



MARJORIE TATIANA DEL AGUILA RIOS

**ENTOMOPATHOGENIC POTENTIAL OF *Xenorhabdus*
SYMBIOTIC BACTERIA ASSOCIATED WITH
ENTOMOPATHOGENIC NEMATODES AGAINST *Spodoptera*
*frugiperda***

**LAVRAS-MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para obtenção do título de Mestre.

Prof. Dr. Khalid Haddi
Orientador

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**POTENCIAL ENTOMOPATOGÊNICO DE BACTÉRIAS SIMBIÓTICAS
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*Spodoptera frugiperda***

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Prof. Dr. Khalid Haddi
Orientador

**LAVRAS-MG
2026**

Dedicado a Deus por sempre me acompanhar e me dar forças neste processo.

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RESUMO GERAL

A lagarta-do-cartucho, *Spodoptera frugiperda*, é uma praga polífaga que ameaça à segurança alimentar, causando sérios danos às culturas. Esta praga é tradicionalmente controlada com inseticidas sintéticos ou plantas transgênicas que expressam toxinas Bt. Entretanto, a evolução da resistência a esses métodos de controle tem impulsionado a busca por alternativas mais eficazes, seguras e sustentáveis. Nesse contexto, os nematoides entomopatogênicos (NEP) do gênero *Steinernema* e suas bactérias simbióticas do gênero *Xenorhabdus* destacam-se como agentes promissores para o controle biológico de insetos-praga. O presente estudo teve como objetivo avaliar o potencial entomopatogênico de bactérias simbióticas do gênero *Xenorhabdus* associadas a nematoides entomopatogênicos contra *S. frugiperda*. Inicialmente, foi avaliada a patogenicidade de diferentes espécies de NEP em larvas da praga. Em seguida, as bactérias simbióticas foram isoladas e caracterizadas por meio de análises fenotípicas, testes bioquímicos, sequenciamento do gene *recA* e análise filogenética. Também foram determinadas as curvas de crescimento bacteriano *in vitro* e *in vivo*, bem como a atividade entomopatogênica de suspensões celulares e sobrenadantes livres de células por injeção e ingestão oral.

A análise molecular identificou as bactérias associadas a *S. carpocapsae* e *S. brazilense* como *Xenorhabdus nematophila* e *X. doucetiae*, respectivamente. As cepas apresentaram diferenças nos padrões de crescimento, porém ambas demonstraram elevada virulência quando injetadas em larvas de *S. frugiperda*. As suspensões celulares promoveram mortalidades superiores às observadas para os sobrenadantes livres de células, embora estes também tenham exibido atividade inseticida. Os resultados confirmam o potencial entomopatogênico das bactérias simbióticas de *Steinernema* e reforçam sua relevância como fontes de metabólitos bioativos para o desenvolvimento de novas estratégias de controle biológico compatíveis com programas de manejo integrado de pragas.

Palavras-chave: bactérias entomopatogênicas; NEP; lagarta-do-cartucho; patogenicidade.

GENERAL ABSTRACT

The fall armyworm, *Spodoptera frugiperda*, is a polyphagous pest that threatens food security by causing serious crop damages. This pest is traditionally controlled with synthetic insecticides or transgenic plants expressing Bt toxins. However, the evolution of resistance to these control methods has driven the search for more effective, safe, and sustainable alternatives. In this context, entomopathogenic nematodes (EPN) of the genus *Steinernema* and their symbiotic bacteria of the genus *Xenorhabdus* have emerged as promising agents for the biological control of insect pests. The aim of this study was to evaluate the entomopathogenic potential of *Xenorhabdus* symbiotic bacteria associated with entomopathogenic nematodes against *S. frugiperda*. Initially, the pathogenicity of different EPN species was assessed in larvae of the pest. Subsequently, the symbiotic bacteria were isolated and characterized through phenotypic analyses, biochemical tests, *recA* gene sequencing, and phylogenetic analysis. In addition, bacterial growth curves were determined both *in vitro* and *in vivo*, and the entomopathogenic activity of bacterial cell suspensions and cell-free supernatants was evaluated by injection and oral administration.

Molecular analyses identified the bacteria associated with *S. carpocapsae* and *S. brazilense* as *Xenorhabdus nematophila* and *X. doucetiae*, respectively. Although the strains exhibited distinct growth patterns, both showed high virulence when injected into *S. frugiperda* larvae. Bacterial cell suspensions caused higher mortality than cell-free supernatants, although the latter also exhibited insecticidal activity. These findings confirm the entomopathogenic potential of *Steinernema*-associated symbiotic bacteria and highlight their relevance as sources of bioactive metabolites for the development of novel biological control strategies compatible with integrated pest management programs.

Keywords: entomopathogenic bacteria; EPN; fall armyworm; pathogenicity.

INDICADORES DE IMPACTO

A busca por soluções sustentáveis para o controle de pragas na agricultura tornou-se um desafio fundamental atualmente, especialmente devido ao aumento da resistência aos inseticidas e às restrições ambientais associadas aos métodos tradicionais de controle. Nesse contexto, os nematoides entomopatogênicos e suas bactérias associadas ganharam relevância como sistemas biológicos de grande interesse, pois oferecem uma combinação de eficácia ecológica, especificidade e um notável potencial em biotecnologia. A análise dessas interações abre novas possibilidades para a descoberta de compostos bioativos e o desenvolvimento de tecnologias microbianas inovadoras. Este estudo focou nas bactérias do gênero *Xenorhabdus*, que se relacionam com nematoides entomopatogênicos, investigando suas características fenotípicas, moleculares e funcionais, com ênfase em sua atividade contra a praga agrícola *Spodoptera frugiperda*, que é uma das mais relevantes mundialmente. A pesquisa forneceu informações valiosas sobre os mecanismos de patogenicidade das bactérias e as complexas interações entre o nematoide, a bactéria e o inseto ao integrar métodos microbiológicos, moleculares e biológicos. Os resultados alcançados têm importância tanto científica quanto prática, demonstrando o potencial das bactérias simbióticas como fontes promissoras de metabólitos com propriedades inseticidas. Além disso, elas impulsionam o progresso de estratégias de controle mais seguras e amigáveis ao meio ambiente. Dessa forma, essa investigação se encaixa perfeitamente nos Objetivos de Desenvolvimento Sustentável (ODS), principalmente no ODS 2 Fome Zero e Agricultura Sustentável, no ODS 12 Consumo e Produção Responsáveis e no ODS 15 Vida Terrestre, fornecendo opções que fomentam a sustentabilidade na produção e atenuam a necessidade de inseticidas sintéticos. Nesse cenário, o estudo não só aumenta o conhecimento científico sobre microrganismos entomopatogênicos, como também destaca a biotecnologia microbiana como um pilar fundamental para impulsionar uma agricultura mais duradoura e forte.

IMPACT INDICATORS

The search for sustainable pest-control solutions in agriculture has become a fundamental challenge, especially because of increased insecticide resistance and environmental restrictions associated with traditional control methods. In this context, entomopathogenic nematodes and their associated bacteria have gained prominence as biological systems of great interest because they offer a combination of ecological efficacy, specificity, and significant potential in biotechnology. Analyzing these interactions opens new possibilities for discovering bioactive compounds and developing innovative microbial technologies. This study focused on bacteria of the genus *Xenorhabdus*, which are associated with entomopathogenic nematodes, investigating their phenotypic, molecular, and functional characteristics, with an emphasis on their activity against the agricultural pest *Spodoptera frugiperda*, one of the most relevant worldwide. The research provided valuable insights into the pathogenic mechanisms of these bacteria and the complex interactions among nematodes, bacteria, and insects, integrating microbiological, molecular, and biological methods. The results are both scientifically and practically significant, demonstrating the potential of symbiotic bacteria as promising sources of metabolites with insecticidal properties. In addition, they advance safer and more environmentally friendly control strategies. Thus, this research aligns perfectly with the Sustainable Development Goals (SDGs), particularly SDG 2 Zero Hunger and Sustainable Agriculture, SDG 12 Responsible Consumption and Production, and SDG 15 Life on Land, providing options that promote sustainable production and mitigate the need for synthetic insecticides. In this scenario, the study not only increases scientific knowledge about entomopathogenic microorganisms but also highlights microbial biotechnology as a fundamental pillar for promoting more sustainable and robust agriculture.

LIST OF FIGURES

Figure 1: Tail of male <i>Steinernema carpocapsae</i> showing the mucron (arrow).	20
Figure 2: Infectious juvenile stage of <i>Steinernema carpocapsae</i>	20
Figure 3: Posterior part of first-generation males of <i>Steinernema brazilense</i> . (A) Tail termination. (B) Spicules.	21
Figure 4: Tail of an infectious juvenile showing the anus (arrow) and hyaline portion.	21
Figure 5: Life cycle of <i>Steinernema</i>	23
Figure 6: Cultivation of symbiotic bacteria. (A) Phase I colonies. (B) Phase II colonies.....	25
Figure 7: Coloring of <i>Xenorhabdus nematophila</i> colonies. Phase I (blue) and phase II (red). ..	25
Figure 8: Phylogenetic trees of <i>Xenorhabdus</i> bacteria and <i>Steinernema</i> EPN.....	27

SUMMARY

1 INTRODUCTION	14
2 THEORETICAL FRAMEWORK.....	16
2.1 <i>Spodoptera frugiperda</i>	16
2.2 Current control alternatives	17
2.3 Entomopathogenic nematodes.....	18
2.3.1 Genus <i>Heterorhabditis</i>	19
2.3.2 Genus <i>Steinernema</i>	19
2.3.2.1 <i>Steinernema carpocapsae</i> (Weiser, 1955).....	20
2.3.2.2 <i>Steinernema brazilense</i> (Nguyen, 2010).....	21
2.4 Life cycle of <i>Steinernema</i> EPN.....	22
2.5 Symbiotic entomopathogenic bacteria of <i>Steinernema</i>	23
2.5.1 Phase variant.....	24
2.5.2 Phylogeny	26
2.5.3 Pathogenicity.....	27
REFERENCES	30
Article I - Phenotypic and molecular characterization of <i>Xenorhabdus</i> bacteria associated with <i>Steinernema</i> and evaluation of their entomopathogenic potential against <i>Spodoptera frugiperda</i>.....	39

1 INTRODUCTION

Lepidopteran insect pests are a significant source of damage to agricultural crops (Tomar et al., 2023). Within this group, the genus *Spodoptera* includes numerous polyphagous species with a wide distribution, many of which pose a significant threat to various crops (Moisan et al., 2024). *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is a highly polyphagous pest that causes significant economic losses in agriculture by damaging crops such as corn, rice, wheat, sorghum, beans, potatoes, and cotton (Acharya et al., 2020; Fallet et al., 2022). This species has a wide host range, with more than 350 host species, which increases its impact and its ability to adapt to different agricultural systems. As a result, it poses several threats to global food security and has significant socioeconomic repercussions for farmers and their families (Fallet et al., 2022).

In response, insecticides have been repeatedly applied to control this pest, providing only partial control. However, this approach has led to a range of consequences, including resistance development in *S. frugiperda* populations (Acharya et al., 2020) and adverse effects on non-target organisms, human health, and the environment (Fallet et al., 2022; Tomar et al., 2023; Moisan et al., 2024).

Another widely adopted strategy for controlling *S. frugiperda* is the use of entomopathogenic microorganisms or their bioproducts, such as bacteria of the species *Bacillus thuringiensis* (Bt), which produce proteins toxic to insects (Ffrench-Constant; Bowen, 2000). Although Bt is widely used as a biological control agent, *S. frugiperda* has developed resistance to several of its Bt toxins (Acharya et al., 2020; Yang; Wang; Kerns, 2022), further reducing the number of effective tools available for controlling this pest.

Consequently, there is a need to develop strategies to control *S. frugiperda*, and biological control is an effective, safe, and sustainable alternative (Acharya et al., 2020; Fallet et al., 2022; Moisan et al., 2024). Biological control relies on natural enemies such as parasitoids, predators, and pathogens (Acharya et al., 2020). In this sense, entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* are among the potential effective biological control agents (Ben Husin; Port, 2021; Tomar; Thakur; Yadav, 2022; Kamou et al., 2024; Moisan et al., 2024). These EPN are soil pathogens that affect a wide variety of herbivores across insect orders, including Lepidoptera (Moisan et al., 2024).

As of 2024, the genus *Steinernema* comprises 114 valid species (Půža; Machado; Malan, 2025). In South America, new species of EPN have been identified in several countries, including Argentina, Uruguay, Colombia, Chile, Venezuela, and Brazil (Bhat; Chaubey; Askary, 2020). In Brazil, a new species, *Steinernema brazilense*, isolated in Mato Grosso, has been described; likewise, the presence of *Steinernema carpocapsae* has also been reported (Nguyen et al., 2010). *Steinernema carpocapsae* has a wide geographic distribution and numerous strains (Poinar, 1990), and is used in a range of commercial products available in several countries worldwide for the control of target insect pests (Nurashikin-Khairuddin et al., 2022; Irsad et al., 2023).

A fundamental feature of *Steinernematidae* and *Heterorhabditidae* nematodes is their close symbiosis with bacteria, which explains their entomopathogenic nature, as pathogenicity toward the host insect depends mainly on the action of these symbionts (Gozel; Gozel, 2016; Tarasco et al., 2023). *Steinernema* symbiotic bacteria belong to the genus *Xenorhabdus* (Boemare; Akhurst, 1988; Forst; Neelson, 1996; Del Valle et al., 2016) and are pathogenic to insects, even in the absence of nematodes (Forst; Neelson, 1996; Da Silva et al., 2020). These bacteria produce a variety of secondary metabolites, such as toxic compounds and proteins (Isaacson; Webster, 2002; Shi; Bode, 2018; Muangpat et al., 2020; Tomar; Thakur; Yadav, 2022), during the late exponential growth phase and the early stationary phase (Palma et al., 2024). These metabolites include compounds with insecticidal, cytotoxic, acaricidal, antibacterial, and antifungal activities (Fukruksa et al., 2017; Muangpat et al., 2020; Abebew et al., 2022; Cimen et al., 2022; Kamou et al., 2024). In addition, bioactive compounds have been reported to cause toxicity by ingestion and injection (Palma et al., 2024; Tomar et al., 2024). For this reason, recent studies have highlighted the enormous capacity and potential of entomopathogenic bacteria (EPB) as biological control agents for integrated pest management programs (Eivazian; Sharifi; Mohammadi, 2025), whose use is based on their low environmental impact, selectivity, and biodegradable nature (Namsena; Bussaman; Rattanasena, 2016; Tomar et al., 2024). To deepen our understanding of potential biological control agents, it is necessary to identify and characterize specific EPB and analyze their pathogenicity (Thanwisai et al., 2024; Eivazian; Sharifi; Mohammadi, 2025).

Xenorhabdus bacterial bioproducts, such as cell suspensions and cell-free filtrates, show great potential for identifying new active ingredients (Vicente-Díez et al., 2021) and have received increasing attention in recent years because they offer an alternative to insecticides (Cimen et al., 2022; Kamou et al., 2024).

In this context, the present study focused on identifying and evaluating symbiotic bacteria associated with *Steinernema* species to explore the potential of their products, such as cell suspensions and cell-free supernatants. These products are a rich source of bioactive compounds with potential applications in the biological control of agricultural pests, offering alternatives to the growing problem of resistance and helping to reduce environmental impact. To achieve this objective, the pathogenicity of several strains of entomopathogenic nematodes was evaluated; the symbiotic bacteria associated with the entomopathogenic nematodes *S. carpocapsae* and *S. brazilense* were phenotypically and molecularly characterized; and the pathogenicity of these symbiotic bacteria in *S. frugiperda* larvae was evaluated. Based on the above, the hypothesis was formulated that symbiotic bacteria of the genus *Xenorhabdus* associated with *Steinernema* species exhibit significant entomopathogenic activity against *S. frugiperda*, both in cell suspensions and in cell-free supernatants, constituting a viable alternative for biological control.

2 THEORETICAL FRAMEWORK

2.1 *Spodoptera frugiperda*

The fall armyworm *Spodoptera frugiperda* (J.E. Smith, 1797) belongs to the order Lepidoptera and the family Noctuidae. It is native to the tropical and subtropical regions of the Americas (Acharya et al., 2020). The pest's high voracity and remarkable dispersal capacity have made it a serious threat to food security (Babendreier et al., 2020; Fallet et al., 2022). Since 2016, *S. frugiperda* has spread from the American continent to more than 70 countries across three continents (Fallet et al., 2022). In addition, its short life cycle complicates its control, management, and eradication (Bakry; Abdel-Baky, 2023).

It can attack a variety of plant species, but it shows a strong preference for maize (Tambo et al., 2023). *Spodoptera frugiperda* larvae are capable of causing defoliation (Makgoba et al., 2021) because, at this stage, they feed mainly on young leaves and shoots, particularly buds, where the greatest consumption of plant tissue occurs (Paredes-Sánchez et al., 2021). However, third-stage larvae cause only about 2% of the damage, while those in the final stages are the most voracious, leaving crops ragged and destroyed, thereby reducing yields (Makgoba et al., 2021). In addition, larger larvae that lodge in the tassels can attack the ears or kernels of maize

(Makgoba et al., 2021; Sisay et al., 2019). Due to their high invasiveness and devastating effects, they can cause major economic losses and threaten food security in the future (Bakry; Abdel-Baky, 2023; Barkessa et al., 2024).

