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ORIGINAL ARTICLE

Evaluation of cell-free supernatants from the symbiotic bacteria of entomopathogenic nematodes for controlling the colorado potato beetle [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)]

Esengül Erdem[1] · Ebubekir Yüksel[2] · Ramazan Canhilal[2]

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Abstract
The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, poses a significant threat to potato crops globally and has developed resistance to numerous insecticides. Entomopathogenic nematodes (EPNs), from the genera *Steinernema* and *Heterorhabditis*, are promising biocontrol agents. This study aimed to identify the bacterial symbionts of ten native EPN isolates, evaluate the efficiency of cell free supernatants (CFSs) from their symbiotic bacteria against different developmental stages of CPB, and determine the effect of these CFSs on CPB developmental stages and lifespan. The recA gene region was utilized to determine the symbiotic bacteria of ten local EPNs. CFSs from these bacteria were applied orally and through contact to CPB's various developmental stages (*L1*/*L2*, *L3*/*L4* larval stages, and adults). Mortalities, developmental transition times, and lifespans of adults were observed. The CFSs showed significant toxicity to CPB, with higher efficiency against young larvae. The CFSs exhibited cumulatively lethal effects over time, particularly on *L1*/*L2* larval stages. CFSs from *X. bovienii* exhibited the highest efficacy. In all cases, where larvae received CFSs orally or by contact, they failed to develop into pupae and adults. In contrast, the transition periods of old larvae to pupal and adult stages were comparable to those of the control group. Lifespans of adults differed based on bacterial isolates and application methods. This study shows the potential efficacy of CFSs from *Xenorhabdus* and *Photorhabdus* as biocontrol agents against CPB, particularly in its young larval stages. Further research is needed to unravel the mechanisms behind these effects and examine the impact on CPB mating and oviposition behaviors.

Keywords Entomopathogenic nematodes · Cell-free bacterial supernatant · Biological control · Colorado Potato Beetle

Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a notorious defoliator of potato crops worldwide. Its distribution spans approximately 16 million square kilometers, covering regions in Asia, Europe, and North America (Ditner and Ciszewska 2004; Alyokhin et al. 2008; Hsiao and Fraenkel 1968). In addition to its primary host, the CPB exhibits a diverse diet, targeting crops such as tomatoes, eggplants, tobacco, peppers, and cabbage, along with common weeds (Hare 1990; Riddick et al. 2013; Weber and Ferro 2016). The CPB's polyphagous nature facilitates its persistence in fields even when potato plants are absent (Ditner et al. 2019). Remarkably, a single CPB can consume a minimum of 100 cm^2 of potato foliage during its lifetime, causing considerable yield losses when control measures are absent (Zehnder et al. 1994; Ferro et al. 1997).

Current control strategies for CPB encompass an array of methods, including cultural practices, biological, and chemical controls (Sewell and Alyokhin 2005; Szendrei et al. 2012). However, chemical control with insecticides represent a cornerstone of CPB management (Grafius 1995; Boiteau et al. 2009). This reliance on chemical control has had a dramatic impact on the insecticide industry's development (Alyokhin et al. 2008, 2015). The CPB's documented resistance to a broad range of insecticides (Alyokhin et al. 2008; Alyokhin et al. 2012; Park et al. 2002, Grafius and

✉ Esengül Erdem
eozdemir@sirnak.edu.tr

[1] Faculty of Agriculture, Plant Protection Department, Şırnak University, 73000 Şırnak, Turkey

[2] Faculty of Agriculture, Plant Protection Department, Erciyes University, 38030 Melikgazi, Kayseri, Turkey

Douches 2008; Whalon and Mota-Sanchez 2017; Kaplanoglu et al. 2017; Schoville et al. 2018) shows the urgent need for alternative control methods. One of the promising alternatives is the utilization of entomopathogenic nematodes (EPNs), notably those belonging to the *Steinernema* and *Heterorhabditis* genera. EPNs are recognized for their rapid efficacy, active host-seeking behavior, and compatibilities with pesticides (Shapiro-Ilan et al. 2018; Campos-Herrera et al. 2013; Moens et al. 2019). Their pathogenicity is attributed to symbiotic relationship with *Xenorhabdus* and *Photorhabdus* bacteria (Georgis et al. 2006; Koppenhöfer and Kaya 1998).

Prior studies have demonstrated that both cell suspensions and cell-free supernatants (CFSs) derived from *Xenorhabdus* spp. and *Photorhabdus* spp. exhibit potent lethality against economically significant agricultural pests (Bussaman et al. 2006; Hinchliffe 2010; Tobias et al. 2017; Shehata et al. 2019; Shapiro-Ilan et al. 2020; Askary and Abd-Elgawad 2021; Abd-Elgawad 2022; Yuksel et al. 2022a, b). These symbiotic bacteria have garnered significant attention among researchers for their crucial role in augmenting the virulence of EPNs.

Nevertheless, as far as our knowledge extends, no study has specifically examined the efficacy of CFSs from *Xenorhabdus* or *Photorhabdus* against the CPB. This conspicuous gap in the literature prompted our current study, which seeks to evaluate the efficacy CFSs from local isolates against CPB. We particularly concentrate on exploring their contact and oral efficacy within the controlled Petri dish environment. Additionally, we aim to examine the influence of CFSs on various developmental stages of CPB and its overall lifespan, thereby contributing valuable insights into potential biocontrol strategies for this pest.

Materıals and methods

Entomopathogenic nematodes

Entomopathogenic nematode isolates, which were previously isolated from Kayseri and Nevşehir provinces, were used in the study. Nematodes were produced on the last instar larvae of *Galleria mellonella* (Woodring and Kaya 1988). Larvae of *G. mellonella* (Lepidoptera: Pyralidae) were cultured on artificial diet at 28 °C and in the dark (Mohammad and Coppel 1983). Information about the isolates is given in Table 1.

