper	Slope $\pm$ S.E.	χ^2	Df	p
<i>X. miraniensis</i> (bMH16.1_TH)	Whole-cell	0.670 a	0.352–0.711	0.56 ± 0.38	1.90	4	0.219
	Supernatant	0.616 a	0.475–0.740	0.74 ± 0.13	2.25	4	0.062
	Cell pellet	2.197 b	1.178–2.560	0.54 ± 0.27	5.42	4	0.113
<i>P. akhurstii</i> (bSBR11.1_TH)	Whole-cell	1.526 m	1.102–1.707	0.57 ± 0.77	4.92	4	0.153
	Supernatant	1.720 m	1.220–2.401	0.52 ± 0.17	5.72	4	0.170
	Cell pellet	2.170 n	1.765–2.357	0.80 ± 0.23	1.28	4	0.261

Different lowercase letters indicate statistical significance ($p < 0.05$) between the bacterial suspensions within the bacterial isolate.

As larval maturity increased to the fifth instar, the time required to achieve LT_{50} generally increased (Table 2), and the pattern of significant differences varied among bacterial fractions. The whole-cell treatment of *X. miraniensis* (bMH16.1_TH) remained the most effective, showing the lowest LT_{50} at 1.748 days (95% fiducial limit: 1.351–1.748 days), followed by the cell pellet fraction (1.891 days) (95% fiducial limit: 0.923–2.185 days) and supernatant fraction (1.915 days) (95% fiducial limit: 1.539–1.915 days) (Table 2). However, the overlapping fiducial limits and statistical comparisons indicated that the whole-cell suspension and cell supernatant were not significantly different ($p > 0.05$).

Similar to those in the younger larvae, *P. akhurstii* (bSBR11.1_TH) exhibited a higher LT_{50} value. Among the bacterial fractions, the cell pellet fraction showed the lowest LT_{50} value of 2.128 days (95% fiducial limit: 1.847–2.302 days), followed by the supernatant fraction, with a value of 2.182 days (95% fiducial limit: 1.974–2.249 days) ($p > 0.05$), whereas the whole-cell treatment showed the highest LT_{50} value at 2.551 days (95% fiducial limit: 2.326–2.703 days). The whole-cell suspension did not differ significantly from the cell pellet fraction ($p > 0.05$), but differed significantly from the supernatant treatment ($p < 0.05$). These results suggested that *p. akhurstii* (bSBR11.1_TH) was not the fastest-acting preparation, and numerical differences among fractions should be interpreted in conjunction with the statistical comparisons.

Table 2. LT_{50} values of fifth instar of fall armyworm (*S. frugiperda*) larvae exposed to two isolates of symbiotic bacteria of entomopathogenic nematodes, *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH).

Bacterial Isolates	Bacterial Fractions	T_{50} (Days)	Fiducial Limit (95%), Lower–Upper	Slope $\pm$ S.E.	χ^2	Df	p
<i>X. miraniensis</i> (bMH16.1_TH)	Whole-cell	1.748 a	1.351–1.748	0.47 ± 0.10	1.80	4	0.090
	Supernatant	1.915 ab	1.539–1.915	0.69 ± 1.67	4.92	4	0.059
	Cell pellet	1.891 b	0.923–2.185	0.93 ± 1.15	3.93	4	0.088
<i>P. akhurstii</i> (bSBR11.1_TH)	Whole-cell	2.551 m	2.326–2.703	0.42 ± 0.13	4.97	4	0.006
	Supernatant	2.182 n	1.974–2.249	0.34 ± 0.51	1.27	4	0.041
	Cell pellet	2.128 mn	1.847–2.302	0.31 ± 0.14	3.42	4	0.294

Different lowercase letters indicate statistical significance ($p < 0.05$) between the bacterial suspensions within the bacterial isolate.