The most common strategies of *S. frugiperda* control are chemical and the use of genetically modified plants that produce Bt insecticidal proteins (Van Den Berg; Du Plessis, 2022). However, the continued use of a wide variety of chemical insecticides has led to rapid pest resistance, a phenomenon also observed with Bt insecticidal proteins (Amaral et al., 2023; Boaventura et al., 2020). There have been 272 reported cases of resistance to 47 active ingredients used to control *S. frugiperda*, including chemical compounds and Bt proteins (Mota-Sanchez; Wise, [S.d.]). This underscores the urgent need to develop and implement alternative control strategies for this agricultural pest that use alternative active compounds (Amaral et al., 2023).

2.2 Current control alternatives

The early stages of *S. frugiperda* larvae are more susceptible to various control strategies (Paredes-Sánchez et al., 2021). Conventional control of this pest in maize and other crops in South America relies mainly on synthetic insecticides (Horikoshi et al., 2021). However, excessive and inappropriate use of these compounds is a major driver of resistance evolution in *S. frugiperda* populations (Gutiérrez-Moreno et al., 2019; Paredes-Sánchez et al., 2021).

In Brazil, insecticides are applied 3 to 8 times during the maize crop cycle to control this pest (Van Den Berg; Du Plessis, 2022). Various *S. frugiperda* populations have developed resistance to multiple insecticide groups, including DDT, cyclodiene organophosphates, carbamates, and pyrethroids (Paredes-Sánchez et al., 2021). Historical exposure to different insecticides has favored the emergence of cross-resistance, influencing the dynamics of resistance evolution. This process reduces treatment effectiveness and leads to lower-than-expected control efficiency, even when insecticides are applied according to label recommendations (Van Den Berg; Du Plessis, 2022). In addition, the intensive use of chemical insecticides contributes to soil contamination and bioaccumulation in the environment (Matuska-Łyżwa et al., 2024).

Furthermore, the adoption of Bt crops has been a significant advance in managing *S. frugiperda*, contributing to a substantial reduction in the use of synthetic insecticides. In the United States, insecticide use declined by 47.8% after the introduction of this technology (Brookes; Barfoot, 2018; Van Den Berg; Du Plessis, 2022). Among the main benefits of Bt crops are control of target pests, reduced impact on non-target organisms, and reduced dependence on chemical insecticides (Bernardi et al., 2015). In Brazil, agriculture has been consolidated through the incorporation of these technologies, with the cultivation of Bt maize hybrids standing out since their approval in 2007 (Bernardi et al., 2015).

However, the continuous expression of *cry* genes in Bt plants exerts constant selection pressure on insect pest populations, thereby promoting resistance evolution (Bernardi et al., 2015). To delay this process, resistance management strategies have been implemented, such as gene pyramiding (Orozco-Restrepo et al., 2024), which combines two or more toxins with different modes of action to manage *S. frugiperda* (Santos-Amaya et al., 2025). However, selection for resistance to combinations of Cry toxins can reduce susceptibility to other toxins in the same group, indicating cross-resistance (Santos-Amaya et al., 2015). In this context, cases of *S. frugiperda* resistance to maize hybrids expressing Cry1A.105 + Cry2Ab have been reported in Brazil (Orozco-Restrepo et al., 2024), as well as practical resistance to the Cry1A.105 + Cry2Ab2 + Cry3Bb1 event in Colombia (Santos-Amaya et al., 2025). In this regard, the search for complementary or alternative strategies to control pests that rely exclusively on Bt toxins, such as the use of biological control agents, is especially relevant.

2.3 Entomopathogenic nematodes

Since the 1980s, entomopathogenic nematodes (EPN) have been recognized as effective biological control agents against a wide range of agricultural insect pests, including species that feed on leaves, live on the soil surface, and live underground (Liu et al., 2020). EPN are distributed worldwide, except in Antarctica (Gozel; Gozel, 2016; Yimthin et al., 2021; Cimen et al., 2022; Tomar; Thakur; Yadav, 2022). These nematodes primarily inhabit soil (Forst et al., 1997; Cimen et al., 2022) and maintain mutualistic relationships with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, associated with *Steinernema* and *Heterorhabditis* entomopathogenic nematodes, respectively (Boemare; Akhurst, 1988; De Brida et al., 2017; Shinde et al., 2022; Stock; Hazir, 2025). Because of this close bacterial symbiosis, these EPN

are not considered entomoparasites but rather entomopathogenic nematodes, as their pathogenicity toward the host insect depends fundamentally on the action of their symbiotic bacteria (Tarasco et al., 2023).

By the end of 2024, 114 valid species of the genus *Steinernema* and 21 of the genus *Heterorhabditis* had been described, although new species continue to be identified (Půža; Machado; Malan, 2025). In general, each species of entomopathogenic nematode is usually associated with a single symbiotic bacterial species, while the same bacteria can establish relationships with more than one species of nematode (Chandra; Lee; Kim, 2019). Knowledge of this diversity and the interactions between ENP and their symbiotic bacteria has broadened prospects for developing new biotechnological applications (Stock; Hazir, 2025).

2.3.1 Genus *Heterorhabditis*

The genus *Heterorhabditis* is classified into three predominant clades: Indica, Bacteriophora, and Megidis (Nguyen; Shapiro-Ilan; Mbata, 2008). The infective juveniles (IJ) of this genus are characterized by a dorsal tooth, a structure that enables them to actively penetrate the host insect's intersegmental membrane (Griffin; Boemare; Lewis, 2005; Tarasco et al., 2023; Trejo-Meléndez; Ibarra-Rendón; Contreras-Garduño, 2024). Bacteria of the genus *Photorhabdus* are found in the anterior region of the *Heterorhabditis* intestine or distributed throughout it (Trejo-Meléndez; Ibarra-Rendón; Contreras-Garduño, 2024). In Brazil, *H. amazonensis* is considered a native species, and *H. indica* and *H. baujardi* have also been isolated in the state of Rondônia. In the state of Minas Gerais, *H. amazonensis*, *H. baujardi*, *H. indica* have also been isolated (De Brida et al., 2017).

2.3.2 Genus *Steinernema*

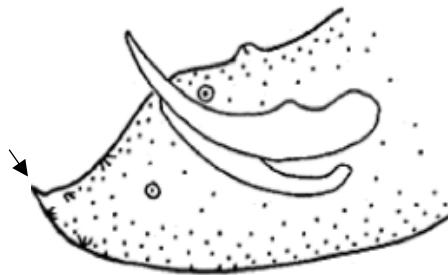
This genus usually locates its host insect using an “ambush” search strategy, characterized by standing and jumping behaviors. However, some species may also employ a “cruiser” strategy, in which they actively move around the environment in search of their host (Ndereyimana et al., 2019). The genus *Steinernema* was initially grouped into five main clades based on early phylogenetic analyses. However, as the number of described species has

increased, these clades have been reorganized into a more detailed system that recognizes twelve multispecies clades (Affine, Bicornutum, Cameroonense, Carpocapsae, Costaricense, Feltiae, Glaseri, Karii, Khoisanae, Kushidai, Longicaudum, and Monticola) and three single-species clades (*S. neocurtillae*, *S. unicornum*, and *S. rarum*) (Půža; Machado; Malan, 2025). Among the species of the genus *Steinernema*, *S. carpocapsae* belongs to the Carpocapsae clade, which comprises 16 species, whereas *S. brazilense* belongs to the Glaseri clade, which includes 14 species (Půža; Machado, 2024).

2.3.2.1 *Steinernema carpocapsae* (Weiser, 1955)

Steinernema carpocapsae is widely distributed (Poinar, 1990). Males adopt a C-shape when exposed to heat and have a smooth cuticle. The tail is short (Torrini et al., 2014) and is characterized by a mucron (1 to 4 μm long) at its tip and yellowish-gray spicules (Figure 1) (Poinar, 1990). The infectious juvenile (Figure 2) has a slender body enclosed in a sheath (Torrini et al., 2014). It measures 438–650 μm , with an average of 558 μm , and the excretory pore is located in the anterior part of the nerve ring (Poinar, 1990). The nerve ring is located at 68% of the length of the pharynx (Torrini et al., 2014).

Figure 1: Tail of male *Steinernema carpocapsae* showing the mucron (arrow).



Source: Poinar (1990).

Figure 2: Infectious juvenile stage of *Steinernema carpocapsae*.

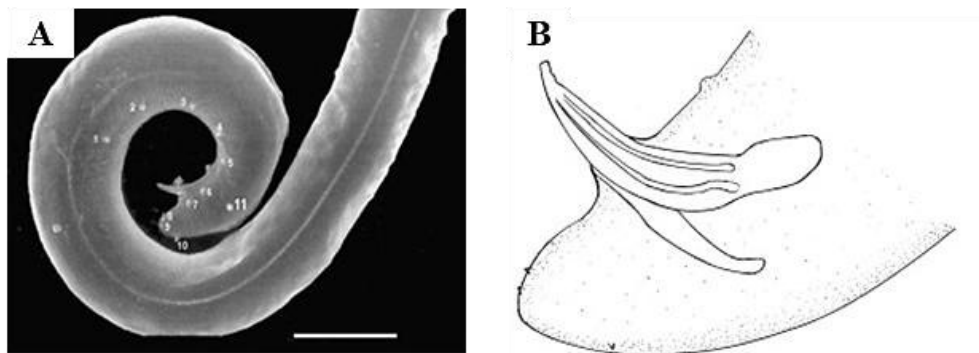


Source: Tomar et al. (2023).

2.3.2.2 *Steinernema brazilense* (Nguyen, 2010)

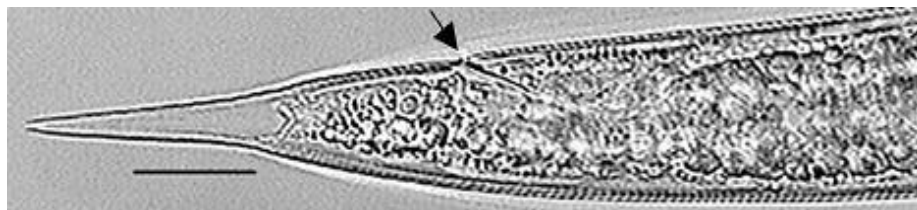
The posterior part of the body of first-generation males is curved, J-shaped when exposed to heat (Figure 3A). The cuticle is marked by slight transverse striations. The tail is elongated, with a blunt, rounded tip, and lacks a mucron (Figure 3B). Males are distinguished by a spicule length of 75 to 89 μm , with an average of 83 μm . In addition, the spicule head is longer than it is wide (Nguyen et al., 2010). The infectious juvenile has an elongated body enclosed in a sheath that corresponds to the second-stage cuticle, although this sheath may be lost during storage. It is 1157 μm (1023 – 1284) long in average. The distance from the anterior end to the excretory pore is 95 μm (87 – 102) in average. From the anterior end to the end of the esophagus, it is 139 to 153 μm , with an average of 148 μm . The tail measures 80 to 104 μm , with an average of 85 μm . The excretory pore is located anterior to the nerve ring and the middle esophagus. The tail gradually narrows toward the tip, and approximately 55% of its length is formed by the hyaline portion (Figure 4) (Nguyen et al., 2010).

Figure 3: Posterior part of first-generation males of *Steinernema brazilense*. (A) Tail termination. (B) Spicules.



Source: Nguyen et al. (2010).

Figure 4: Tail of an infectious juvenile showing the anus (arrow) and hyaline portion.



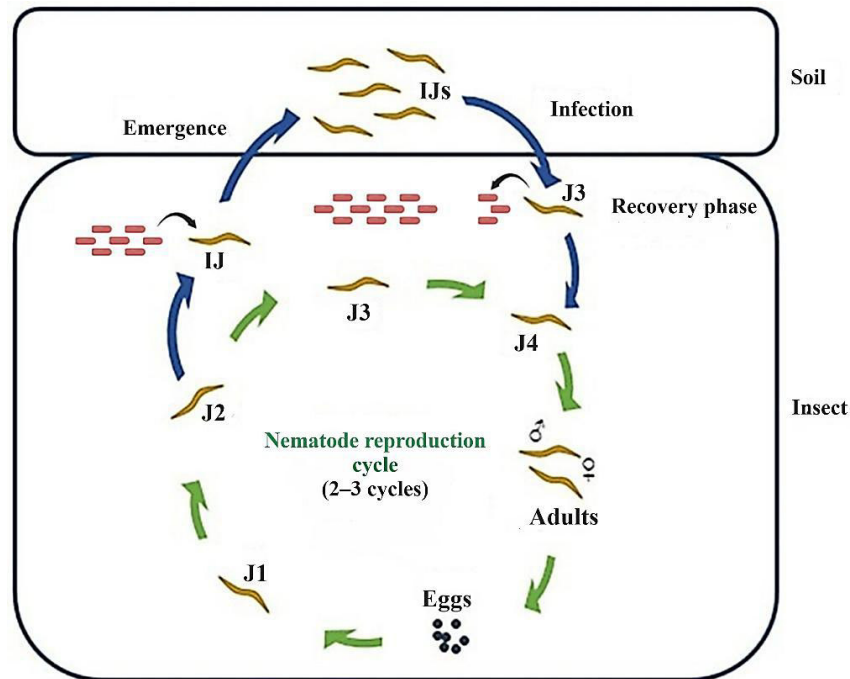
Source: Nguyen et al. (2010).

2.4 Life cycle of *Steinernema* EPN

The life cycle of EPN comprises three stages: egg, juvenile, and adult (Manochaya et al., 2022). It is characterized by the infection and death of insects, whose carcasses then provide a favorable environment for the multiplication of symbiotic bacteria and the nematodes themselves (Ffrench-Constant; Bowen, 2000). This process involves a symbiotic stage, in which bacteria of the genus *Xenorhabdus* are transported in a specialized bilobulated intestinal vesicle present in IJ (Forst; Nealson, 1996; Cortés-Martínez; Chavarría-Hernández, 2020), and a pathogenic stage, in which the death of the susceptible insect results from the joint action of the nematode and the bacteria (Forst et al., 1997). Depending on the species, the life cycle can last between one and three weeks (Gozel; Gozel, 2016; Cimen et al., 2022).

Nematodes can enter the insect's larval-stage hemocoel through natural openings such as the mouth, anus, or respiratory spiracles (Forst; Nealson, 1996; Fukruksa et al., 2017; Shi; Bode, 2018; Cimen et al., 2022; Palma et al., 2024). In the hemocoel, *Xenorhabdus* bacteria are released into the hemolymph via defecation (Dreyer; Malan; Dicks, 2018; Cortés-Martínez; Chavarría-Hernández, 2020; Da Silva et al., 2020) and proliferate until they dominate the microbial flora. In addition to killing insects, these bacteria serve as a food source for nematodes. Thus, *Steinernema* nematodes in the IJ stage recover and reproduce optimally, developing to the fourth juvenile stage, at which point they become adults. Therefore, reproduction requires the presence of males and females (Boemare; Akhurst, 1988; Forst; Nealson, 1996; Ffrench-Constant; Bowen, 2000; Gozel; Gozel, 2016).

When food runs out and nematode density is high, they enter the IJ or Dauer stage, and bacteria reassociate to emerge from the insect carcass (Forst; Nealson, 1996; Shi; Bode, 2018; Cortés-Martínez; Chavarría-Hernández, 2020). The IJ stage is the only free-living stage in which nematodes live in the soil (Cortés-Martínez; Chavarría-Hernández, 2020; Da Silva et al., 2020; Moisan et al., 2024) and do not feed, relying on stored energy reserves until they find a new host (Figure 5) (Kim et al., 2021). This feeding blockage is caused by a retained cuticle from the second stage that obstructs the mouth and anus in the third stage (Awori, 2022).

Figure 5: Life cycle of *Steinernema*.

Source: Dreyer; Malan; Dicks (2018) modified.

2.5 Symbiotic entomopathogenic bacteria of *Steinernema*

The family Steinernematidae contains symbiotic bacteria of the genus *Xenorhabdus* (Tomas and Poinar 1979), which belong to the class Gammaproteobacteria, order Enterobacterales, and family Morganellaceae, despite being catalase-negative (Awori, 2022; Palma et al., 2024). They are motile, Gram-negative bacilli that are facultative anaerobes (Forst et al., 1997; Thanwisai et al., 2012; Da Silva et al., 2020).

Therefore, based on its etymology, *Xenorhabdus* refers to pathogenic, rod-shaped bacteria (Parte, 2014). To date, 39 species and 11 subspecies of *Xenorhabdus* have been reported, of which 29 have valid, published names, and 30 have valid names, including synonyms (Parte, 2014). Although associations are usually species-specific, one species of *Xenorhabdus* can infect more than one species of *Steinernema* (Dreyer; Malan; Dicks, 2018). *Xenorhabdus* lives in the gut of EPN during the infectious juvenile stage in the soil (Thanwisai et al., 2012, 2024); however, they can grow as free-living organisms under normal laboratory conditions, sustained by the enriched nutrients provided by culture media and the absence of competition that usually exists in the soil (Forst; Nealson, 1996). In addition, the growth

medium accumulates pigments that bacteria tend to produce as secondary metabolites and proteins that are toxic to insects (Forst; Nealson, 1996).