The colorado potato beetle *Leptinotarsa decemlineata* Say

Potato production was carried out in an area of 3 decares in order to ensure the continuous supply of the insect cultures and potato plants throughout the study period. In order to increase CPB populations in this field, none of the standart control medhods was applied. Only maintenance practices such as irrigation and fertilization were carried out in the field.

The populations obtained in the field were collected and left on the potato plants in pots. For the experiments, only individuals without any visible symptoms of infection or sickness were collected.

Isolation of symbiotic bacteria

In order to obtain symbiotic bacteria from each entomopathogenic isolate, approximately 500 recently emerged infective juveniles (IJs) were utilized. To ensure the exclusion of external contaminants, a sterile Ringer's solution containing 10% sodium hypochlorite by volume was employed for immersing these IJs over a period of 10 min. Following this decontamination step, the IJs underwent two subsequent rinses in sterile Ringer's solution, after which they were

Table 1 Entomopathogenic nematodes used in the study

Entomopathogenic nematode	Location	References
Steinernema feltiae E-76	Kayseri	Canhilal et al. (2017)
Steinernema feltiae AKS-1	Kayseri	Canhilal et al. (2017)
Steinernema bicornotum MGZ-4-S	Kayseri	Canhilal et al., (2017)
Steinernema carpocapsae KCS-4-S	Kayseri	Canhilal et al. (2017)
Heterorhabditis bacteriophora FLH-4-H	Kayseri	Canhilal et al. (2017)
Steinernema feltiae KBÇ-4	Nevşehir	Yuksel and Canhilal (2019)
Heterorhabditis bacteriophora AVB-15	Nevşehir	Yuksel and Canhilal (2019)
Steinernema feltiae ÜTP-5	Nevşehir	Yuksel and Canhilal (2019)
Steinernema feltiae MÇB-8	Nevşehir	Yuksel and Canhilal (2019)
Steinernema feltiae DDKY-11	Nevşehir	Yuksel and Canhilal (2019)

crushed in 1 cc of sterile phosphate-buffered saline (PBS). The resulting suspension was evenly streaked in 10-µl aliquots onto Nutrient Bromothymol Blue Triphenyltetrazolium Agar (NBTA) plates, following the methodology as described by Akhurst and Boemare (1988). These Petri dishes were then incubated at 28 °C for 48 h, and the ensuing colony-forming units were set aside for molecular analysis.

To extract genomic DNA, the GeneMATRIX tissue and bacterial DNA purification kit (EURx) was employed. Subsequently, the recombinase A gene (recA) was amplified using the recA F (5′-GCTATTGATGAAAATAAACA-3′) and recA R (5′-RATTTT RTCWCCRTTRTAGCT-3′) primers, aligning with the methodology outlined by Tailliez et al. (2010). The PCR amplification protocol consisted of an initial denaturation step at 94 °C for 12 min, followed by 30 cycles comprising denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The PCR products underwent verification through electrophoresis and were subsequently sent for sequencing to Macrogen, Inc. (South Korea). The resulting sequences were then deposited in GenBank (Table 2).

Preparation of cell-free bacterial supernatants from the symbiotic bacteria

Initially, the bacterial isolates were streaked onto NBTA plates and allowed to incubate for 24 h. Subsequently, a loopful of the bacterial culture was carefully transferred into a 300 ml conical flask containing 100 ml of Nutrient Broth (Merck, Darmstadt-Germany). The bacterial culture was placed on a rotary incubator, set at 150 rpm, for an incubation duration of 144 h. The entire incubation process was meticulously conducted in a dark environment, maintaining a temperature of 28 °C.

After the incubation period, the bacterial cultures were transferred into 50-ml centrifuge tubes and subjected to centrifugation at 20,000 rpm for 15 min at 4 °C. This centrifugation effectively separated the bacterial cells from the culture, resulting in a clear supernatant. To further ensure the purity of the obtained cell-free supernatant, the they were filtered through a 0.22 µm Millipore filter. The flow-through from this filtration process was designated as the cell-free supernatant. To confirm the complete absence of any residual bacterial cells, a portion of the flow-through was aseptically streaked onto NBTA agar plates, following the rigorous procedure as previously described by Bussaman et al. (2012) and Hazir et al. (2016).

The obtained supernatants were subsequently transferred into sterile 50 ml centrifuge tubes, sealed, and safely stored at − 20 °C until they were to be employed for the experimental purposes, adhering to the protocol delineated by Muangpat et al. (2017).

Determination of oral effects of bacterial supernatants on different stages of CPB

In order to determine the oral effect, each CFS used in the study was applied to the *L*1/*L*2, *L*3/*L*4 and adult stages of the CPB. This involved immersing potato plant leaves in 1 mL of the respective bacterial supernatant, allowing them to dry in 9 cm diameter Petri dishes. Subsequently, five CPB individuals were placed on each treated leaf, and the Petri dishes were sealed with parafilm. These dishes were then placed in an incubator set at 25 °C for a duration of 96 h. Each application was replicated three times, and the entire experiment was conducted twice. Control leaves were treated solely with Nutrient broth. The Petri dishes were checked at 24, 48, 72, and 96 h post-application, and dead insects were counted. Determination of insect mortality involved gently probing the insects with a fine-tipped needle; those that displayed no movement upon contact were considered dead. Upon concluding the application period, we recorded the time it took for surviving larvae to progress to the pupal and adult stages. This allowed us to assess the impact of bacterial supernatants on developmental stages and lifespan.