4. Discussion

The current study demonstrated that *Xenorhabdus* and *Photorhabdus*, two symbiotic bacteria derived from entomopathogenic nematodes, possess insecticidal efficacy against *S. frugiperda*. There were remarkable differences in pathogenicity among bacterial isolates, bacterial fractions, and larval developmental stages. This finding is consistent with previous studies indicating that *Xenorhabdus* and *Photorhabdus* possess bioactive potential and can produce toxins, enzymes, and immunosuppressive compounds that contribute to rapid host mortality [10,11]. Throughout all larval stages and bacterial preparations, *X. miraniensis* (bMH16.1_TH) continuously displayed greater virulence than *P. akhurstii* (bSBR11.1_TH), as indicated by higher mortality rates and lower LT₅₀ values. Variations in virulence among bacterial isolates may be associated with differences in the composition and expression of these metabolites [26,27]. These results were consistent with previous studies showing that *Xenorhabdus* species can produce highly potent insecticidal substances that disrupt insect midgut function, suppress immune responses, induce septicemia, and eventually cause host death [11]. The production of toxin complexes (Tc toxins) and other virulence-associated compounds, which are common in *Xenorhabdus* and *Photorhabdus* species, may be responsible for the rapid insecticidal activity observed in this study [28,29]. Tc toxins specifically damage insect midgut epithelium and lumen, disrupting nutrient absorption and causing progressive tissue destruction [30]. In this study, differences in insecticidal activity between *X. miraniensis* (bMH16.1_TH) and *P. akhurstii* (bSBR11.1_TH) were observed, suggesting that pathogenicity among symbiotic bacteria of entomopathogenic nematodes is highly species- and isolate-specific. The superior virulence of *X. miraniensis* (bMH16.1_TH) likely reflects isolate-specific differences in the expression and diversity of these virulence-associated compounds, though the exact metabolite profiles of this Thai isolate remain to be fully characterized. Future metabolomic analyses would help elucidate which specific compounds drive the observed insecticidal potency. However, since this study emphasizes only two Thai local bacterial isolates, further investigations involving additional species of *Xenorhabdus* and *Photorhabdus* should be considered in order to expand the possibility of using symbiotic bacteria as biopesticides.

Whole-cell suspensions of both bacterial isolates showed the highest and fastest insecticidal activity across all bacterial isolates and larval developmental stages. These results coincide with previous research demonstrating that whole-cell fractions contain both extracellular and intracellular bioactive compounds, which may enhance pathogenicity by continuing the synthesis of toxins, enzymes, and immunosuppressive factors within the host body [16,17]. In addition, the whole-cell suspension used in this study was suspended in its original culture medium, which may have allowed continued bacterial metabolism and the production of additional extracellular metabolites during the bioassay. Consequently, the observed insecticidal activity likely reflects the combined effects of viable bacterial cells and their naturally occurring metabolites rather than bacterial cells alone. In addition, the whole-cell suspension used in the present study was standardized to 1×10^8 CFU/mL prior to fractionation, corresponding to approximately 5×10^7 CFU per treatment, which is within the range commonly applied for *Xenorhabdus* and *Photorhabdus* bioassays against insect pests [15,31]. Nevertheless, comparisons with previous studies on lepidopteran pests remain limited because bacterial inocula are frequently reported as culture broth or optical density rather than viable cell counts (CFU/mL) [32,33]. Standardized dose-response assays, standardized buffer-based preparations, and quantification of individual bioactive compounds are needed to improve experimental reproducibility and facilitate comparison among studies. Considering that the supernatant fraction contains mainly extracellular metabolites, whereas the cell pellet fraction contains intracellular and membrane-associated compounds, resulting in reduced and delayed insecticidal effects [34,35], the insecticidal

activity observed in the cell supernatant is likely associated with a complex mixture of extracellular bioactive compounds rather than a single component. In addition to secreted protein toxins and secondary metabolites, Gram-negative bacteria (*Xenorhabdus* and *Photorhabdus*) also release lipopolysaccharides into the extracellular environment, which may contribute to biological activity [36]. However, the present study did not characterize or quantify individual extracellular components; therefore, the relative contribution of lipopolysaccharides and other secreted metabolites could not be determined.