2.5.1 Phase variant

Symbiotic bacteria of the genus *Xenorhabdus* can exist in two phases, known as phase I (primary) and phase II (secondary) (Akhurst, 1980). These phases differ morphologically and, in their ability to adsorb dyes from culture media and to produce antimicrobial compounds (Boemare; Akhurst, 1988).

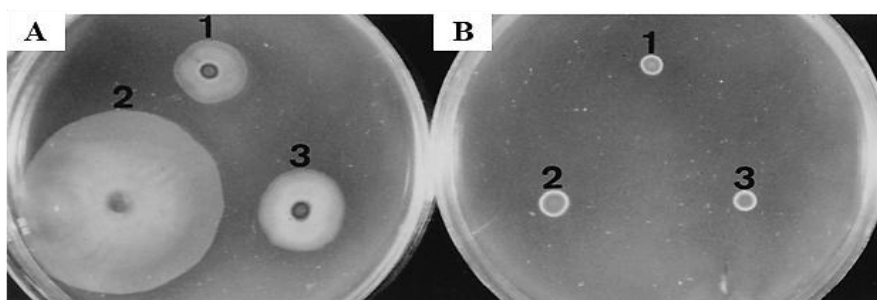
Phase I can be found in nematodes in the infectious juvenile stage (Akhurst, 1980; Forst et al., 1997) and is more efficient at supplying nutrients to nematodes, both in insects and in monoxenic culture media (Boemare; Akhurst, 1988), which facilitates nematode maturation and accelerates reproduction (Akhurst, 1980). In addition, phase I bacteria may remain in a medium enriched with nutrients and salt from the insect's hemocoel (Forst et al., 1997). Therefore, phase I specializes in producing compounds with antibiotic activity, dye absorption, and the generation of lipase, phospholipase, and protease. Bacterial cells are larger (Figure 6A) (Forst et al., 1997; Dreyer; Malan; Dicks, 2018; Tomar; Thakur; Yadav, 2022) and pleomorphic, with 80 to 90% rod-shaped and the remaining 10 to 20% spheroplasts. They are motile due to peritrichous flagella and exhibit coordinated movement known as swarming, which allows them to form an extensive layer when cultured on suitable agar (Forst et al., 1997).

The characteristics of bacterial colonies are more evident in agar containing triphenyltetrazolium chloride and bromothymol blue (Akhurst, 1980), the main components of the culture medium NBTA, a selective and differential medium (Muangpat et al., 2020). This culture medium is suitable because, after 3 to 5 days, it allows differential staining of bacteria in phase I to be observed. This occurs because bromothymol blue is absorbed and triphenyltetrazolium chloride is reduced, resulting in colonies with a red core covered by dark blue, with a clear surrounding zone (Figure 7) (Akhurst, 1980; Dreyer; Malan; Dicks, 2018). In addition, the colonies have a convex or umbonate surface (Thanwisai et al., 2012; Fukruksa et al., 2017; Kamou et al., 2024). However, when bacteria are cultured for more than 7 days under anaerobic conditions, some colonies develop secondary morphology (Akhurst, 1980; Forst et al., 1997; Tarasco et al., 2023).

Phase II cells are smaller than phase I cells (Figure 6B) (Forst et al., 1997; Tomar; Thakur; Yadav, 2022). They provide less favorable nutritional conditions for the nematode, and their formation occurs under low-salinity conditions (Forst et al., 1997). This stage may co-occur with phase I in both monoxenic cultures started with phase I and in the hemocoel of infected *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvae. *Galleria mellonella* larvae are often used in EPN studies or EPN production because of their high susceptibility, small size, short life cycle, ease of rearing on artificial diets with various ingredients, adaptability to different temperatures, and high nematode production (Kotchofa; Baimey, 2019).

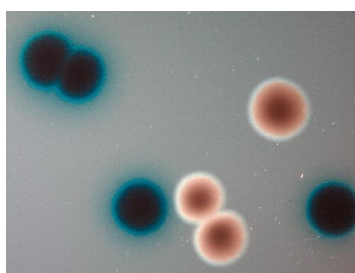
In addition, nematodes in the infectious juvenile stage contain only phase II bacteria when phase I development does not occur (Akhurst, 1980). In agar culture media such as NBTA, phase II colonies are red to brown due to the presence of blue agar and do not show a clear surrounding zone (Figure 7). This is due to the lack of bromothymol blue absorption (Akhurst, 1980). Although the death of *G. mellonella* requires can be caused by few bacterial cells (Forst et al., 1997), studies with *Galleria* and *Manduca sexta* have shown that phase II cells of *X. nematophilus* are much less virulent than phase I cells and, in some cases, show no virulence at all (Akhurst, 1980; Forst et al., 1997).

Figure 6: Cultivation of symbiotic bacteria. (A) Phase I colonies. (B) Phase II colonies.



Source: Volgyi et al. (1998).

Figure 7: Coloring of *Xenorhabdus nematophila* colonies. Phase I (blue) and phase II (red).

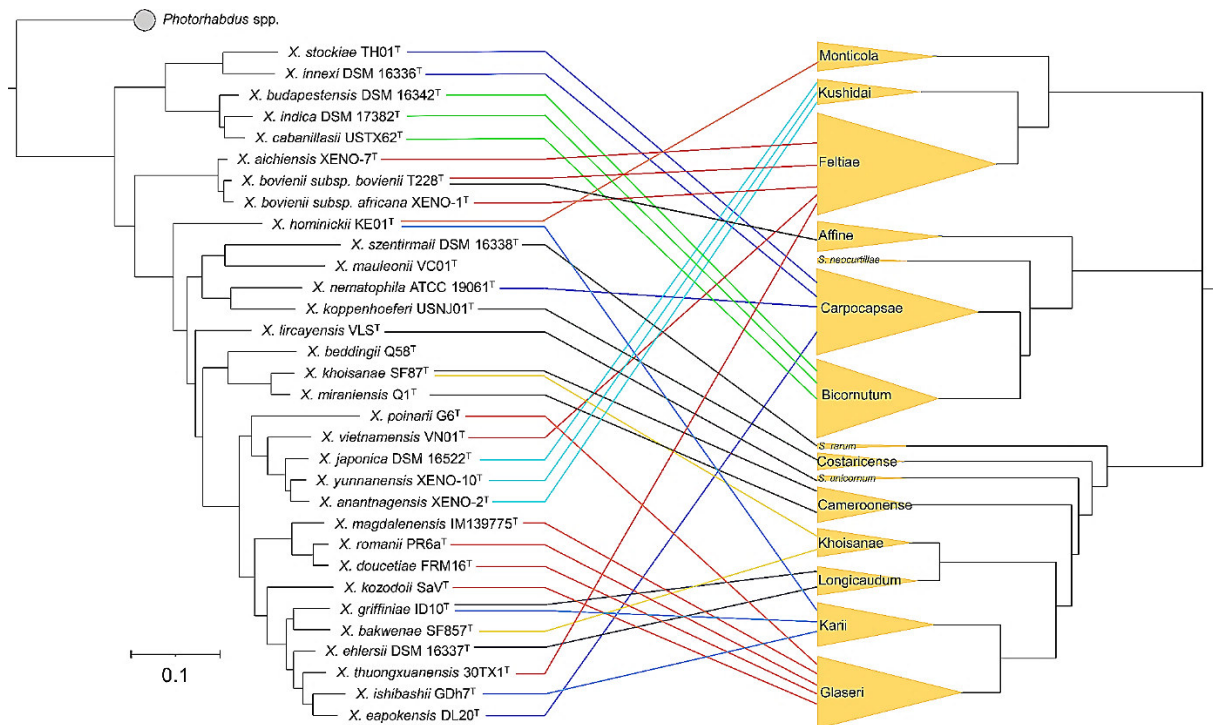


Source: Cambon (2018).

2.5.2 Phylogeny

Phylogenetic analysis of specific regions of the bacterial genome is used to assess phylogenetic relationships within a bacterial group, as in the case of *Xenorhabdus* species, which show significant diversification (Forst et al., 1997) within the family Morganellaceae. Over the years, phylogenetic analysis of these symbiotic bacteria has provided insight into their evolution and the relationships between species based on genetic similarities, providing a clearer understanding of their diversity. Similarly, it allows the identification of new species and the understanding of the associations of symbiotic bacteria with EPN (Figure 8) (Thanwisai et al., 2012; Fukruksa et al., 2017; Půža; Machado, 2024). Methods based on nucleotide sequence analysis are currently the primary tools for delimiting prokaryotic species and enable reliable identification of new isolates and the revision of previously described taxa (Awori, 2022).

Based on the analysis of the 16S rDNA sequence of six *Xenorhabdus* strains, these bacteria are distinguished by a pair of UC at position 450 (according to *Escherichia coli* numbering) (Forst et al., 1997). They can also be differentiated at positions 208-211 (*E. coli*), where *Xenorhabdus* has TTCG in a region of the 16S rRNA gene (Thanwisai et al., 2012). However, the 16S rRNA gene does not allow to clearly defining the evolutionary relationships at the deepest nodes of the phylogenetic trees within the genus *Xenorhabdus* (Tailliez et al., 2010). Many studies use the *recA* (recombinase A) gene to identify and perform phylogenetic analyses of this genus (Thanwisai et al., 2012; Fukruksa et al., 2017; Muangpat et al., 2020; Yimthin et al., 2021; Subkrasae et al., 2022; Kamou et al., 2024). This gene encodes a DNA recombination protein and supports phylogenetic nodes that unite subclades (Tailliez et al., 2010). Another advantage is that this gene is present in all sequenced bacterial organisms (Klasson; Andersson, 2004). Therefore, the high conservation in size and sequence of *recA* allows for almost unambiguous alignments (Eisen, 1995).

Figure 8: Phylogenetic trees of *Xenorhabdus* bacteria and *Steinernema* EPN.

Source: Půža; Machado (2024).

2.5.3 Pathogenicity

The bacteria are released into the host's hemolymph within 5 hours after invasion (Forst; Neilson, 1996) and cause septicemia that quickly kill the host insect larvae within 48 hours (Fukruksa et al., 2017; Cimen et al., 2022; Tomar; Thakur; Yadav, 2022; Palma et al., 2024). The carcasses of *G. mellonella* insects infected with *Xenorhabdus* exhibit characteristic coloration, usually turning grayish or creamy yellow (Tarasco et al., 2023). This may occur because healthy *G. mellonella* larvae have a light, homogeneous cream-colored appearance, with no distinct areas of melanization (Pereira; Rossi, 2020). However, when infected by pathogenic microorganisms, the pigmentation progressively darkens due to the action of hemocytes, which produce the enzyme phenoloxidase, which catalyzes the production of melanin. As a result of the accumulation of hemocytes in the dorsal vessel of the larvae, signs of dark pigmentation are more evident in the dorsal region. Consequently, melanization often leads to the complete darkening of the larvae and their subsequent death (Pereira; Rossi, 2020).

The bacteria can kill the insect when they reach 10^6 cells/mL, the threshold considered to indicate septicemia (Huot et al., 2019). When the insect dies, the number of bacterial cells in

the hemolymph increases to 10^8 cells/mL (Forst et al., 1997). Therefore, bacteria produce potent toxins that damage the insect and do not need to proliferate rapidly within the hemolymph during the infectious phase (Forst et al., 1997).

Phagocytosis is the first line of defense of the insect immune system against bacterial infection in the hemolymph. As the bacterial load increases, this response is accompanied by the formation of nodules, structures composed of hemocytes that sequester bacteria and limit their dissemination (Forst et al., 1997). Although the insect immune system produces bactericidal proteins and antimicrobial peptides, species of the genus *Xenorhabdus* can proliferate intensely in the hemolymph. This ability has been associated with resistance to these molecules or with the capacity to suppress the host's defense mechanisms. For instance, *X. nematophila* exhibits cell surface features that interfere with hemocyte activity, thereby enhancing bacterial survival within the insect host (Forst et al., 1997).

Similarly, phenoloxidase activation is an important component of the insect humoral immune response. However, bacteria of the genus *Xenorhabdus* have evolved strategies to evade this pathway, primarily by inhibiting the conversion of prophenoloxidase to phenoloxidase. This effect has been linked to the presence of bacterial lipopolysaccharides, which are regarded as key virulence factors (Forst; Nealson, 1996; Forst et al., 1997; Dreyer; Malan; Dicks, 2018).

In addition, insects possess other molecules that play key roles in inducing humoral and cellular immune responses, such as eicosanoids, which mediate immune signaling against a wide range of pathogens. However, *Xenorhabdus* has been reported to induce immunosuppression in *Spodoptera exigua* by inhibiting phospholipase A2 (PLA2), thereby disrupting the main catalytic step in eicosanoid biosynthesis and ultimately triggering septicemia (Shrestha; Kim, 2010; Dreyer; Malan; Dicks, 2018).

In phase I, most *Xenorhabdus* strains can be distinguished from other members of the family Morganellaceae by their ability to produce crystalline inclusion bodies during the stationary phase, which are not observed in exponentially growing cells (Forst; Nealson, 1996; Forst et al., 1997; Dreyer; Malan; Dicks, 2018; Tomar; Thakur; Yadav, 2022). These bacteria also release a variety of bioactive compounds into the hemolymph, including secondary metabolites that affect insect tissues and create favorable conditions for nematode growth and reproduction (Shrestha; Kim, 2010; Tomar; Thakur; Yadav, 2022; Tarasco et al., 2023). They also produce toxic proteins with strong insecticidal activity (Thanwisai et al., 2012; Eivazian;

Sharifi; Mohammadi, 2025), as well as enzymes such as chitinases and proteases (Forst; Neelson, 1996; Palma et al., 2024). Additionally, antimicrobial compounds synthesized by *Xenorhabdus* inhibit the growth of competing microorganisms, thereby preventing secondary infections (Shrestha; Kim, 2010; Thanwisai et al., 2012; Cimen et al., 2022). Notable metabolites reported for *Xenorhabdus* include ambactin, benzylideneacetone, cabanillasin, fabclavine, indole, nematophin, PAX peptides, rhabdopeptide, rhabduscin, szentiamide, xenocoumacin, xenorhabdin, xenortide, and xenotetrapeptide (Cimen et al., 2022).

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Article I - Phenotypic and molecular characterization of *Xenorhabdus* bacteria associated with *Steinernema* and evaluation of their entomopathogenic potential against *Spodoptera frugiperda*

Abstract

Spodoptera frugiperda is one of the most important agricultural pests worldwide, and controlling it has become increasingly challenging due to resistance to chemical insecticides and Bt proteins. In this context, entomopathogenic nematodes (EPN) and their symbiotic bacteria represent a promising, environmentally safe alternative. The objective of this study was to phenotypically and molecularly characterize the symbiotic bacteria associated with *Steinernema carpocapsae* and *S. brazilense* and to evaluate their pathogenicity against *S. frugiperda* larvae. To achieve this objective, the bacteria were isolated from infected larvae and characterized both phenotypically and molecularly using the *recA* gene. Their growth was evaluated *in vitro* and *in vivo*, and their pathogenicity was assessed by injection and oral bioassays, using both bacterial cell suspensions and cell-free supernatants. Molecular analysis identified the strains as *Xenorhabdus nematophila* (bsSC) and *X. doucetiae* (bsSB). Both exhibited typical characteristics of the genus and high virulence upon injection, reaching more than 50% mortality at 48 h at concentrations of 10^5 and 10^8 CFU/mL. *Xenorhabdus nematophila* exhibited faster growth, while *X. doucetiae*, although growing more slowly, achieved mortality rates of 80% at the highest concentration. Oral administration was less effective, but ingestion of the bacteria by second-stage larvae was confirmed. Cell-free supernatants had a moderate insecticidal effect, although less than that of cell suspensions. In this sense, the results show that symbiotic bacteria of *Steinernema* have great potential as entomopathogenic agents against *S. frugiperda*, even without the direct intervention of the nematodes.

Keywords: bacterial pathogenicity; biological control; *Xenorhabdus*.

1 INTRODUCTION

The fall armyworm *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is one of the most economically important polyphagous pests worldwide (Boaventura et al., 2020; Lalramnghaki et al., 2021; Oliveira et al., 2024; González et al., 2025). This pest can damage more than 350 cultivated plant species, including maize, rice, cotton, soybeans, and sugarcane (Acharya et al., 2020; Lalramnghaki et al., 2021; Mohamed; Shairra, 2023; Oliveira et al., 2024). It also exhibits high dispersal capacity, high oviposition rates, long-distance migration, and a remarkable ability to adapt to diverse climatic conditions (Sayed; Ibrahim; El-

Gepaly, 2022; Mohamed; Shairra, 2023). Due to these characteristics, *S. frugiperda* was declared a quarantine pest globally in 2018 by the Food and Agriculture Organization of the United Nations (FAO) (Sayed; Ibrahim; El-Gepaly, 2022).

Among the most environmentally friendly technologies for controlling this pest is the use of *Bacillus thuringiensis* (Bt). However, its adoption presents limitations in small-scale agricultural systems, associated with factors such as uncertainty about its effectiveness under variable climatic conditions, the high cost of seeds, unfavorable soil conditions, traditional agricultural practices, and limited availability of agricultural extension services (González et al., 2025).