Table 2 Molecularly identified symbiotic bacterial species of nematode isolates belonging to *Steinernema and Heterorhabditis*

Isolate	Symbiotic bacteria	Accession number
Steinernema feltiae E-76	*Xenorhabdus bovienii*	MZ688376
Steinernema feltiae AKS-1	*Xenorhabdus bovienii*	MZ688378
Steinernema bicornotum MGZ-4-S	*Xenorhabdus budapestensis*	MW403817
Steinernema carpocapsae KCS-4-S	*Xenorhabdus nematophila*	MZ688381
Heterorhabditis bacteriophora FLH-4-H	*Photorhabdus luminescens* subsp. *kayaii*	MZ688382
Steinernema feltiae KBC-4	*Xenorhabdus bovienii*	MZ688379
Heterorhabditis bacteriophora AVB-15	*Photorhabdus luminescens* subsp. *kayaii*	MW403818
Steinernema feltiae UTP-5	*Xenorhabdus bovienii*	MZ688375
Steinernema feltiae MCB-8	*Xenorhabdus bovienii*	MZ688377
Steinernema feltiae DDKY-11	*Xenorhabdus bovienii*	MZ688380

Determination of the contact effects of bacterial supernatants on different stages of CPB

To assess contact toxicity, we exposed the potato beetles to 1 ml of bacterial supernatant for specified time intervals and then transferred them to Petri dishes containing potato leaves. The control group, on the other hand, was immersed solely in Nutrient broth medium and placed in Petri dishes. In order to evaluate the contact effect on the larvae, we conducted inspections at 24, 48, 72, and 96 h post-application, during which we counted the dead insects. Determination of insect mortality was same as for the oral effect bioassays. Similar to the study assessing oral efficacy, we recorded the time it took for surviving larvae to progress to the pupal and adult stages after 96 h, and we also noted the time of death for adult beetles.

Statistical analyzes

Since there were no mortalities in the control groups, no correction were made to the mortality rates. The analysis was conducted using IBM SPSS Statistics version 20.0 for Windows (SPSS Inc., Chicago, IL, USA), and the mortalities underwent arcsine transformation prior to analysis. In the bioassays for oral and contact efficacy, the effects of main factors and their interactions was determined at 0.05 level ($P \leq 0.05$). Factorial repeated measures ANOVA were applied to all data, utilizing a General Linear Model. The data of transition time and adult lifespan were subjected to one-way ANOVA. Tukey's multiple range tests were used to categorize mean differences ($P \leq 0.05$).

Results

Molecular characterization of symbiotic bacteria

Based on the cladogram created using symbiotic bacteria obtained from 10 native entomopathogenic nematode isolates and representative data from the recombinase A (recA) gene region sourced from GenBank, we observed that *Xenorhabdus* species and strains (specifically *X. bovienii, X. nematophila,* and *X. budapestensis*) were prevalent across all locations. These *Xenorhabdus* species were found to be closely related to other selected *Xenorhabdus* species, with *X. bovienii* exhibiting significant intraspecific polymorphism and substantial divergence from other species, supported by high bootstrap values of 99%. Based on the cladogram, it can be concluded that *X. bovienii* demonstrates paraphyletic characteristics. Similarly, species *X. nematophila* and *X. budapestensis* clustered together, forming a distinct paraphyletic group. In terms of *Photorhabdus* species, *P. luminescens* subsp. *kayaii* was identified as descending from *P. temperata* subsp. *temperata* (Fig. 1).

Oral efficacies of bacterial supernatants on different stages of CPB

The differences in the mortalities observed in the CPB larvae were significantly affected by all the main factors of interest (Supernatant [S], Exposure Time [T], and Developmental Stage [D]) (Table 3). Moreover, the interactions of the main factors, specifically the three-way interaction; T*D*S also exhibited the statistical significance ($P < 0.001$). Namely, all CFSs showed statistically significant toxic effect on CPB.

When we assessed the mortalities induced by the CFSs, a notable trend emerged. As the developmental stage of the target insects advances, we observed that there is a discernible reduction in the efficacy of CFSs, particularly in the context of younger larval stages. Furthermore, the impact of CFSs on adult insects appears to be relatively limited.

Additionally, upon closer examination of the evolving effectiveness of CFSs over time, a noteworthy pattern becomes apparent. Over time, the efficacy of CFSs tends to increase, demonstrating a cumulative lethal effect. This augmentation in their lethal effect is particularly conspicuous on *L1*/*L2* larval stages.

Comparing the efficacies of CFSs from various isolates at specific time intervals, we observe the highest efficacy against the young larval stage in isolates *X. bovienii* UTP-5 and *X. nematophila* KCS-4-S, resulting in 15% mortality. However, the mortalities of the remaining 10 isolates did not display statistically significant differences.

Following a 48-h application period, the evaluation of the effectiveness on young larvae exhibited remarkable results. Notably, the *X. bovienii* KBC-4 isolate exhibited the highest efficacy, inducing a35% mortality. Subsequently, *X. bovienii* AKS-1, *X. bovienii* UTP-5, and *X. bovienii* MCB-8 isolates exhibited comparable effectiveness, with mortalities ranging around 30%.

Upon expiration of the 72-h application period, a switch in efficacy patterns became apparent. *X. bovienii* AKS-1 showed a substantial increase in efficacy, resulting in high mortality of 60%. Both *P. luminescens* subsp. *kayaii* FLH-4-H and *X. bovienii* UTP-5 isolates exhibited comparable efficacy, causing 57.5% mortality against the young larvae. These findings emphasize the dynamic nature of activity over time and between various isolates (Table 4).