Because the supernatant fraction used in this study was not membrane-filtrated before the bioassay, the possible presence of residual viable bacterial cells cannot be completely excluded. Hence, we might assume that part of the observed insecticidal activity may have resulted from both extracellular metabolites and a limited number of remaining bacterial cells. This would be a limitation of the study. So, we suggest that further studies on insecticidal activity of bacterial supernatant should involve filtration to distinguish the effects of secreted metabolites from those of live bacteria.

Larval developmental stage also played a critical role in determining susceptibility to bacterial infection. Compared to fifth-instar larvae, second-instar larvae were more vulnerable, as evidenced by higher mortality rate and lower LT_{50} values. According to previous research, younger insect stages are typically more susceptible to pathogen infections due to their thinner cuticles, less developed immune systems, and higher physiological sensitivity [37,38]. Additionally, early instars of *S. frugiperda* have been reported to be more susceptible to entomopathogenic nematode infections [39]. Tolerance in older larval instars is often attributed to more developed physiological and immunological defense, including stronger physical barriers such as thicker cuticles. Furthermore, they can enhance cellular encapsulation and increase antimicrobial peptide production, which collectively reduce pathogen efficacy and delay death [40–42]. In addition to mortality results, the median lethal time values (LT_{50}) provided additional quantitative results of the speed of bacterial action. In *X. miraniensis* (bMH16.1_TH) treatment, lower LT_{50} values, particularly in second-instar larvae (0.616 days), indicated faster action against the insect host, which is a desired property for biological control agents. According to [29], rapid host mortality minimizes feeding damage and reduces the likelihood of resistance development. This plays an important role as a control strategy, especially for *S. frugiperda*, as they can cause severe yield losses in later feeding stages [2]. However, fiducial limits showed some considerable overlap, suggesting that differences between some treatments might not be statistically significant. This finding suggests that both whole-cell suspension and cell supernatant fractions may contain sufficient insecticidal factors to produce a similar rate of larval mortality. Additional studies under field conditions are required to confirm their relative performance against *S. frugiperda*.

Nevertheless, as our bioassays were conducted under controlled laboratory conditions, the results may not fully reflect bacterial performance in different conditions. In complex and variable environments of agricultural fields, factors such as temperature fluctuations, UV radiation, and competition with soil microorganisms can substantially affect bacterial viability and efficacy. Hence, field trials should be conducted to validate the efficacy of these isolates under realistic agricultural conditions. In addition, metabolomic profiling of active bioactive compounds should be pursued to identify and characterize the specific insecticidal compounds responsible for the observed pathogenicity. To implement this bacterial-based biopesticide, the stability of these bacterial preparations needs to be investigated.

5. Conclusions

This study demonstrated the significant insecticidal activity of two symbiotic bacteria isolated from entomopathogenic nematodes, *X. miraniensis* (bMH16.1_TH) and *P. akhurstii*

(bSBR11.1_TH), against *S. frugiperda* larvae. *X. miraniensis* (bMH16.1_TH) showed higher pathogenicity across all larval instars and suspension fractions. Of the three bacterial fractions tested, whole-cell suspensions were the most effective, followed by cell pellet and cell supernatant fractions. The mortality rate of younger (second-instar) larvae increased rapidly after infection, and this larval stage was more susceptible to bacterial infection than older (fifth-instar) larvae. These results indicate the potential of *X. miraniensis* (bMH16.1_TH) as a promising biological control agent against *S. frugiperda*, with both whole-cell suspension and cell supernatant fractions demonstrating comparable insecticidal efficacy. Further investigations should focus on clarifying the active insecticidal compounds produced by this bacterium, evaluating its efficacy and stability under field conditions, and assessing its safety to non-target organisms, in order to integrate the bacteria-based biopesticide into sustainable biological control programs.

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