In Brazil, *S. frugiperda* primarily affects maize and cotton crops (Okuma et al., 2018; Boaventura et al., 2020), where control is based mainly on chemical insecticides and Bt crops. However, the intensive use of these strategies has led to the development of resistance to insecticides such as lambda- cyhalothrin, spinosad, chlorpyrifos, and lufenuron (Okuma et al., 2018; Acharya et al., 2020), as well as to Bt proteins such as Cry1F, Cry1Ab, and Cry1A.105 + Cry2Ab2 (Amaral et al., 2020).

For this reason, there is a need to explore more sustainable control alternatives. Entomopathogenic nematodes (EPN) and their symbiotic bacteria are promising options because they can quickly eliminate insect pests and have a low environmental impact. These nematodes belong mainly to the genera *Steinernema* and *Heterorhabditis* and maintain mutualistic associations with the bacteria *Xenorhabdus* and *Photorhabdus*, respectively (Shinde et al., 2022; Yüksel, 2022). It has been shown that some *Xenorhabdus* species can induce mortality in host insects even in the absence of their nematode vectors (Kim et al., 2017; McMullen et al., 2017). These bacteria have attracted growing interest due to their fundamental role in the virulence of EPN; therefore, bacterial cell suspensions and their cell-free supernatants have been used to evaluate their pathogenicity against insect pests (Yüksel, 2022; Erdem; Yüksel; Canhilal, 2024). Symbiotic bacteria can suppress the host's immune responses, primarily by producing natural products involved in insect pathogenicity (Tobias; Shi; Bode, 2018; Kenney et al., 2019).

Numerous studies have reported the symbiotic association between *S. carpocapsae* and *X. nematophila* and its high pathogenicity against a wide range of insects (Acharya et al., 2020; Tomar et al., 2023; Erdem; Yüksel; Canhilal, 2024; Kamou et al., 2024), which translates into its ability to infect approximately 200 insect hosts (Serra et al., 2019). In contrast, few studies

have examined the pathogenicity of *S. brazilense*, a species native to Mato Grosso do Sul (Brazil), against *S. frugiperda*, and no previous reports have characterized its symbiotic bacteria. In this context, the present study aimed to characterize phenotypically and molecularly the symbiotic bacteria associated with entomopathogenic nematodes of the genus *Steinernema* and evaluate their pathogenicity against *S. frugiperda* larvae.

2 MATERIALS AND METHODS

2.1 Rearing of *S. frugiperda*

Spodoptera frugiperda colony used in the experiments was initially field-collected and then maintained in the Molecular Biology and Ecotoxicology Laboratory (MEET) of the Entomology Department (DEN) at the Federal University of Lavras (UFLA), under controlled conditions at 25 ± 2 °C, relative humidity of $65 \pm 10\%$, and a photoperiod of 12 h.

The adults were kept in cages made from polyvinyl chloride (PVC) cylinders 17 cm high and 15 cm in diameter, covered at the top with organza fabric and sealed at the bottom with white paper; these cages were placed on Petri dishes ($\varnothing = 17$ cm). The inner walls of the cages were also completely covered with white paper to encourage oviposition. The adults were fed a 10% aqueous honey solution applied to cotton, which was placed in a Petri dish ($\varnothing = 5$ cm) inside the cage.

The eggs were collected every 2 days; the paper used as an oviposition surface was removed and replaced with new paper. The areas containing egg masses were carefully cut out, and the eggs laid on the organza fabric were collected with a fine brush and transferred to Petri dishes ($\varnothing = 15$ cm) covered with cling film. Pieces of an artificial diet for the newly hatched hatchlings were placed inside the Petri dishes. The artificial diet, made from beans, wheat germ, and brewer's yeast, was prepared according to the method of Menezes et al. (2020). Upon reaching the third larval stage, the larvae were separated into 50 mL plastic cups to minimize cannibalism. Two larvae were placed in each cup containing approximately 2 cm³ of artificial diet, and they developed into pupae. The pupae were collected and placed in 500 mL plastic containers until adult emergence, after which they were returned to the cages.

2.2 Obtaining and multiplying entomopathogenic nematodes

The EPN were obtained from the Pathogen Bank of the Laboratory of Insect Pathology and Microbial Control (LPCMI) at UFLA, which contains native and exotic isolates of the genera *Steinernema* and *Heterorhabditis*. The isolates were stored in aqueous suspensions in

700 mL tissue culture bottles and maintained in a BOD incubator at 16 ± 1 °C, relative humidity of $70 \pm 10\%$, in darkness.

The nematodes were multiplied *in vivo*, following the method described by Woodring and Kaya (1988), using sixth-instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) from a colony established at LPCMI (Macedo, 2024). Two filter papers ($\varnothing = 9$ cm) and 10 *G. mellonella* larvae were placed at the bottom of a Petri dish ($\varnothing = 9$ cm), to which 1.8 mL of the isolate was applied (Macedo, 2024). The dish was then sealed with Parafilm, and two dishes were prepared per isolate. The plates were incubated in a BOD incubator at 25 ± 1 °C, relative humidity of $70 \pm 10\%$, and a photoperiod of 12 h.

After 4 days of incubation, the filter papers were removed, and the plates were dried to eliminate the presence of the EPN. Subsequently, after 7 days, the larvae showing symptoms of infection were transferred to a modified White trap (White, 1927). This consisted of a plastic Petri dish ($\varnothing = 9$ cm) with a piece of acrylic fixed in the center, covered with filter paper ($\varnothing = 9$ cm), and with the addition of distilled water, allowing the infective juveniles (IJ) to emerge from the insect carcass and migrate through the moist paper, attracted by the moisture gradient (Macedo, 2020). The White trap was washed daily with a wash bottle to collect EPN, and the recovered material was collected in a beaker. For subsequent tests, the IJ collected over 3 days were used.

2.3 Bioassay 1: Pathogenicity of entomopathogenic nematodes

The pathogenicity of EPN was evaluated using isolates from *Heterorhabditis* and *Steinernema*, including *H. amazonensis* NEPET 11 (isolated from Palmeiras das Missões, Rio Grande do Sul, Brazil), *H. amazonensis* RSC05 (isolated from Benjamin Constant, Amazonas, Brazil), *H. bacteriophora* (isolated from New Jersey, USA), *S. carpocapsae* All (isolated from North Carolina, USA), and *S. brazilense* (isolated from Porto Murtinho, Mato Grosso, Brazil).

The EPN suspension recovered in the beakers were adjusted to a known volume with distilled water. For counting infectious juveniles, a test tube was prepared by adding 1 mL of nematode suspension to 9 mL of distilled water, and the mixture was distributed evenly into 100 μ L per well of an ELISA plate. Ten (10) wells were used. The IJ in each well were counted under a stereoscope (SMZ-168 Series), and the concentration was adjusted to 1000 IJ/mL.

For each EPN isolate, 30 Petri dishes ($\varnothing = 8$ cm) were used, each containing filter paper at the bottom, a fifth-instar *S. frugiperda* larva, and a block of artificial diet ($\approx 2 \times 1 \times 1$ cm; length $\times$ width $\times$ height). The filter paper was moistened with 1 mL of nematode suspension at

1000 IJ/mL. The control consisted only of distilled water. The plates were sealed with Parafilm and incubated in a BOD incubator (25 ± 1 °C, relative humidity of $70 \pm 10\%$, and a photoperiod of 12 h). Mortality was assessed every 24 hours for 96 hours. After 7 days, larval death was confirmed by opening the carcasses with stylets and examining for EPN under a stereoscope.

2.4 Bioassay 2: Isolation and characterization of symbiotic bacteria associated with entomopathogenic nematodes *S. carpocapsae* and *S. brazilense*

2.4.1 Isolation of symbiotic bacteria

The symbiotic bacteria used were from the genus *Xenorhabdus*, associated with the strains *Steinernema carpocapsae* and *S. brazilense*, which are distinguished by their pathogenicity and use in biological control. *S. carpocapsae* was used as a positive control due to the well-established pathogenicity of its symbiotic bacteria. *S. brazilense* was included to evaluate a previously uncharacterized symbiotic association. The bacteria were isolated from the hemolymph of last-instar *Tenebrio molitor* larvae that died after infection. These larvae were from a colony maintained at the LPCMI.

First, two filter papers ($\varnothing = 9$ cm) and 15 *T. molitor* larvae, characterized by a hardened cuticle, were placed in a Petri dish ($\varnothing = 9$ cm), and 1.8 mL of aqueous nematode suspension was added. The dish was sealed with Parafilm. After 2 days, the dead larvae were disinfected in 95% ethanol for 3 min, then rinsed with distilled water for 3 min. Hemolymph was extracted from 3 larvae by puncturing the 5th-6th lateral abdominal segments with a 30G 5/16" needle and a disposable insulin syringe (1 mL). A drop of hemolymph was plated onto Petri dishes ($\varnothing = 9$ cm) containing nutrient agar with bromothymol blue and triphenyltetrazolium chloride (NBTA). The plates were incubated in a BOD at 28 °C in the dark (Kamou et al., 2024).

The NBTA components were prepared according to the protocols described by Kamou et al. (2024) and Thanwisai et al. (2024). The culture medium consisted of 8 g/L nutrient agar (Kasvi), 25 mg/L bromothymol blue (Synth), and 4 mg/L 2,3,5-triphenyltetrazolium chloride (TTC) (Dinâmica). The nutrient agar and bromothymol blue were adjusted to pH 8 and autoclaved at 121 °C for 15 minutes. The TTC was prepared as a 0.4% stock solution in a 50 mL Falcon tube, protected from light with aluminum foil, and then sterilized by filtration through a 0.20 µm syringe filter (LLG Labware). The TTC was added once the medium reached approximately 45-50 °C, using 1 mL of the stock solution per 100 mL of prepared medium, resulting in a final concentration of 4 mg/L (0.004%). After 4 days, one colony of each bacterial

strain was subcultured onto NBTA medium to ensure isolation, and the isolates were then maintained in Luria-Bertani (LB) broth (Kasvi) supplemented with 20% glycerol at -80 °C (Fukruksa et al., 2017).

2.4.2 Phenotypic characterization of symbiotic bacteria

The selection of bacteria of the genus *Xenorhabdus* was based on the morphological characteristics of the colonies, which were identified after 4 days of incubation on NBTA medium plates by direct observation (Thanwisai et al., 2012). Parameters such as colony size, shape, elevation, texture, and color were observed (Pervez; Lone; Pattnaik, 2020). The catalase test was performed by applying a drop of 1% (v/v) hydrogen peroxide onto a bacterial colony placed on a microscope slide (Kazimierzak et al., 2016). The bacterial isolates were subjected to Gram staining according to the procedure described by Thanwisai et al. (2024), which consisted of applying crystal violet for 1 min, iodine fixative solution for 1 min, 95% alcohol for 30 s, and finally safranin for 1 min. The bacterial colonies were documented using a Leica stereomicroscope (M205 C, Leica Microsystems) equipped with an integrated K3C digital camera.

In addition, bacterial morphology was examined by scanning electron microscopy (SEM) using a modified method Nogueira et al. (2021). A 48-h-old bacterial colony was transferred to an Eppendorf tube (1.5 mL) containing 1 mL of modified Karnovisk fixative solution (2.5% glutaraldehyde, 2% formaldehyde, 0.001 mol/L CaCl₂, and 0.05 mol/L cacodylate buffer, pH 7.2 (Sigma-Aldrich®)) and stored at 4°C until processing. Subsequently, 0.1% poly-L-lysine was spread onto a coverslip and allowed to air-dry for 5 min. Visible samples were transferred to the coverslip and allowed to air-dry. The samples were washed with 0.05 M sodium cacodylate buffer (pH 7.2) for 10 min, then dehydrated in a graded series of acetone concentrations (25%, 50%, 75%, 90%, and 100%) for 10 min at each stage. The 100% concentration was performed twice. The samples were dried in a critical-point dryer (Bal-tec CPD 030) and then coated with a thin layer of gold using a metallizer (Bal-tec SCD 050). They were then stored in a container with silica gel until observation. Electron micrographs were obtained using a scanning electron microscope (Leo EVO 40XVP-Carl Zeiss) at a working distance of 20 mm from the samples at the Electron Microscopy and Ultrastructural Analysis Laboratory (LME) at UFLA.

2.4.3 Molecular characterization of symbiotic bacteria

2.4.3.1 DNA extraction, PCR amplification, and Sequencing

DNA extraction and amplification from the isolated bacterial strains were performed using the modified methodology of Muangpat et al. (2020). A 48-h-old bacterial colony was inoculated into 3 mL of LB broth in a 15 mL Falcon tube, sealed with a cotton plug, and incubated in the dark at 28 °C and 180 rpm on an orbital shaker (Luca-222, Lucadema) for 24 h. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. A partial 890 bp region of the *recA* gene was amplified by Polymerase Chain Reaction (PCR) in a Unicycler thermocycler (Uniscience), using the primers *recA_F* (5'-GCTATTGATGAAAATAAACA-3') and *recA_R* (5'-RATTTTRTCWCCRTTRTAGCT-3'). The reaction was carried out in a final volume of 20 µL, containing 9 µL of GoTaq® G2 Green Master Mix, 8 µL of nuclease-free water, 1 µL of each primer (10 µM), and 1 µL of DNA. The amplification conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. The amplified products were visualized by electrophoresis on a 1.2% agarose gel at 100 V for 50 min, then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified products were quantified using 1 µL of sample in a NanoDrop One (Thermo Scientific). Sequencing the purified PCR products was performed at the René Rachou Institute (Fiocruz Minas).

2.4.3.2 Bioinformatics and Phylogenetic Analysis

Nucleotide sequences were edited and assembled using Sequencher™ software (<https://www.genecodes.com/>), which allowed refinement of the reads and generation of consensus sequences from four reads per strain (F and R from two samples). *Xenorhabdus* species were identified using BLASTN (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) against the NCBI nucleotide database. Sequences with $\geq 97\%$ similarity were considered to belong to the same species (Yimthin et al., 2021). Reference sequences for *Xenorhabdus* were downloaded from NCBI and, together with the sequences obtained in this study (Table S1), were converted to FASTA format using BioEdit v7.7 software (<https://bioedit.software.informer.com/>). Subsequently, the sequences were aligned and trimmed to 646 bp using Clustal W in Mega v12.1 software (<https://www.megasoftware.net/>) (Thanwisai et al., 2012).

The phylogenetic tree was constructed using the Maximum Likelihood (ML) method with the GTR+G substitution model, selected as the most suitable based on the corrected Akaike Information Criterion (AICc). Clade robustness was assessed using Bootstrap with 1000

replicates (Yimthin et al., 2021), using Mega 12.1 software. The phylogenetic tree was edited using iTOL v7 (Letunic; Bork, 2024).

2.4.4 *In vitro* and *in vivo* growth of symbiotic bacteria

In vitro growth of *Xenorhabdus* strains was evaluated using a modified methodology of Kim et al. (2017). After 24 h of growth, bacterial cultures in LB broth were adjusted to an optical density (OD) of 0.1 at 600 nm using a spectrophotometer (Multiskan SkyHigh, Thermo Scientific). Subsequently, 200 µL of bacterial culture was added to each well of a 96-well ELISA plate, with nine wells prepared for each *Xenorhabdus* species. The control treatment consisted of LB broth. The plate was incubated in the spectrophotometer at 25 °C with constant shaking for 19 h, and OD₆₀₀ was recorded every hour. The values obtained for each species were averaged and plotted with the standard error of the mean.

In vivo growth of *Xenorhabdus* strains was evaluated by inoculating fifth-instar *S. frugiperda* larvae. The larvae were pre-anesthetized by exposure to 4 °C for 3 min and injected with 10 µL of a bacterial cell suspension at 10⁵ CFU/mL using a 30G 5/16" needle and a disposable insulin syringe (1 mL). The injection was made into the 5th–6th lateral abdominal segment. Infected larvae were transferred to Petri dishes (Ø = 8 cm) containing filter paper and an artificial diet block (≈ 2 × 1 × 1 cm; length × width × height), and the dishes were sealed with Parafilm. The larvae were maintained under controlled conditions (25 ± 2 °C, relative humidity of 65 ± 10%, and a photoperiod of 12 h). To determine the number of colony-forming units (CFU) in the infected larvae, hemolymph was collected at 0, 6, 12, 24, and 48 h post-infection. Larvae were previously disinfected with 95% ethanol and sterile distilled water for 3 min each. Two larvae were used at each evaluation time. The hemolymph obtained was subjected to serial decimal dilutions (1:10) in sterile distilled water, and two consecutive dilutions were spread onto Petri dishes (Ø = 9 cm) with NBTA medium using a sterile glass rod. The plates were incubated for 3 days in a BOD at 28 °C in darkness. After incubation, viable colonies were counted using a colony counter, and the bacterial concentration was expressed as CFU/mL, calculated using the following equation (Martini et al., 2024):

$$\text{CFU/mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{planted volume (mL)}}$$

Bacterial counts were expressed as log₁₀(CFU/mL) for analysis and graphical representation.

Furthermore, the specific *in vitro* and *in vivo* growth rates were calculated from data collected during the exponential phase and at 6 h post-infection (HPI), respectively, using the following equation: $\ln(N_2/N_1) = k(t_2 - t_1)$, where N_1 is the number of CFU at the initial time (t_1) and N_2 represents the number of CFU recovered at the final time (t_2) (Kim et al., 2017).