In a parallel set of observations focused on determining the transitional timelines of surviving larvae to pupal and adult stages, as well as the times of mortality in adult insects 96 h post-application, intriguing findings were obtained. Notably, for the surviving individuals in the early larval stage, it was consistently observed that they

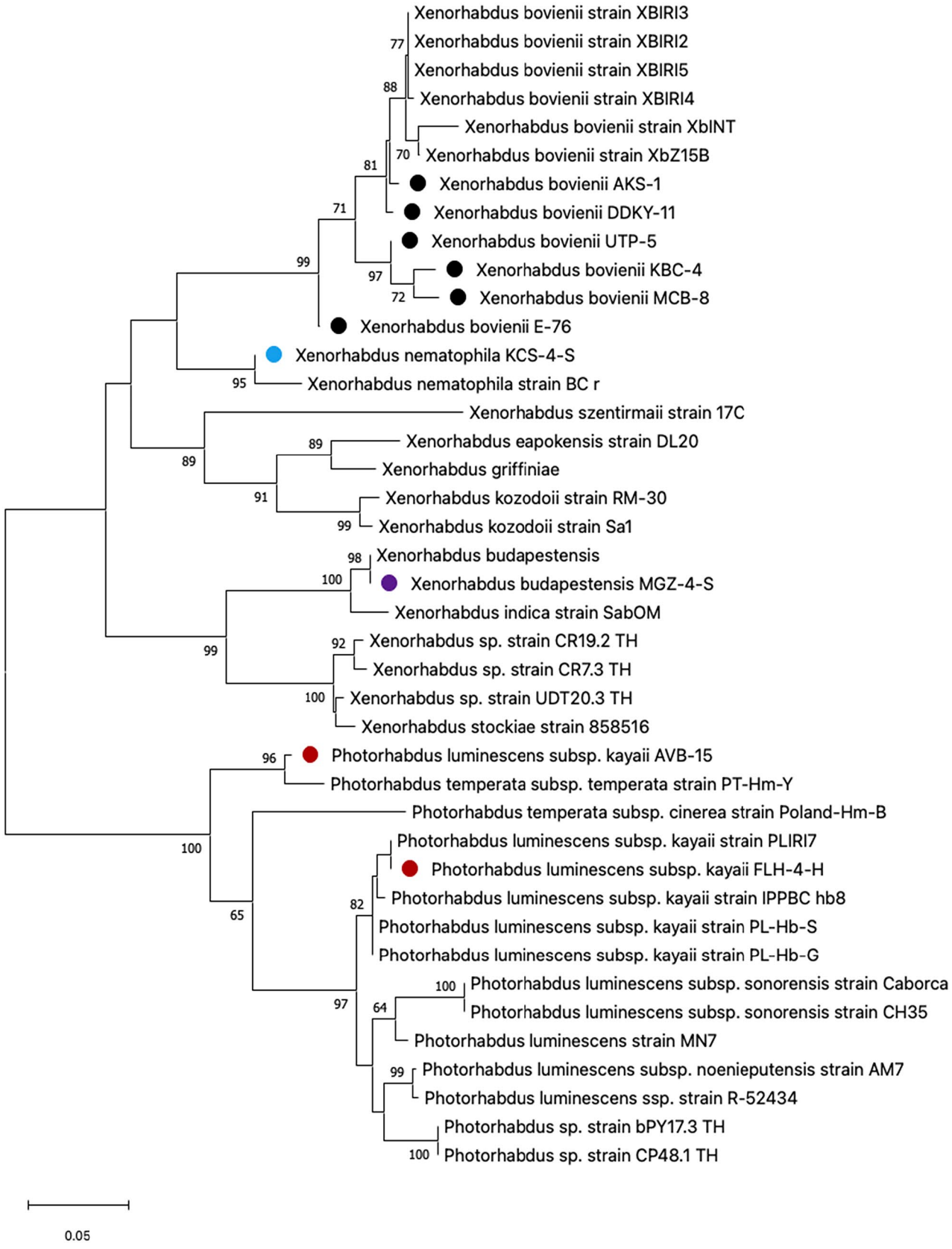

Fig. 1 Cladogram of phylogenetic relationships of *Xenorhabdus* and *Photorhabdus* isolates based on sequence analysis of recombinase A (recA) gene region

Table 3 ANOVA results for the oral efficacies of the CFSs of symbiotic bacteria on CPB (*L1*/*L2*, *L3*/*L4*, and Adult)

Sources	Df	*F*-Value	*P*-Value
T	3	541.844	< 0.001
S	10	10.863	< 0.001
D	2	641.937	< 0.001
T*S	30	504.647	< 0.001
T*D	6	233.771	< 0.001
S*D	20	6.923	< 0.001
T*D*S	60	3.390	< 0.001

*T Exposure time, *S* Supernatant, *D* Developmental stage

succumbed to mortality before progressing to the pupal stage across all CFSs applications. In contrast, concerning individuals in the old larval stage survived (Table 5), *X. bovienii* E-76, *X. bovienii* AKS-1, and *X. bovienii* KBC-4 treated larvae significantly accelerated the transition from larvae to pupae compared to the control group. This finding suggests that these specific treatments effectively hasten the larval-pupal transition phase. Conversely, treatments MGZ-4-S, *X. bovienii* KCS-4-S, and *X. bovienii* UTP-5 demonstrated moderately longer transition times, yet these were still comparable to the control. Notably, *X. bovienii* MCB-8 and *X. bovienii* DDKY-11 exhibited the longest durations for this transition, aligning closely with the control group's results, thereby indicating these treatments did not significantly alter the larval-pupal transition in comparison to the control.

Regarding the pupal-adult transition, treatment *X. bovienii* E-76 remarkably shortened the transition period relative to the control group. This indicates a potent effect of *X. bovienii* E-76 in accelerating the pupal to adult development. Other treatments, including *X. bovienii* AKS-1, *P. luminescens* subsp. *kayaii* FLH-4-H, *X. bovienii* UTP-5, *X. Budapestensis* MGZ-4-S, *X. bovienii* KCS-4-S, and *X. bovienii* DDKY-11 presented varied transition times. However, these were predominantly shorter or similar to those observed in the control group, suggesting their moderate effectiveness in expediting this developmental stage. In contrast, treatments *X. bovienii* KBC-4, *P. luminescens* subsp. *kayaii* AVB-15, and *X. bovienii* MCB-8, respectively, showed transition times that were longer or akin to the control group, indicating a negligible or absent influence in accelerating the pupal to adult transition.

Overall, the data elucidates that certain treatments significantly hasten developmental transitions in insects, particularly from larvae to pupae and from pupae to adults, in comparison to untreated controls. Other treatments, however, exhibited limited efficacy or mirrored the natural developmental timeline observed in the control group.

Table 4 Effect of oral application of CFSs of symbiotic bacteria on mortalities (Means ± SE) of different developmental stages of CPB on the first, second, third and fourth day after treatment

Mortality (%)												
CFSs	*L1/L2*				*L3/L4*				Adult			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
E-76	5.0 ± 5.7 Aa	15.0 ± 12.9Ba	53.0 ± 22.7Bbc	62.5 ± 9.5Bbc	0.0 ± 0.0Aa	0.0 ± 0.0Aa	10.0 ± 11.5Bb	15.0 ± 10.0Bb	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	5.0 ± 10.0Bb	10.0 ± 11.5Bb
AKS-1	12.5 ± 9.5Ab	30.0 ± 14.1Bc	60.0 ± 20.0Cc	75.0 ± 12.9Cc	0.0 ± 0.0Aa	0.0 ± 0.0Aa	5.0 ± 10.0Bab	5.0 ± 10.0Bab	0.0 ± 0.0Aa	0.0 ± 0.0Aa	5.0 ± 10.0Bb	10.0 ± 11.5 b
MGZ-4-S	12.5 ± 5.0 Ab	25.0 ± 5.7 Ac	42.5 ± 5.0Bb	52.5 ± 5.0Bb	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	5.0 ± 10.0Bab	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0Aa	10.0 ± 11.5Bb
KCS-4-S	15.0 ± 10.0 Ab	27.5 ± 5.0 Ac	35.0 ± 10.0ABb	55.0 ± 5.7Bb	0.0 ± 0.0Aa	5.0 ± 10.0Bb	5.0 ± 10.0Bab	10.0 ± 11.5Bb	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	10.0 ± 11.5 Bb
FLH-4-H	5.0 ± 5.7 Aa	15.0 ± 5.7Ab	57.5 ± 12.5Bc	72.5 ± 5.0Bc	0.0 ± 0.0Aa	0.0 ± 0.0Aa	5.0 ± 10.0Bab	15.0 ± 19.1Cb	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Bb	10.0 ± 11.5 Bb
KBC-4	7.5 ± 5.0 Aab	35.0 ± 12.9Bc	40.0 ± 14.1 Bb	50.0 ± 8.1Cb	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	10.0 ± 11.5Bb	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
AVB-15	10.0 ± 8.1 ab	20.0 ± 8.1Abc	52.5 ± 9.5 Bbc	65.0 ± 10.0 Bbc	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	10.0 ± 11.5Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	10.0 ± 11.5 Bb
UTP-5	15.0 ± 5.7 Ab	30.0 ± 8.1Bc	57.5 ± 5.0 Cc	57.5 ± 5.0Cb	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	10.0 ± 11.5Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Aa
MCB-8	7.5 ± 9.5 Aab	30.0 ± 8.1Bc	47.5 ± 5.0 Cbc	60.0 ± 8.1Dbc	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	5.0 ± 10.0Bab	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Aa
DDKY-11	12.5 ± 5.0 Ab	22.5 ± 5.0ABbc	37.5 ± 9.5 Bb	47.5 ± 9.5Bb	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	5.0 ± 10.0Bab	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
Control	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 5.7 Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa

Table 5 Transition times of old larvae (*L3*/*L4*) to the next developmental stages in oral efficacy studies (25 °C, 60% RH, 16:8)

Treatment	Pupae	Adult
E-76	8.0 ± 3.5a	8 ± 1.1a
AKS-1	9.0 ± 2.9a	9 ± 3.1ab
MGZ-4-S	10.0 ± 2.7	11 ± 1.5b
KCS-4-S	11 ± 3.1b	10 ± 1.4b
FLH-4-H	9 ± 3.9ab	11 ± 3.3b
KBC-4	8 ± 4.1a	14 ± 1.1c
AVB-15	9 ± 2.6ab	12 ± 2.8bc
UTP-5	10 ± 2.2b	10 ± 3.1b
MCB-8	13 ± 1.2c	12 ± 3.1bc
DDKY-11	12 ± 2.1bc	9 ± 3.3ab
Control	11 ± 1.9bc	13 ± 4.5c

Table 6 ANOVA results for the contact efficacies of the CFSs of symbiotic bacteria on CPB (*L1*/*L2*, *L3*/*L4*, and Adult)

Source*	df	F	*P*
T	3	326.175	< 0.001
S	10	16.381	< 0.001
D	2	981.835	< 0.001
T*S	30	4.014	< 0.001
T*D	6	171.722	< 0.001
S*D	20	14.137	< 0.001
T*D*S	60	2.743	< 0.001

**T* Exposure time, *S* Supernatant, *B* Developmental stage

Contact effects of bacterial supernatants on different stages of CPB

Using repeated measures ANOVA, a thorough examination of the interaction table involving factors (Supernatant [S], Exposure Time [T], and Developmental Stage [D]) revealed statistically significant findings. Specifically, the triple interaction among these factors (T*D*S) demonstrated a high degree of statistical significance ($P < 0.001$), as outlined in Table 6. This statistical outcome highlights a significant interplay among the considered factors in our analysis.

Upon scrutinizing the mortalities resulting from the contact effect of CFSs (Table 7), it becomes clear that their impact on young larval stages is notably pronounced. In the first 72 h, the maximum mortality observed in mature larvae reaches 10%, but notably rises to 30% when the *P. luminescens* subsp. *kayaii* FLH-4-H isolate. In contrast, the contact effects of CFSs across all isolates on the adult stage demonstrated considerably lower activity, with a maximum 5% mortality ($P < 0.001$). This observation applies to *P. luminescens* subsp. *kayaii* FLH-4-H, *X. bovienii* AKS-1, *X. bovienii* E-76, and *X. bovienii* DDKY-11.