2.5 Bioassay 3: Pathogenicity of symbiotic bacteria in *S. frugiperda* larvae

2.5.1 Obtaining bacterial cell suspensions and cell-free filtrate

Bacterial cell suspensions and cell-free filtrates were prepared according to the modified methodology of Kamou et al. (2024). Isolated 48-h-old *Xenorhabdus* colonies were inoculated into 40 mL of LB broth in 125 mL Erlenmeyer flasks and incubated in the dark for 96 h at 28 °C and 180 rpm on an orbital shaker, during which the OD at 625 nm ranged from 2.5 to 3.5. The cultures were centrifuged at 4000 rpm for 10 min to obtain bacterial cell suspensions. The resulting pellet was resuspended in sterile distilled water and adjusted to 10^8 CFU/mL ($OD_{625} = 0.7$) using a spectrophotometer (Multiskan SkyHigh, Thermo Scientific). To obtain the cell-free filtrate, the supernatant from the initial centrifugation was subjected to a second centrifugation at 8000 rpm for 10 min, then filtered through a 0.20 µm syringe filter. Finally, an aliquot of the suspensions was plated on NBTA agar plates to verify bacterial viability, while the supernatants were plated on the same medium to confirm the absence of cells.

2.5.2 Mortality of *S. frugiperda* in different concentrations of bacterial suspension

The entomopathogenic potential of the bacterial strains was evaluated using three concentrations of bacterial cell suspensions (low, medium, and high). The stock suspension (10^8 CFU/mL- high) was diluted in sterile distilled water to obtain final concentrations of 10^5 (medium) and 10^3 (low) CFU/mL. A block of artificial diet ($\approx 2 \times 1 \times 1$ cm; length $\times$ width $\times$ height) was placed in Petri dishes ($\varnothing = 8$ cm) lined with filter paper. Fifth-instar larvae of *S. frugiperda* were anesthetized by exposure to 4 °C for 3 min and then injected with 10 µL of each bacterial suspension concentration (Machado et al., 2024; Thanwisai et al., 2024) into the 5th-6th lateral abdominal segment using a 30G 5/16" needle and a disposable insulin syringe (1 mL). After injection, the larvae were transferred to the corresponding Petri dishes with diet, which were sealed with Parafilm. The control treatment followed the same procedure and consisted of sterile distilled water. The experiment was performed with 40 repeats, and larval mortality was assessed every 24 h for 72 h. Larvae were considered dead in the absence of movement when stimulated by contact with a needle (Jung; Kim, 2006). During the experiment,

larvae were maintained under controlled conditions (25 ± 2 °C, relative humidity of $65 \pm 10\%$, and a photoperiod of 12 h).

To confirm that mortality was caused by bacteria, nine additional larvae were injected with each concentration of the bacterial cell suspension, following the same procedure described previously. During each evaluation period, hemolymph was extracted from three larvae that died after treatment. The hemolymph was plated onto Petri dishes ($\varnothing = 9$ cm) containing NBTA medium and incubated at 28 °C in a BOD incubator in the dark. Bacterial growth was evaluated after four days of incubation.

2.5.3 Mortality of *S. frugiperda* by injection

The pathogenicity of bacterial cell suspensions (10^8 CFU/mL) and their cell-free filtrates (100 %) was evaluated by injecting them into fifth-instar *S. frugiperda* larvae. A filter paper and an artificial diet block ($\approx 2 \times 1 \times 1$ cm; length $\times$ width $\times$ height) were placed in Petri dishes ($\varnothing = 8$ cm). The larvae were anesthetized by exposure to 4 °C for 3 min. Each larva was injected with 10 μ L of the treatment (Machado et al., 2024; Thanwisai et al., 2024) into the 5th-6th lateral abdominal segment using a 30G 5/16" needle and a disposable insulin syringe (1 mL), and then transferred to the corresponding Petri dish with diet, which was sealed with Parafilm. Control treatments followed the same procedure and consisted of sterile distilled water and filtered LB broth. Each treatment was performed with 40 repeats (larvae), and larval mortality was assessed every 24 h for 72 h. Larvae were considered dead in the absence of movement when stimulated by contact with a needle (Jung; Kim, 2006). Larvae were maintained under controlled conditions (T° : 25 ± 2 °C, RH: $65 \pm 10\%$, and a photoperiod of 12 h).

Additionally, nine larvae were injected into each treatment group, using the same procedure described previously, with the bacterial cell suspension and the LB broth control to confirm that mortality was caused by bacteria. During each evaluation period, hemolymph was collected from three larvae that died after the suspensions were applied. In the control treatment, hemolymph was extracted from live and/or dead larvae to verify the absence of bacteria. The hemolymph was plated onto Petri dishes ($\varnothing = 9$ cm) containing NBTA medium and incubated in a BOD at 28 °C in the dark. Bacterial growth was evaluated after four days of incubation.

2.5.4 Mortality of *S. frugiperda* via oral route (ingestion)

The pathogenicity of bacterial cell suspensions (10^8 CFU/mL) and their cell-free filtrates (100%) was evaluated by oral administration in second-instar *S. frugiperda* larvae, a stage

known to be more susceptible, following a modified methodology described by Kamou et al. (2024). Young maize leaves used for larval feeding/rearing were collected from the MEET-UFLA greenhouse and washed beforehand. Petri dishes ($\varnothing = 5$ cm) with filter paper were used, and a piece of maize leaf ($\approx 5 \times 2$ cm; length $\times$ width) was sprayed on both sides with approximately 2 mL of bacterial suspensions or their filtrates and placed in a dish. A piece of moistened cotton was placed at the edge of each leaf to prevent dehydration. A larva was introduced into each dish, and the dishes were sealed with Parafilm. Control treatments followed the same procedure and consisted of sterile distilled water and filtered LB broth. Treatments were reapplied up to two additional times to leaves that showed desiccation during the bioassay. The experiment was conducted with 30 repeats (larvae), and larval mortality was assessed every 24 h for 96 h. Larvae were considered dead in the absence of movement when stimulated by contact with a needle (Jung; Kim, 2006). During the experiment, larvae were maintained under controlled conditions (25 ± 2 °C, relative humidity of $65 \pm 10\%$, and a photoperiod of 12 h).

At the end of the evaluation period, hemolymph was extracted from three dead larvae from the bacterial suspension treatment and plated onto Petri dishes ($\varnothing = 9$ cm) containing NBTA medium to confirm that mortality was caused by bacteria. The plates were incubated in a BOD at 28 °C in the dark, and bacterial growth was assessed after 4 days.

2.6 Statistical analysis

The assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Data on *in vitro* growth and pathogenicity of nematodes and bacteria were analyzed using two-way analysis of variance (ANOVA) (factors: time and treatment). Nonparametric tests were used when the assumptions were not met. Subsequently, multiple pairwise comparisons were performed using the Holm-Sidak post hoc test at the 0.05 significance level. Mortality data over time in the injection and oral trials were subjected to regression analysis. The most appropriate model was selected based on parsimony, the lowest standard errors, and a pronounced increase in R^2 with model complexity. Regression analysis was performed using curve fitting. Statistical analyses and figures were generated using SigmaPlot 14 (Systat Software Inc., San Jose, CA, USA).

3 RESULTS

3.1 Bioassay 1: Pathogenicity of entomopathogenic nematodes

Larval mortality of *S. frugiperda* differed significantly between *Heterorhabditis* and *Steinernema* isolates ($F = 175.44$; $df = 5$; $p < 0.001$) during the different evaluation period ($F = 157.00$; $df = 3$; $p < 0.001$). All isolates caused higher mortality than the control ($p < 0.001$) and reached $\geq 80\%$ mortality at 48 h, after which no significant differences were observed between treatments. For each EPN isolate, significant differences were observed between the 24 h evaluation and those at 48, 72, and 96 h ($p < 0.001$). At 24 h, no significant differences were observed between the control and *H. bacteriophora* (HB), in which no mortality was recorded; likewise, the isolates *H. amazonensis* NEPET 11 (HA Nepet) and *H. amazonensis* RSC05 (HA RSC-05) did not differ significantly from each other, presenting mortality rates of $23.30 \pm 6.50\%$ and $36.70 \pm 8.50\%$, respectively (Figure 1).

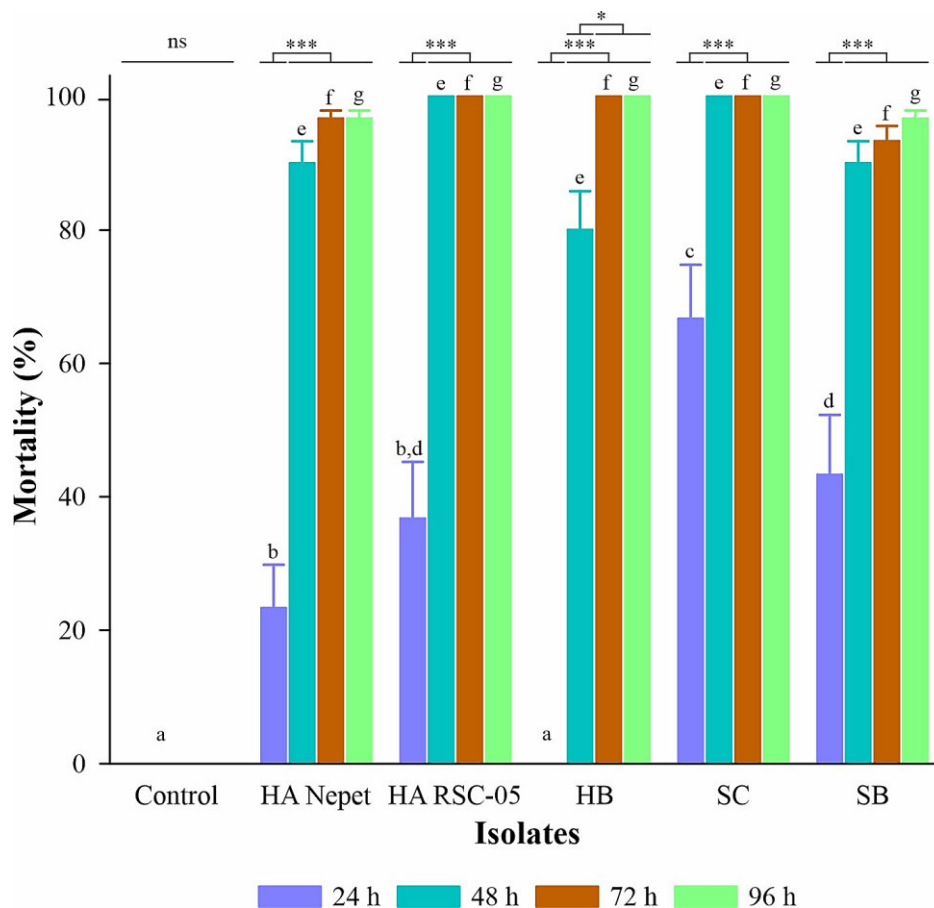


Figure 1. Mortality (%) caused by different EPN isolates in fifth-instar *Spodoptera frugiperda* larvae at different time intervals. HA Nepet = *Heterorhabditis amazonensis* NEPET 11, HA RSC-05 = *Heterorhabditis amazonensis* RSC05, HB = *Heterorhabditis bacteriophora*, SC = *Steinernema carpocapsae* All, and SB = *Steinernema brazilense*. Asterisks (* $p < 0.05$; *** $p < 0.001$).

0.001) indicate significant differences between time points within the same treatment. Different lowercase letters indicate significant differences at the same time between treatments, while “ns” indicates no significant differences, according to the Holm-Sidak test.

After 7 days of exposure, larval mortality was confirmed to be caused by EPN, as IJ were observed in all isolates that invaded insect corpses (Figure 2). No mortality or EPN were detected in the control treatment.

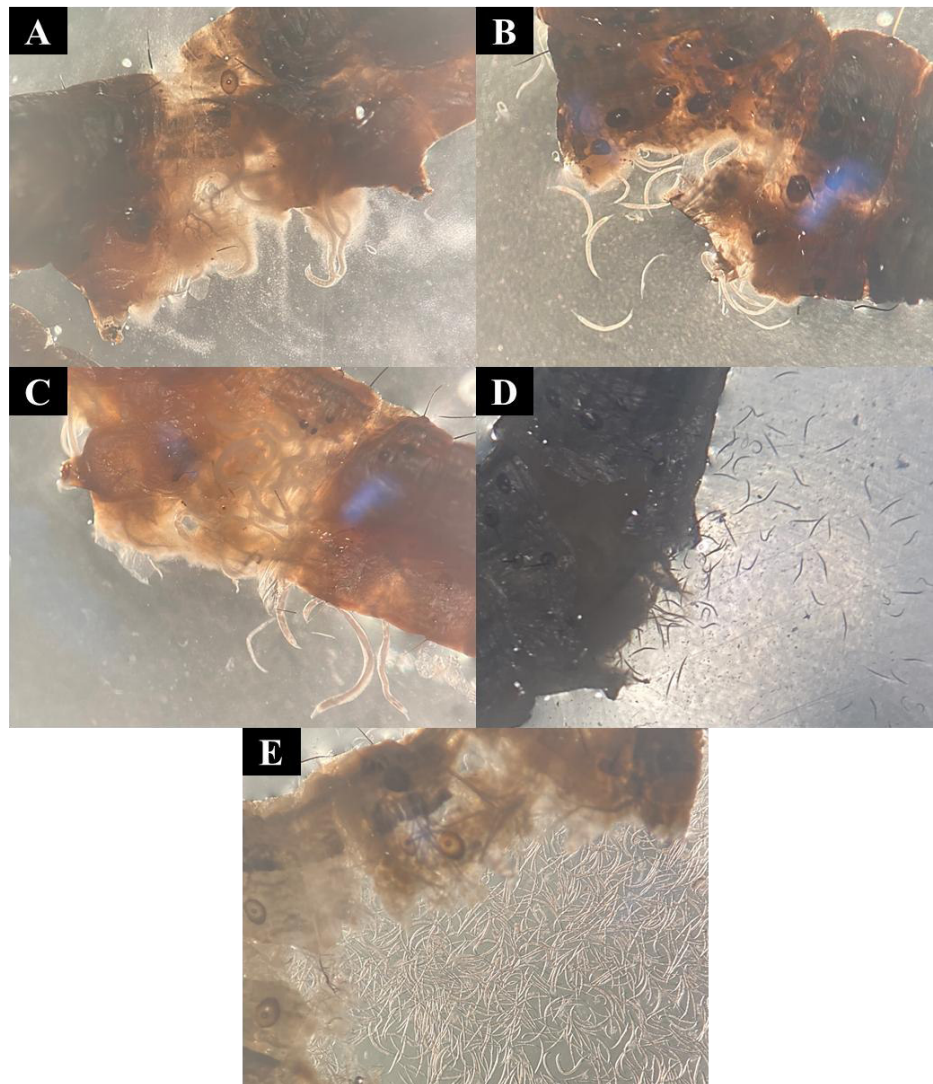


Figure 2. Images of EPN presence in fifth-instar *Spodoptera frugiperda* larvae after 7 days of exposure to *Heterorhabditis amazonensis* NEPET 11 (A), *Heterorhabditis amazonensis* RSC05 (B), *Heterorhabditis bacteriophora* (C), *Steinernema carpocapsae* All (D), and *Steinernema brazilense* (E).

3.2 Bioassay 2. Isolation and characterization of symbiotic bacteria associated with entomopathogenic nematodes *S. carpocapsae* and *S. brazilense*

3.2.1 Phenotypic characterization of symbiotic bacteria

For each nematode species, bacterial strains were isolated and named bsSC and bsSB, corresponding to *S. carpocapsae* All and *S. brazilense*, respectively. Both strains exhibited similar characteristics when cultured on NBTA medium. After 4 days of growth, the colonies were circular and convex, with slightly irregular margins and a smooth surface. Colony size ranged from 0.85 to 3.00 mm for bsSC and from 0.90 to 3.20 mm for bsSB. The colonies adsorbed bromothymol blue, displaying a dark blue or green coloration surrounded by a clear zone, characteristic of the primary phase (Table 1; Figure 2). Both strains were Gram-negative, exhibiting rod-shaped cells (Table 1; Figures 3 and 4). They were also negative for the catalase test, as evidenced by the absence of bubble formation (Table 1).

Table 1. Phenotypic characteristics of symbiotic bacteria isolated from *Steinernema carpocapsae* All and *Steinernema brazilense*.