Table 7 Mortalities (%) of CPB in contact activity studies of symbiotic bacteria isolated from EPNs

Mortality (%)													
Application	*L1/L2*				*L3/L4*				Adult				
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	
E-76	10.0 ± 8.1 Aab	30.0 ± 8.1 Bb	60.0 ± 24.4 Cc	72.5 ± 12.5 Dbc	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	15.0 ± 10.0 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Bb
AKS-1	17.5 ± 12.5 Ab	30.0 ± 8.1 Ab	65.0 ± 12.9 Bc	85.0 ± 5.7 Cc	0.0 ± 0.0 Aa	5.0 ± 10.0 Ab	5.0 ± 10.0 Ab	10.0 ± 11.5 Ba	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Bb
MGZ-4-S	12.5 ± 9.5 Aab	27.5 ± 5.0 Bb	57.5 ± 9.5 Cc	70.0 ± 8.1 Cbc	0.0 ± 0.0 Aa	5.0 ± 10.0 Ab	5.0 ± 10.0 Ab	15.0 ± 10.0 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
KCS-4-S	12.5 ± 9.5 Aab	32.5 ± 5.0 Bb	57.5 ± 9.5 Cc	77.5 ± 5.0 Dc	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Ba	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
FLH-4-H	10.0 ± 8.1 Aab	17.5 ± 12.5 Aab	40.0 ± 8.1 Bbc	65.0 ± 12.9 Cb	0.0 ± 0.0 Aa	5.0 ± 10.0 Ab	10.0 ± 11.5 Ab	30.0 ± 11.5 Bc	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Bb
KBC-4	27.5 ± 9.5 Ab	37.5 ± 5.0 Ab	72.5 ± 5.0 Bc	87.5 ± 5.0 Bc	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	10.0 ± 20.0 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
AVB-15	10.0 ± 8.1 Aab	22.5 ± 5.0 Aab	32.5 ± 9.5 Bb	60.0 ± 8.1 Cb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Aa	15.0 ± 19.1 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
UTP-5	17.50 ± 5.0 Ab	22.5 ± 5.0 Aab	45.0 ± 5.7 Bbc	62.5 ± 12.5 Cb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	10.0 ± 11.5 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
MCB-8	7.5 ± 9.5 Aa	25.0 ± 10.0 Bab	47.5 ± 5.0 Cbc	60.0 ± 8.1 Db	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	15.0 ± 10.0 Bb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa
DDKY-11	15.0 ± 12.0 Ab	27.5 ± 9.5 ABb	45.0 ± 5.7 Bbc	62.5 ± 5.0 Cb	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Ba	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 10.0 Bb
Control	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	5.0 ± 5.7 Ba	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa

Analyzing the temporal changes in the contact activities of supernatants from all isolates, it is discernible that the activity exhibited an increase over time ($P < 0.05$). However, in alignment with the outcomes of oral activity tests, it is apparent that the *L1*/*L2* larval stages manifest greater susceptibility to CFSs.

Comparing the efficacy of supernatants across isolates at specific time intervals, the highest efficacy against young larval stages was observed 24 h post-application, particularly when the supernatant of the *X. bovienii* KBC-4 isolate was administered, resulting a mortality of 27.5%. In contrast, *X. bovienii* MCB-8 caused a 10% mortality, representing the least active isolate. Nevertheless, the differences in contact toxicities of CFSs among the other nine isolates, excluding the application of *X. bovienii* MCB-8 supernatant, did not attain statistical significance ($P > 0.05$).

Examining activities on young larvae 48 h after application, the *X. bovienii* KBC-4 isolate emerged as the most effective, inducing a 37.5% mortality, followed by *X. nematophila* KCS-4-S with 32.5%, and *X. bovienii* DDKY-11 with 27.5%. While *P. luminescens* subsp. *kayaii* FLH-4-H demonstrated the lowest effect at 17.5%, it did not represent a statistically significant difference ($P > 0.05$).

Finally, after a 72-h application period, *X. bovienii* KBC-4 caused a 72.5% mortality, while the supernatant application of isolate *P. luminescens* subsp. kayaii AVB-15 demonstrated the lowest effect and yielded a 32.5% mortality. After 96 h post-application, the highest mortality was achieved at 87.5% with *X. bovienii* KBC-4, closely followed by *X. bovienii* AKS-1 (85%) and *X. nematophila* KCS-4-S (77.5%). These differences are proportional, and no statistically significant distinction was observed among the activities of the isolates ($P > 0.05$).

When evaluating the impact of supernatants on old larvae, no significant effect was discernible within the first 72 h after application. However, the CFS derived from the *P. luminescens* subsp. *kayaii* FLH-4-H isolate demonstrated the highest efficacy with a 30% mortality. Correspondingly, similar to the oral effect studies, CFSs were found to exert no significant effect on adult insects.

In the part of the study to determine the timing of developmental changes in larvae that survived application of CFSs, we observed a consistent and surprising pattern developed. Specifically, across all CFS treatments, individuals that survived the early larval stage always perished before reaching the pupal stage.

In addition, it became evident that the transition to the pupal stage happened more rapidly in mature larvae compared to the control group (Table 8). The treatments *X. bovienii* E-76, *P. luminescens* subsp. *kayaii* FLH-4-H, *X. bovienii* KBC-4, and *P. luminescens* subsp. *kayaii* AVB-15, demonstrated a significant acceleration in transition times compared to the control group. This indicates the effectiveness of these treatments in hastening the larval to pupal transition phase. In contrast, *X. bovienii* AKS-1 and *X. bovienii* MCB-8, respectively, exhibited prolonged transition times. Additionally, treatments *X. budapestensis X. budapestensis* MGZ-4-S, *X. bovienii* KCS-4-S, and *X. bovienii* DDKY-11 showed moderately extended transition periods, albeit within the range of the control group.