Characteristics	bsSC	bsSB
Colony size (mm)	0.85-3.00	0.90-3.20
Color	Blue or dark green	Blue or dark green
Shape	Circular	Circular
Elevation	Convex	Convex
Texture	Smooth	Smooth
Margin	Slightly irregular	Slightly irregular
Surface	Shiny	Shiny
Density	Translucent	Translucent
Gram staining	-	-
Catalase	-	-

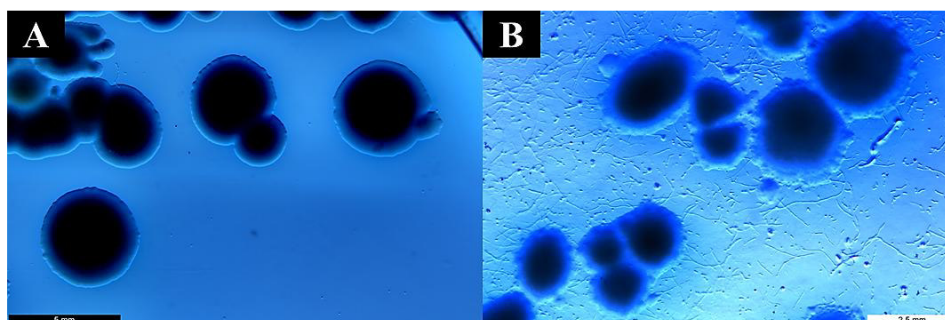


Figure 2. Images of bacterial colonies obtained with a stereomicroscope: (A) bsSC and (B) bsSB.

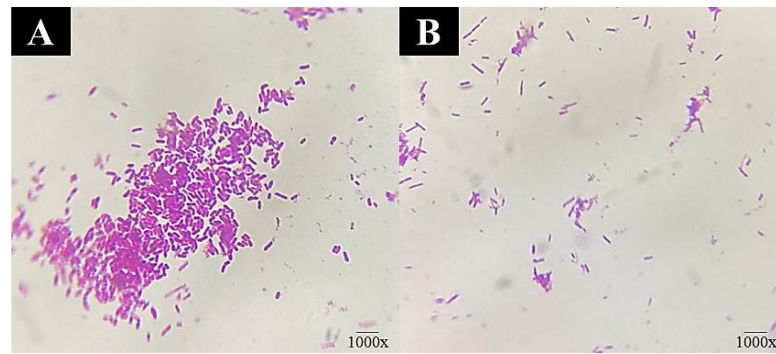


Figure 3. Gram stain of the isolated symbiotic bacteria at 1000x: (A) bsSC and (B) bsSB.

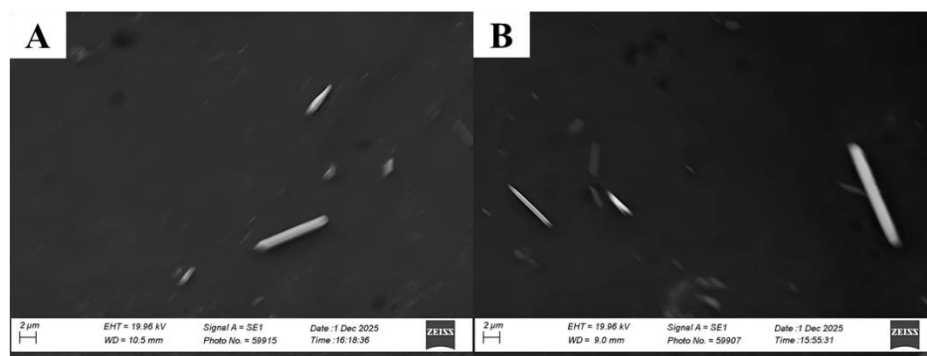


Figure 4. Scanning electron microscopy images of bacterial cells from the isolated strains: (A) bsSC and (B) bsSB. Scale bar = 2 µm.

3.2.2 Molecular characterization of symbiotic bacteria

The products amplified using the *recA* gene were approximately 900 bp in size for both bacterial strains. BLASTN analysis of a partial region of the *recA* gene identified the bsSC strain as *Xenorhabdus nematophila* ATCC 19061 (100% identity and coverage) and the bsSB strain as *X. doucetiae* LWD1 (100% identity and coverage).

Phylogenetic analysis of 646 bp of the *recA* region showed that the bsSC strain clustered with *X. nematophila*, including the type strain ATCC 19061, with high bootstrap support (100% and 94%). In contrast, the bsSB strain clustered with the *X. doucetiae* strain LWD1, with moderate bootstrap support (63%), clearly distinct from the *X. nematophila* clade (Figure 5).

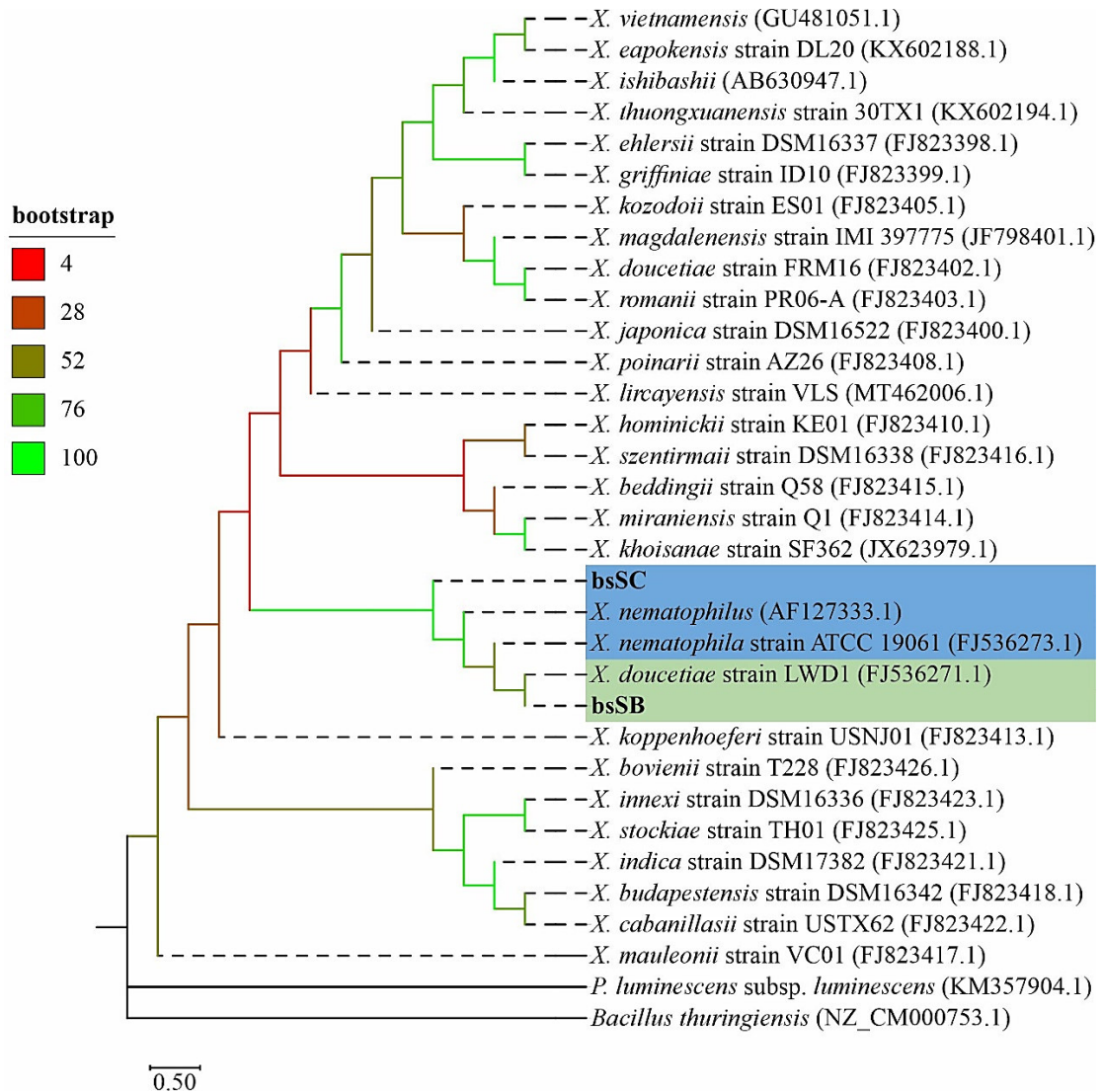


Figure 5. Maximum-likelihood tree of *Xenorhabdus* bacterial strains based on 646 bp of the *recA* region, including sequences downloaded from NCBI. Bootstrap values are based on 1000 replicates. The bar indicates 0.50 nucleotide substitutions per sequence position.

3.2.3 *In vitro* and *in vivo* growth of symbiotic bacteria

The growth curves of the *Xenorhabdus* strains in LB broth differed significantly from one another and from the control ($p < 0.001$), as determined by multiple pairwise comparisons using the Holm-Sidak method. *Xenorhabdus nematophila* (bsSC) showed clear exponential growth, beginning at approximately 7 h of incubation, reaching maximum OD₆₀₀ values between 14 and 16 h, and then declining. In contrast, *X. doucetiae* (bsSB) maintained virtually constant OD₆₀₀ values throughout the evaluation period, indicating limited growth and a slower specific growth rate ($\mu = 0.015 \pm 0.003 \text{ h}^{-1}$) than *X. nematophila* (bsSC) ($\mu = 0.128 \pm 0.003 \text{ h}^{-1}$) during the exponential phase. The control LB broth did not show any increase in OD₆₀₀.

Multiple pairwise comparisons using the Holm-Sidak method showed that differences in OD₆₀₀ between the bacterial strains became significant at 12 h ($p < 0.05$) and remained significant until the final evaluation time (Figure 6).

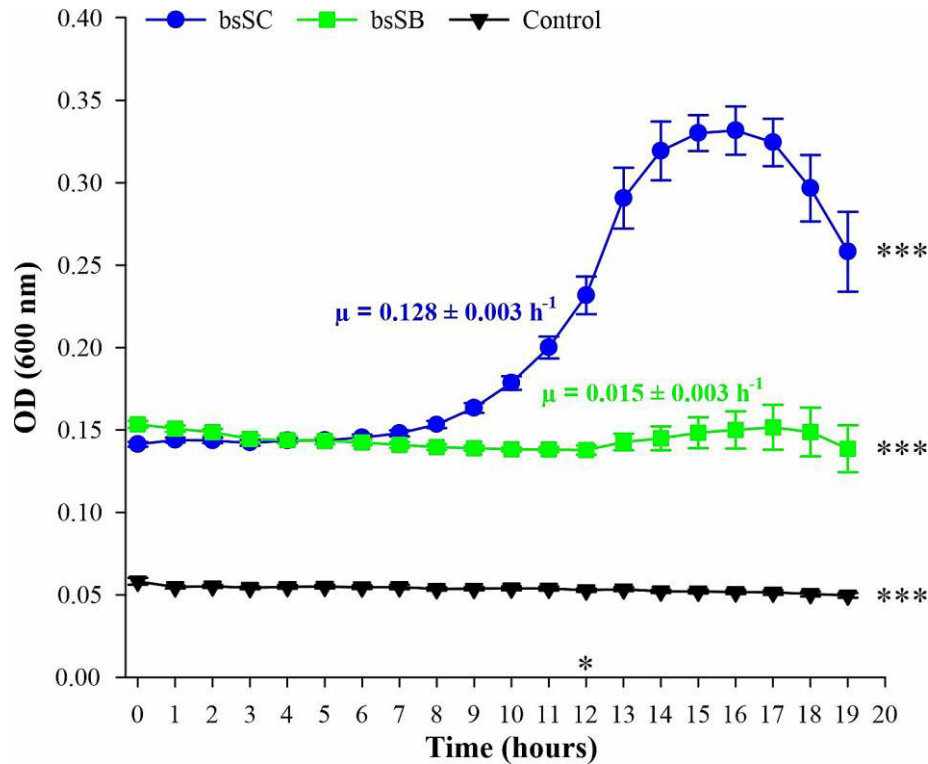


Figure 6. *In vitro* growth of the bacterial strains bsSC (*Xenorhabdus nematophila*) and bsSB (*Xenorhabdus doucetiae*) in LB broth for 19 h. The asterisk (*) indicates significant differences between strains at the same time point ($p < 0.05$), and (***) indicates significant differences between strains ($p < 0.001$) according to the Holm-Sidak test. The μ values indicate the specific growth rate, calculated during the exponential phase.

In vivo bacterial growth dynamics differ temporarily between *X. nematophila* (bsSC) and *X. doucetiae* (bsSB). During the first 6 h post-infection (HPI), both bacteria increased in density, with *X. nematophila* (bsSC) exhibiting a higher specific growth rate ($\mu = 1.129 \text{ h}^{-1}$) than *X. doucetiae* (bsSB) ($\mu = 0.882 \text{ h}^{-1}$). At 12 HPI, *X. nematophila* (bsSC) reached 6.75×10^8 CFU/mL, whereas *X. doucetiae* (bsSB) showed lower growth at 7.00×10^7 CFU/mL. However, at 48 h post-infection, *X. doucetiae* (bsSB) surpassed *X. nematophila* (bsSC), reaching 6.25×10^{10} CFU/mL, whereas *X. nematophila* (bsSC) remained at 8.00×10^9 CFU/mL (Figure 7).

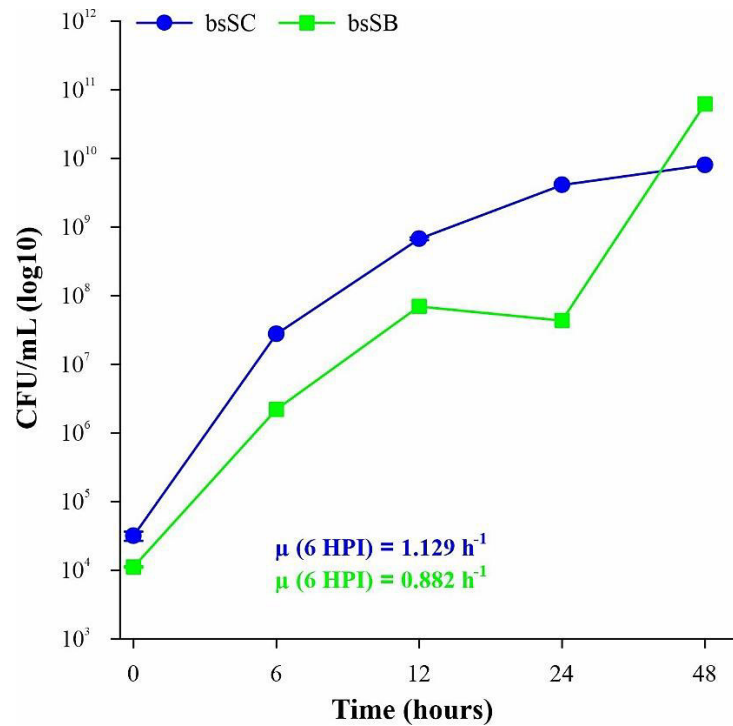


Figure 7. *In vivo* growth of the bacterial strains bsSC (*Xenorhabdus nematophila*) and bsSB (*Xenorhabdus doucetiae*) in infected *Spodoptera frugiperda* larvae, expressed as CFU/mL (log10) at different hours post-infection (HPI). The μ values indicate the specific growth rate calculated at 6 HPI.

3.3 Bioassay 3: Pathogenicity of symbiotic bacteria in *S. frugiperda* larvae

3.3.1 Mortality of *S. frugiperda* in different concentrations of bacterial suspension

The pathogenicity of the bacteria *X. nematophila* (bsSC) and *X. doucetiae* (bsSB) was affected by both concentration and time (Figure 8). *Xenorhabdus nematophila* (bsSC; Figure 8A) showed a dose-dependent significant difference among treatments after 72 hours (two-way ANOVA: $F = 67.77$; $df = 3$; $p < 0.001$). Multiple pairwise comparisons using the Holm-Sidak method showed that high and medium concentrations differed significantly from each other and from the control ($p < 0.001$), except for the 10^3 CFU/mL (low) concentration, which did not differ from the control ($p = 0.071$) at any assessment times. Larval mortality increased significantly with bacterial concentration, reaching $82.50 \pm 4.60\%$ at 48 and 72 h at 10^8 CFU/mL, with a significant difference between 24 ($60 \pm 7.60\%$) and 72 h ($p = 0.039$). The 10^5 CFU/mL concentration caused moderate mortality of $52.50 \pm 7.90\%$ at 72 h, whereas at the lowest concentration (10^3 CFU/mL), mortality was limited (less than 20%) and similar to the control ($5 \pm 1.50\%$).

In *X. doucetiae* (bsSB; Figure 8B), mortality differed significantly between treatments (two-way ANOVA: $F = 49.08$; $df = 3$; $p < 0.001$) and over time (two-way ANOVA: $F = 4.46$; $df = 2$; $p = 0.014$), with significant differences between 24 and 48 h ($p = 0.038$) and between 24 and 72 h ($p = 0.022$), as determined by multiple pairwise comparisons using the Holm-Sidak method. All concentrations differed significantly from the control ($p < 0.001$). The 10^8 CFU/mL concentration resulted in $80 \pm 5.10\%$ mortality at 48 h, while the 10^5 and 10^3 CFU/mL concentrations showed intermediate effects, with mortality rates of $67.50 \pm 6.90\%$ and $62.50 \pm 7.40\%$ at 72 h, respectively. In both cases, the control group showed no significant increase in mortality being around $5 \pm 1.50\%$ at 72 hours.