Table 8 Transition times to the next developmental stages in contact efficacy studies (25 °C, 60% RH, 16:8)

Treatment	Pupae	Adult
E-76	9.0 ± 2.4ab	7.0 ± 2.1a
AKS-1	11.0 ± 1.5c	11.0 ± 3.2c
MGZ-4-S	10.0 ± 2.3b	10.0 ± 3.5b
KCS-4-S	10.0 ± 2.4b	11.0 ± 2.7c
FLH-4-H	9.0 ± 3.7ab	10.0 ± 2.5b
KBC-4	7.0 ± 1.6a	10.0 ± 1.9b
AVB-15	7.0 ± 0.7a	11.0 ± 3.9c
UTP-5	8.0 ± 1.2ab	11.0 ± 1.8c
MCB-8	10.0 ± 1.5b	11.0 ± 2.4c
DDKY-11	10.0 ± 2.2b	8.0 ± 2.1a
Control	9.0 ± 3.2ab	10.0 ± 3.1b

Regarding the pupal to adult transition, *X. bovienii* E-76 and *X. bovienii* DDKY-11 considerably shortened the duration of this stage compared to the control group, underscoring their potent influence in accelerating this phase of development. Meanwhile, treatments *P. luminescens* subsp. *kayaii* FLH-4-H, *X. bovienii* KBC-4, and *P. luminescens* subsp. *kayaii* AVB-15 resulted in slightly abbreviated transition times relative to the control. Conversely, treatments *X. bovienii* AKS-1, *X. budapestensis* MGZ-4-S, *X. bovienii* KCS-4-S, *X. bovienii* UTP-5, and *X. bovienii* MCB-8 were associated with longer transition times than those observed in the control group, implying these treatments were less effective or possibly delayed the transition from pupae to adult.

Overall, the findings suggest a notable variability in the impact of different treatments on the developmental transitions from larvae to pupae and subsequently from pupae to adults in older larvae (*L3*/*L4*). The data highlights that while some treatments significantly expedited these developmental processes, others exhibited limited or even a retarding effect on development.when compared to untreated controls.

In our study of the adult lifespans resulting from the contact and oral applications of CFSs (Table 9), several noteworthy trends emerged. For oral application, CFSs from *X. bovienii* E-76 (9 days), *X. bovienii* AKS-1 (10 days), *X. bovienii* UTP-5 (10 days), and *P. luminescens* subsp. *kayaii* FLH-4-H (10 days) resulted in shortened adult lifespans. Conversely, the application of CFSs from *X. bovienii* DDKY-11 (13 days), *X. budapestensis* MGZ-4-S (12 days),

Table 9 Adult lifespan in contact and oral efficacy studies (25 °C, %60 RH, 16:8)

Application	Adult lifespan (Day)	
	Oral application	Contact application
E-76	9.0 ± 1.5a	10.0 ± 1.7ab
AKS-1	10.0 ± 2.4a	8.0 ± 2.6a
MGZ-4-S	12.0 ± 3.1b	12.0 ± 0.9b
KCS-4-S	11.0 ± 1.8ab	11.0 ± 1.4ab
FLH-4-H	10.0 ± 3.4a	8.0 ± 2.6a
KBC-4	11.0 ± 4.1ab	9.0 ± 2.9a
AVB-15	11.0 ± 2.5ab	11.0 ± 3.1ab
UTP-5	10.0 ± 1.9a	10.0 ± 0.7ab
MCB-8	12.0 ± 3.3b	12.0 ± 1.9b
DDKY-11	13.0 ± 2.5c	14.0 ± 0.4c
Control	11.0 ± 2.7ab	12.0 ± 1.1b

and *X. bovienii* MCB-8 (12 days) prolonged the lifespan of adults.

Turning the attention to the findings of contact application, only the application of *X. bovienii* DDKY-11 (14 days) led to an increase in adult lifespan compared to control ($P < 0.05$). However, the application of CFSs from *X. bovienii* AKS-1 (8 days), *P. luminescens* subsp. *kayaii* FLH-4-H (8 days), and *X. bovienii* KBC-4 (9 days) resulted in a decrease in adult lifespan ($P < 0.05$). Notably, a consistent pattern of shortened adult lifespan was observed in *X. bovienii* E-76, *X. bovienii* AKS-1, and *P. luminescens* subsp. *kayaii* FLH-4-H following both oral and contact applications. However, the adults of CPB exhibited a prolonged lifespan in both oral and contact applications of *X. bovienii* DDKY-11($P < 0.05$).

Discussion

We designed this study with three specific objectives. Firstly, we identified the symbiotic bacteria of local entomopathogenic isolates by using recA gene region. This molecular characterization of symbiotic bacteria is consistent with prior studies that utilized genetic markers such as the recA gene region for bacterial identification (Tailliez et al. 2010). Consequently, it is evident that conducting comparative phylogenetic studies, encompassing diverse gene regions, is essential for the comprehensive delineation of subspecies and bacterial races in phylogenetic studies.

Secondly, we evaluated the toxicity of cell-free supernatants (CFSs) from those symbiotic bacteria from local EPNs against different developmental stages of CPB. The best of our knowledge, this approach has not previously been studied in the context of CPB management. This study expands on previous studies regarding the biocontrol potential of EPNs and their associated bacteria. The findings of the current study showing the oral and contact toxicity of CFSs produced by symbiotic bacteria to CPB larvae are consistent with prior research indicating the toxicity of these bacteria and their products to diverse insect hosts (Eleftherianos et al. 2010; Richard and Muñoz-Carpen 2003).