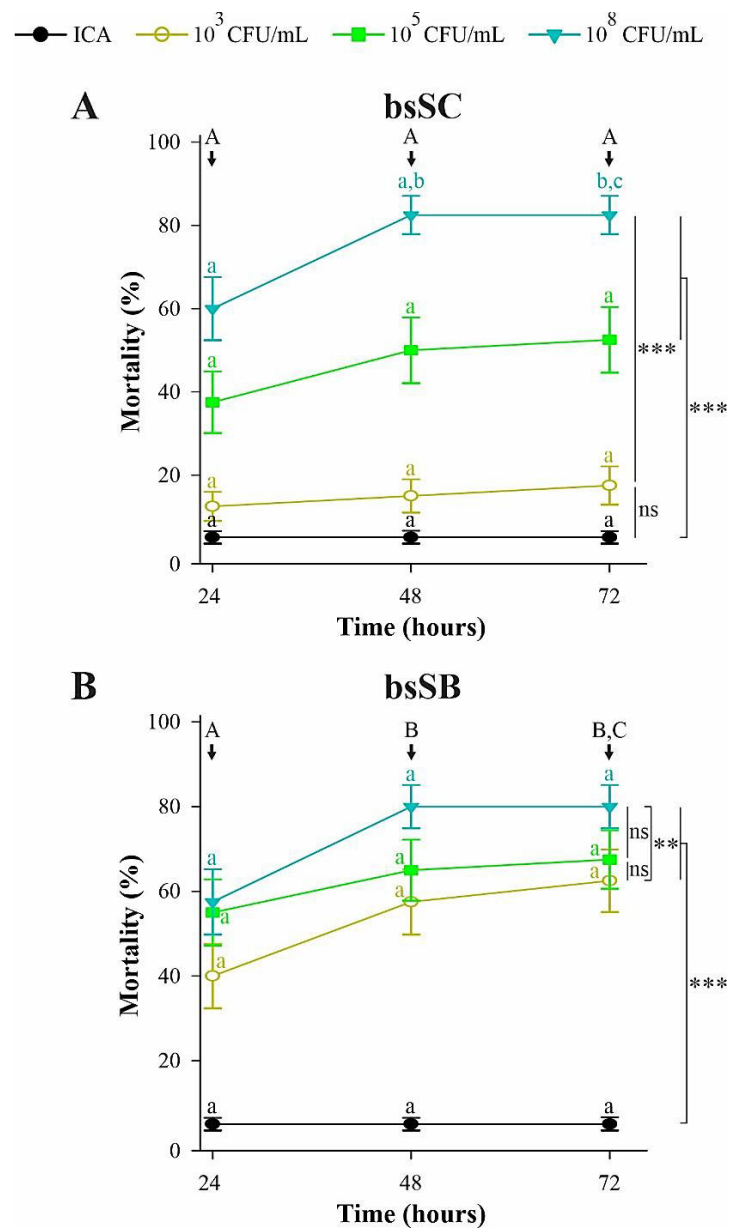


Figure 8. Mortality (%) of *Spodoptera frugiperda* larvae exposed to different concentrations of *Xenorhabdus* bacterial cell suspensions. (A) *Xenorhabdus nematophila* bsSC and (B)

Xenorhabdus doucetiae bsSB, evaluated at different time intervals. The control treatment was ICA. Asterisks (** $p < 0.01$; *** $p < 0.001$) indicate significant differences between treatments. Different capital letters indicate significant differences between time points, while different lowercase letters indicate significant differences between time points within the same treatment. “ns” indicates no significant differences according to the Holm-Sidak test.

The injection of bacterial cell suspensions into *S. frugiperda* larvae was verified by plating on NBTA agar, where bacterial behavior was observed (Figure 9). At a concentration of 10^3 CFU/mL, both *X. nematophila* bsSC (Figure 9A) and *X. doucetiae* bsSB (Figure 9B) showed reduced growth, with colonies of the secondary phase, which are red in color, predominating. By 48 h post-injection, growth of primary-phase bacteria, which are associated with virulence expression, was observed.

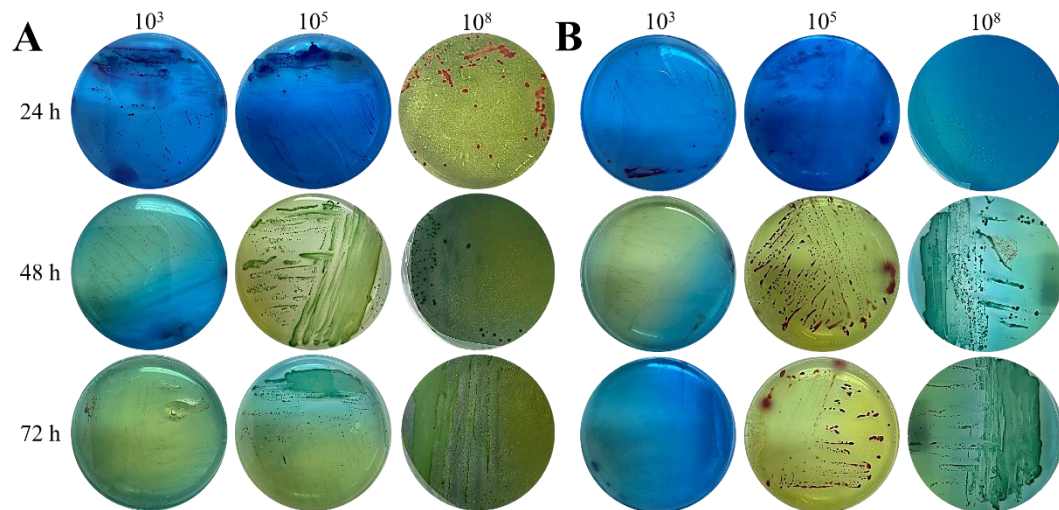


Figure 9. Bacterial growth of *Xenorhabdus* in NBTA medium over the evaluation periods after injection of different concentrations into *Spodoptera frugiperda* larvae. (A) *Xenorhabdus nematophila* bsSC and (B) *Xenorhabdus doucetiae* bsSB.

3.3.2 Mortality of *S. frugiperda* by injection

Results from injecting bacterial suspension (IB) and cell-free supernatant (IS) showed significant differences in treatments (two-way ANOVA: $F = 83.34$; $df = 3$; $p < 0.001$) and in time (two-way ANOVA: $F = 4.08$; $df = 2$; $p = 0.020$) for *X. nematophila* (bsSC; Figure 10A). The difference between 24 and 72 h was significant ($p = 0.028$), as indicated by multiple pairwise comparisons using the Holm-Sidak method. IB treatment caused the highest mortality rates, reaching values above 80% at 48 h, with significant differences compared with the other treatments ($p < 0.001$). IS mortality was initially low but increased progressively over time.

Similarly, in *X. doucetiae* (bsSB; Figure 10B), there was a significant difference among treatments (two-way ANOVA: $F = 54.30$; $df = 3$; $p < 0.001$). IB treatment was the most effective, achieving 80% mortality at 48 h, with a statistically significant difference from the other treatments ($p < 0.001$), as indicated by multiple pairwise comparisons using the Holm-Sidak method. Treatment IS showed a gradual increase in mortality over time.

The ICA (water) and IC (LB broth) controls showed no significant increase in mortality and did not differ significantly from one another (Figure 10).

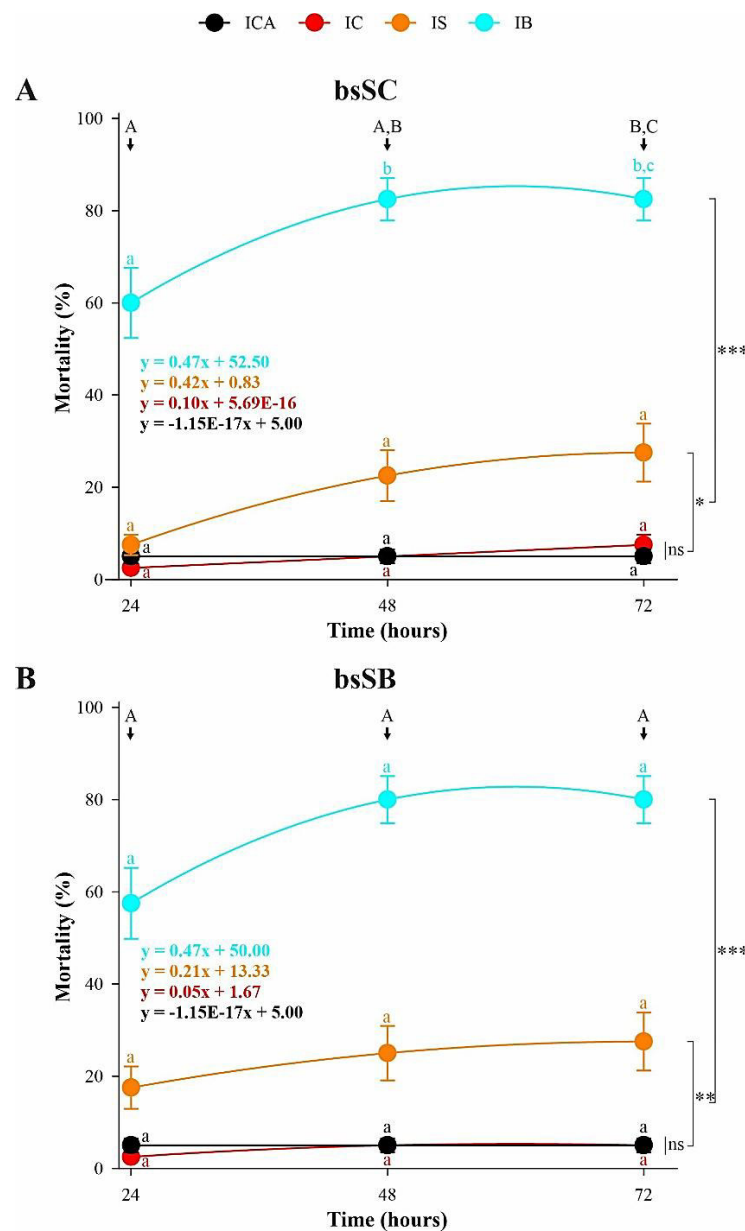


Figure 10. Mortality (%) of *Spodoptera frugiperda* larvae injected with different bacterial preparations of *Xenorhabdus*. (A) *Xenorhabdus. nematophila* bsSC and (B) *Xenorhabdus doucetiae* bsSB, evaluated at different time intervals. Treatments included: ICA (water control),

IC (LB broth control), IS (cell-free filtrate), and IB (bacterial cell suspension). Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant differences between treatments. Different capital letters indicate significant differences between time points, while different lowercase letters indicate significant differences between time points within the same treatment. “ns” indicates not significant according to the Holm-Sidak test. The equation represents the model from the nonlinear regression analysis.

It was also confirmed that the bacterial cell suspension was successfully inoculated into *S. frugiperda* larvae and that these bacteria were responsible for the observed mortality (Figure 11). During the first 24 h post-injection, the predominant bacterial growth corresponded to the secondary phase, whereas at 48 and 72 h, growth of primary-phase colonies was evident. In the control treatment, no growth of bacteria with morphologies characteristic of the genus *Xenorhabdus* was observed.

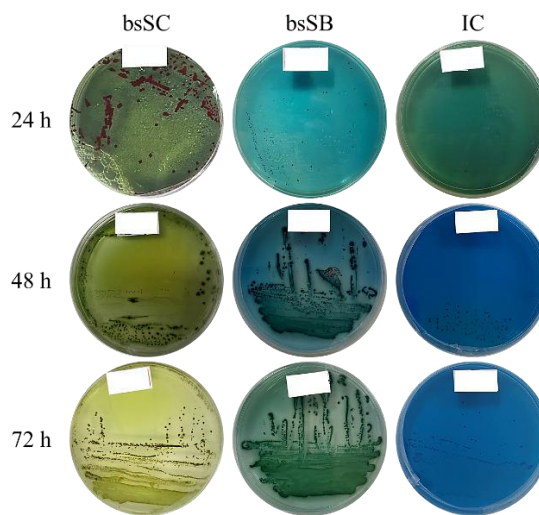


Figure 11. Bacterial growth of *Xenorhabdus* in NBTA medium over the evaluation periods after injection of the bacterial cell suspension into *Spodoptera frugiperda* larvae. bsSC = *Xenorhabdus nematophila*, and bsSB = *Xenorhabdus doucetiae*.

3.3.3 Mortality of *S. frugiperda* via oral route

In *X. nematophila* (bsSC; Figure 12A), larval mortality induced by the different oral treatments differed significantly by treatment type (two-way ANOVA: $F = 29.78$; $df = 3$; $p < 0.001$) and evaluation time (two-way ANOVA: $F = 23.16$; $df = 3$; $p < 0.001$). All time intervals differed statistically from each other, except for 24 and 48 h ($p = 0.061$), as determined by multiple pairwise comparisons using the Holm-Sidak method. The bacterial suspension (OB)

and cell-free supernatant (OS) treatments produced a progressive increase in mortality over time, reaching $36.70 \pm 8.50\%$ and $33.30 \pm 8.10\%$ at 96 h, respectively.

Similarly, *X. doucetiae* (bsSB; Figure 12B) showed significant differences between treatments (two-way ANOVA: $F = 13.88$; $df = 3$; $p < 0.001$) and over time (two-way ANOVA: $F = 8.87$; $df = 3$; $p < 0.001$). The 96-h evaluation differed significantly from that observed at 24 and 48 h ($p < 0.001$), as indicated by multiple pairwise comparisons using the Holm-Sidak method. The OB treatment produced the highest mortality rate of $46.70 \pm 9.10\%$ at 96 h. The OS treatment did not differ significantly from the controls.

The OCA (water) and OC (LB broth) controls maintained minimal mortality, with no significant differences between them (Figure 12).

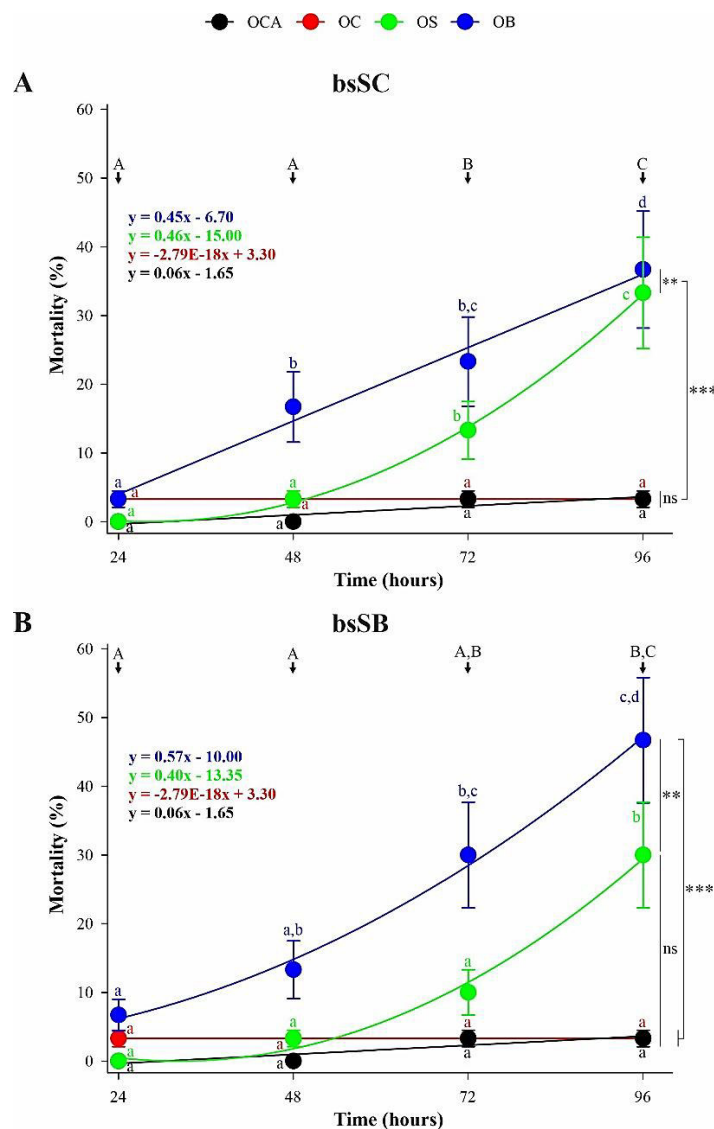


Figure 12. Mortality (%) of *Spodoptera frugiperda* larvae after oral administration of different bacterial preparations of *Xenorhabdus*. (A) *Xenorhabdus nematophila* bsSC and (B)

Xenorhabdus doucetiae bsSB, evaluated at different time intervals. Treatments included: OCA (water control), OC (LB broth control), OS (cell-free filtrate), and OB (bacterial cell suspension). Asterisks (** $p < 0.01$; *** $p < 0.001$) indicate significant differences between treatments. Different capital letters indicate significant differences between time points, while different lowercase letters indicate significant differences between time points within the same treatment. “ns” indicates not significant according to the Holm-Sidak test. The equation represents the model from the nonlinear regression analysis.

The growth of bacterial colonies in NBTA medium confirmed that *S. frugiperda* larvae ingested bacteria while feeding on treated maize leaves. After 96 h, secondary-stage colonies were observed (Figure 13).

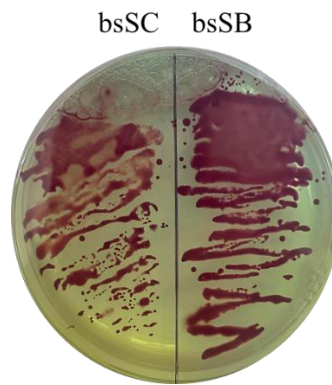


Figure 13. Bacterial growth of *Xenorhabdus* in NBTA medium after oral application of the bacterial cell suspension to *S. frugiperda* larvae. bsSC = *Xenorhabdus nematophila*, and bsSB = *Xenorhabdus doucetiae*, observed 96 h post-application.