Among the various studies, only one study has delved into the potential toxic effect of symbiotic bacteria on the CPB. This particular study solely employed the toxins produced by *Photorhabdus luminescens* bacteria. Remarkably, the study revealed that the toxin complex generated by *P. luminescens* induced mortality rates exceeding 80% among CPB larvae (Blackburn et al. 2005). In our study, we examined two strains of *P. luminescens* subsp. *kayaii*, which, in contact efficacy experiments, resulted in larval mortality rates of 60% and 65% among young larvae, and 65% and 72.5% in oral efficacy experiments. This could be attributed to the rapid infiltration of bacterial toxins into the larvae's midgut. Previous research has indicated that cell suspensions from both *Xenorhabdus* and *Photorhabdus* bacteria proved to be highly effective against the insects under examination when applied to the plants (Mahar et al. 2005; Fukruksa et al. 2017; Vitta et al. 2018). It is well-documented that the efficiency and diversity of secondary metabolites produced by symbiotic bacteria, such as *Photorhabdus* and *Xenorhabdus*, vary not only among different species but also among isolates of the same species (Eroglu et al. 2019). This difference renders symbiotic bacteria and their associated toxins invaluable tools in the search for novel biopesticides. Hence, it is essential to conduct further research focused at identifying specific toxins with insecticidal properties, particularly concerning agriculturally significant pests like CPB. In the current study, CFSs demonstrated a moderate level of insecticidal activity against early CPB larval stages. Mortalities ranged from 47.5 to 87.5% in both oral and contact bioassays. These findings demonstrate the potential of bacterial toxins as a means of CPB control.

Lastly, we evaluated the sublethal effect of the CFSs by assesing the developmental transition periods and adult's lifespan. The efficacy of CFSs on the transition times of the developmental stages and adult lifespan support prior studies on the impacts of EPNs and their associated bacteria on insect physiology (Borgonie et al. 2010). This highlights the complex nature of the relationship between EPNs and their hosts. The differences of findings among contact and oral application suggest that the application methods do not significantly alter the impact once the CFSs have entered the insect's body. However, it is worth considering that bacterial species and strains may have varying effects on lifespan, possibly due to differences in toxic metabolites or target regions. Furthermore, it's important to note that we also observed morphological deformations in adult individuals that emerged from pupae following nearly every application.

These deformations included significantly smaller bodies, predominantly dull coloration in the pronotum region, and color loss in the abdomen. The pattern which was both observed and assessed underscores the importance of future research focused on elucidating the mechanisms by which bacterial toxins influence insect hormones. For a thorough understanding of the underlying processes, it is essential to comprehend these impacts and their target locations.

One of the most important limitation of the study is the mating and oviposition behavior of the surviving adults. If mating occurs, the amount of eggs laid and the percentage of hatching of those eggs were not determined. A second limitation is the lack studying the effect of supernatants applied to the egg stage. of the results by applying supernatants to the egg stage, since the CPB lays its eggs on leaves. The admission of limitations, such as the need for more study into mating and oviposition habits and the effect on CPB eggs, echoes the urge for complete research into biocontrol measures (Grewal et al. 2005). Future research should address these gaps for a more comprehensive understanding.

Conclusion

In conclusion, both previous studies and the findings of the present research underscore the intriguing variability in the virulence of entomopathogenic bacteria, exemplified by *Xenorhabdus* and *Photorhabdus* isolates, particularly in their ability to induce mortality in CPB larvae (Ffrench-Constant et al. 2007). This findings aligns with previous studies that has documented the considerable variation in the pathogenicity of different bacterial strains within the *Xenorhabdus* and *Photorhabdus* genera (Ffrench-Constant et al. 2007). This results emphasize the crucial role played by secondary metabolites (Bode 2009) produced by *Xenorhabdus* spp. and *Photorhabdus* spp. in mediating their insecticidal effects (Sergeant et al. 2003; Proschak et al. 2014). *Xenorhabdus* bacteria are known to secrete a number of secondary metabolites, including XptA1 and XptA2 (Sergeant et al. 2003), Xenocycloins (Proschak et al. 2014), and toxin complexes like as XaxA, XaxB, and the Tcc toxin complexes (Vigneux et al. 2007). These compounds have been demonstrated to serve as potent insecticides (Jarrett et al. 1997; Bowen et al. 1998a,b; ffrench-Constant and Bowen 2000), underlining their significance in the context of biocontrol of insect pests. On the other hand, *Photorhabdus* species produce their own arsenal of insecticidal compounds, including Tca, Tcb, Tcc, Tcd, PirA, PyB, and Mcf toxin complexes (Blackburn et al. 1998; Bowen et al. 1998a,b; Daborn et al. 2002; Waterfield et al. 2005; Dowling and Waterfield 2007; Sheets and Aktories 2017).

These toxin complexes have been well-documented for their efficiency in controlling insect pests and are crucial for the pathogenicity of *Photorhabdus* strains. Interestingly, despite the documented insecticidal activity of both *Xenorhabdus* and *Photorhabdus,* the specific components responsible for their efficiency in cell-free bacterial supernatants remain unclear. This information gap underlines the need for further research to identify and characterize these elusive insecticidal compounds (Jarrett et al. 1997; Blackburn et al. 1998). The identification and characterization of these compounds are not only critical for a more comprehensive understanding of the factors involved their entomopathogenic activities but also for their potential applications in environmentally friendly pest management strategies. The ongoing research in this particular topic may ultimately lead to the development of novel and more effective insecticides based on the compound produced by *Xenorhabdus* and *Photorhabdus* bacteria. Furthermore, a better understanding of the diverse secondary metabolites produced by these bacteria in their interactions with insect hosts can shed light on the intricate world of microbe-insect relationships, with implications for both basic science and practical pest management tactics.

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Declarations

Conflict of interest The authors declare no competing interests.

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