4 DISCUSSION

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are highly relevant biological control agents, whose interest and application have increased considerably in recent years (Shinde et al., 2022). Their effectiveness stems from their ability to infect and kill various pests, especially lepidopteran larvae (Adrianzén et al., 2024). These nematodes are obligate pathogens (Kazimierczak et al., 2018; Khashaba et al., 2020) that establishes a mutualistic symbiosis with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Wattanachaiyingcharoen et al., 2021; Shinde et al., 2022; Yüksel, 2022). In this system, nematodes act as specialized vectors that release the bacteria into the host insect's hemocoel, while the bacteria confer virulence by producing immunosuppressive factors, including toxin complexes, hemolysins, hydrolytic enzymes, and antimicrobial compounds (Mohamed;

Shairra, 2023; Adrianzén et al., 2024 González et al., 2025;). In this context, the present work addresses the entomopathogenic potential of symbiotic bacteria of the genus *Xenorhabdus* associated with entomopathogenic nematodes by characterizing them and evaluating their growth and pathogenicity against *Spodoptera frugiperda*.

The first step in developing biological control programs based on EPN is to evaluate different isolates against a target insect (Ndereyimana et al., 2019). In this regard, Wattanachaiyingcharoen et al. (2021) reported high pathogenicity of *H. indica* (AUT 13.2) and *S. siamkayai* (APL 12.3) against second- and fifth-stage larvae of *S. frugiperda*, while Yuksel and Canhilal (2019) observed mortalities between 63 and 100% at 48 h after application of 100 IJ/mL in larvae of *Galleria mellonella*.

For *H. bacteriophora*, González et al. (2025) reported 25% and 75% mortality at 72 h in fourth- or fifth-instar of *S. frugiperda* larvae exposed to 400 and 1200 IJ/larva, respectively. These results differ from those of the present study, in which 1000 IJ/mL caused total mortality at 72 h; however, they coincide on the onset of mortality, observed at 48 h. This delayed action pattern was also described by Ndereyimana et al. (2019) when 500 IJ/mL were applied to third-instar of *Tuta absoluta* larvae. In agreement, Acharya et al. (2020) reported a prolonged median lethal time (LT₅₀) of 51 h for fifth-stage larvae of *S. frugiperda*.

On the other hand, *S. carpocapsae* has been consistently reported as highly virulent against *S. frugiperda*. Acharya et al. (2020) recorded total mortality at 72 h with an LT₅₀ of 25 h in fifth-instar larvae, while Sayed et al. (2022) observed 77.8% mortality at 96 h with 80 IJ/mL. In this study, *S. carpocapsae* acted more quickly, reaching more than 65% mortality at 24 h and 100% at 48 h.

There are few reports on *H. amazonensis* and *S. brazilense* against *S. frugiperda*. Cecconello (2024) observed mortalities of 90% at 72 h in fifth-instar larvae of *S. cosmioides* exposed to 200 IJ/larva, while Souza et al. (2012) confirmed the pathogenicity of both species in *S. frugiperda*. These results are consistent with those obtained in this study, in which HA Nepet and HA RSC-05 achieved mortalities greater than 96%, and *S. brazilense* showed high mortality, although later. The absence of significant differences between *H. amazonensis* strains coincides with previous reports (Ndereyimana et al., 2019) and could be related to the presence of the same symbiotic bacteria (Yüksel, 2022; Erdem; Yüksel; Canhilal, 2024).

Variations in observed mortality can be attributed to nematode species and strain, as well as to foraging strategy. Species with a "cruiser" strategy, such as *H. amazonensis* and *S.*

carpocapsae, tend to act more quickly, whereas species with an "ambusher" strategy, such as *H. bacteriophora*, show a later onset of pathogenicity (Andaló; Moreira; Moino, 2017).

Overall, the susceptibility of *S. frugiperda* depends on the EPN species, the concentration of IJ, the host species, and the host stage (Acharya et al., 2020; Fallet et al., 2022; Sayed; Ibrahim; El-Gepaly, 2022; Yüksel, 2022). Higher concentrations of IJ generally induce faster mortality because they release more symbiotic bacteria and, consequently, increase bacterial activity (Ndereyimana et al., 2019; Adrianzén et al., 2024). Furthermore, some EPN species show a preference for certain host stages (Acharya et al., 2020), which may be related to the insect's size, body morphology, behavior, and immune defense mechanisms (Mohamed; Shairra, 2023). Host size is a key factor in pathogenicity, as IJ primarily enter through natural openings in the body (Yüksel, 2022). The high efficacy and rapid action observed support the potential of IJ as competitive alternatives to conventional fast-acting insecticides (Ndereyimana et al., 2019).

Based on this integrated system, the present study focused on isolating and characterizing the symbiotic bacteria associated with *S. carpocapsae* and *S. brazilense*. To our knowledge, this is the first study to report symbiotic bacteria associated with *S. brazilense*. Both strains exhibited phenotypic characteristics typical of the genus *Xenorhabdus*, including circular, convex colonies with a smooth texture and blue-green coloration on NBTA medium, a Gram-negative profile, and the absence of catalase activity, which coincides with previous descriptions of symbiotic bacteria in the primary phase (Tailliez et al., 2012; Kuwata et al., 2013; Fukruksa et al., 2017; Pervez; Lone; Pattnaik, 2020; Kamou et al., 2024; Thanwisai et al., 2024). The size of the bacterial colonies ranged from 0.85 to 3.20 mm after 4 days of growth on NBTA medium, values consistent with those reported for species of the genus *Xenorhabdus*. Similarly, Kuwata et al. (2013) recorded diameters of 0.8–2.0 mm in *X. ishibashii* colonies after 3 days of growth on nutrient agar, while Thanwisai et al. (2024) reported diameters between 0.3 and 0.8 cm for *X. bharatensis* at 48 h, as well as similar ranges for *X. entomophaga* and *X. siamensis* after 5–7 days of incubation on LB agar. The variations observed between studies may be attributed to differences in culture medium and incubation time.

Molecular identification and phylogenetic analysis of the *recA* gene indicated that the bacteria isolated from *S. carpocapsae* and *S. brazilense* were similar to *X. nematophila* and *X. doucetiae*, respectively. The *recA* gene has been widely used for molecular characterization because of its high phylogenetic resolution within the genus *Xenorhabdus* (Muangpat et al., 2020; Yimthin et al., 2021; Subkrasae et al., 2022; Erdem; Yüksel; Canhilal, 2024; Kamou et

al., 2024;). The association of *S. carpocapsae* with *X. nematophila* is well documented, supporting the identification obtained in this study. In contrast, *X. doucetiae* has been described mainly as a symbiont of *S. diaprepesi*, a species belonging to the “glaseri” group, to which *S. brazilense* also belongs (Půža; Machado, 2024). This phylogenetic relationship among nematodes explains the observed bacterial association. Furthermore, it has been shown that a single bacterial species can establish symbiosis with different nematode species, which reinforces the validity of the results obtained (Kuwata; Yoshida; Yoshiga, 2017).

In vitro growth curves in LB broth revealed contrasting strategies among the evaluated strains. *Xenorhabdus nematophila* (bsSC) showed a well-defined exponential phase and a higher specific growth rate (μ), whereas *X. doucetiae* (bsSB) showed limited growth and a lower μ . Similar results were reported by Kim et al. (2017), who observed greater growth of *X. nematophila* at 17 h in LB, with an OD₆₀₀ close to 1 and a μ of 0.232 h⁻¹. In that same study, interspecific differences were evident, with μ values of 0.236 and 0.109 h⁻¹ for *X. bovienii* and *X. innexi*, respectively. Similarly, Kim et al. (2017) reported higher *in vivo* growth rates than *in vitro*, with μ (6 HPI) values of 0.479 h⁻¹ for *X. nematophila* and 0.161 h⁻¹ for *X. innexi*. However, these values were lower than those obtained in this study, where *X. nematophila* (bsSC) and *X. doucetiae* (bsSB) reached μ (6 HPI) of 1.129 and 0.882 h⁻¹, respectively. These differences could be attributed to the host used for *in vivo* growth. Indeed, Kim et al. (2017) used *Drosophila melanogaster*, whereas this study used *S. frugiperda*, which may provide greater nutrient availability for bacterial development.

The results of bacterial growth did not directly translate into pathogenicity against *S. frugiperda*. In injection assays, both bacteria showed high virulence, with mortality exceeding 50% at 48 h at concentrations of 10⁵ and 10⁸ CFU/mL. In particular, *X. doucetiae* (bsSB), despite its slower growth, caused high mortality, suggesting a strategy based on persistence and metabolic efficiency rather than rapid proliferation. This may also be because bacteria can produce potent toxins that kill insects.

Some species of the genus *Xenorhabdus* exhibit attenuated virulence when injected into insects, which may reflect genomic specialization geared toward interbacterial competition rather than direct pathogenicity (Bisch et al., 2016; Kim et al., 2017). In contrast, the bacteria isolated in this study showed a highly virulent response after injection, as evidenced by high mortality rates when bacterial suspensions were applied. Similar results were reported by McMullen et al. (2017) when late-stage larvae of *S. littoralis* were injected, in which five strains of *X. bovienii* achieved 95–100% mortality at 48 h with 10³ cells per insect.

In addition, cell-free supernatants were evaluated because they contain natural products with toxic activity against various insect pests (Bussaman et al., 2012; Vicente-Díez et al., 2021). Although cell-free supernatants showed some insecticidal activity, their effect was significantly lower than that of the bacterial suspensions, indicating that the combined action of bacterial proliferation and the continuous release of toxins, enzymes, and other metabolites is essential for successful infection.

In oral trials, efficacy was lower than with direct injection, consistent with the physiological barriers of the insect's digestive tract and the fact that not all bacteria or supernatants can enter the host. However, the growth of bacterial colonies on NBTA medium after feeding on treated maize leaves confirmed that second-instar *S. frugiperda* larvae ingested the bacteria and allowed them to establish internally.

Similar results were reported by Yüksel (2022), who observed that cell-free supernatants from *X. bovienii* caused no more than 45% mortality in oral bioassays with *Tuta absoluta* larvae and that early larval stages were more susceptible than advanced stages. However, in that study, cell suspensions (4×10^8 CFU/mL) caused lower mortality (15–20% at 72 h) than in the present study (20–30% at 72 h), which the authors attributed to low feeding behavior in the early larval stages. Similarly, Yüksel et al. (2022) and Erdem, Yüksel, and Canhilal (2024) reported that early larval stages were more susceptible to cell-free supernatants from *Xenorhabdus* in *Agrotis segetum* and *Leptinotarsa decemlineata*, respectively. In contrast, in the latter study, mortality rates of 47.5–75% were observed at 96 h in 1st/2nd-instar larvae after oral application of cell-free supernatants from *X. nematophila*, *X. bovienii*, and *X. budapestensis*.

Cell-free supernatants can exhibit varying degrees of insecticidal activity across different insect groups, which is associated with variable production of bacterial toxin complexes in terms of quantity and type (Vicente-Díez et al., 2021; Yüksel et al., 2022). Furthermore, insects vary their immune responses by developmental stage, which directly influences their susceptibility to infection. In this context, phenoloxidase activity, a key component of the humoral immune system, is among the first immune responses against microbial infections in insects (Yüksel, 2022). However, bacteria are capable of inhibiting the insect's immune system (Kenney et al., 2019; González et al., 2025).

Furthermore, these results are consistent with those reported by Ogier et al. (2014), who demonstrated that the *X. doucetiae* strain FRM16 is virulent when injected into sixth-stage

larvae of *S. littoralis* and *G. mellonella*, reinforcing the entomopathogenic capacity of this species.

In summary, the results of this study confirm that the high entomopathogenic efficacy initially observed in EPN is due to symbiotic bacteria and that these bacteria can exert a decisive insecticidal effect even independently. Furthermore, the description of two opposing strategies for growth and metabolic efficiency between *X. nematophila* (bsSC) and *X. doucetiae* (bsSB) expands knowledge of functional diversity within the genus *Xenorhabdus*.

Furthermore, the identification of symbiotic bacteria associated with *S. brazilense* should be complemented by more robust genomic studies, which would enable more precise taxonomic identification and analysis of genes encoding toxins and other factors with potential insecticidal activity (Kim et al., 2017; Machado et al., 2024; Palma et al., 2024; Schwarz et al., 2025). In this regard, symbiotic bacteria represent a promising source of natural insecticides. The ongoing identification of toxins and metabolites with insecticidal activity that target economically important pests is a priority, especially given the increasing insect resistance to both chemical insecticides and Bt proteins (Okuma et al., 2018; Amaral et al., 2020; Boaventura et al., 2020).

From an applied perspective, these findings support the use of EPN as an effective biological control tool and suggest the possibility of directly exploiting symbiotic bacteria or their metabolites. Furthermore, future studies could focus on developing formulations based on novel, more efficient, and sustainable natural products derived from bacteria for integrated pest management.

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-SUPPLEMENTARY MATERIAL-

Phenotypic and molecular characterization of *Xenorhabdus* bacteria associated with *Steinernema* with entomopathogenic potential against *Spodoptera frugiperda*

Table S1. Accession numbers of the bacterial sequences used in this study. Sequences generated in this study are shown in bold.

Strain	<i>recA</i>	Genome
<i>Xenorhabdus ishibashii</i>	AB630947.1	—
<i>Xenorhabdus nematophilus</i>	AF127333.1	—
<i>Xenorhabdus doucetiae</i> strain LWD1	FJ536271.1	—
<i>Xenorhabdus nematophila</i> strain ATCC 19061	FJ536273.1	—
<i>Xenorhabdus ehlersii</i> strain DSM16337	FJ823398.1	—
<i>Xenorhabdus griffinae</i> strain ID10	FJ823399.1	—

<i>Xenorhabdus japonica</i> strain DSM16522	FJ823400.1	—
<i>Xenorhabdus doucetiae</i> strain FRM16	FJ823402.1	—
<i>Xenorhabdus romanii</i> strain PR06-A	FJ823403.1	—
<i>Xenorhabdus kozodoii</i> strain ES01	FJ823405.1	—
<i>Xenorhabdus poinarii</i> strain AZ26	FJ823408.1	—
<i>Xenorhabdus hominickii</i> strain KE01	FJ823410.1	—
<i>Xenorhabdus koppenhoeferi</i> strain USNJ01	FJ823413.1	—
<i>Xenorhabdus miraniensis</i> strain Q1	FJ823414.1	—
<i>Xenorhabdus beddingii</i> strain Q58	FJ823415.1	—

<i>Xenorhabdus szentirmaii</i> strain DSM16338	FJ823416.1	–
<i>Xenorhabdus mauleonii</i> strain VC01	FJ823417.1	–
<i>Xenorhabdus budapestensis</i> strain DSM16342	FJ823418.1	–
<i>Xenorhabdus indica</i> strain DSM17382	FJ823421.1	–
<i>Xenorhabdus cabanillasii</i> strain USTX62	FJ823422.1	–
<i>Xenorhabdus innexi</i> strain DSM16336	FJ823423.1	–
<i>Xenorhabdus stockiae</i> strain TH01	FJ823425.1	–
<i>Xenorhabdus bovienii</i> strain T228	FJ823426.1	–
<i>Xenorhabdus vietnamensis</i>	GU481051.1	–

<i>Xenorhabdus magdalenensis</i> strain IMI 397775	JF798401.1	–
<i>Xenorhabdus khoisanæ</i> strain SF362	JX623979.1	–
<i>Xenorhabdus eapokensis</i> strain DL20	KX602188.1	–
<i>Xenorhabdus thuongxuanensis</i> strain 30TX1	KX602194.1	–
<i>Xenorhabdus lircayensis</i> strain VLS	MT462006.1	–
<i>Photorhabdus luminescens</i> subsp. <i>luminescens</i>	KM357904.1	–
<i>Bacillus thuringiensis</i> serovar berliner ATCC 10792	3382191-3383222	NZ_C M000 753.1
<i>Xenorhabdus nematophila</i> bsSC	AACGTGTGCTTTTATTGATGCCGAACATGCCCTTGATCCAGTTTATGCTAAAAAACTGGGCGTAGATATTGATAACCTGTTGTGTTTCGCA GCCTGATACCGGTGAACAGGCGTTGGAAATCTGTGATGCTTTATCCCGTTCCGGCGCAGTGGATGTCATTATTGTTGACTCCGTTGCGG CATTGACACCAAAAAGCGGAAATTGAAGGTGAAATCGGTGATTCTCATATGGGGCTGGCAGCGCGTATGATGAGTCAGGCCATGCGTAA GCTGGCAGGTAACCTTAAAAAATTCAAATACCCTGTTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTTTGGTAACCCTG AAACCACGACGGGTGGTAATGCGCTGAAATTCTATGCGTCTGTCCGTTTGGATATCCGCCGT	

<i>Xenorhabdus doucetiae</i> bsSB	CTGAATCTTCCGGTAAAAACAACGTTAACATTGCAAGTGATCGCTGCTGCCCAGCGTGAAGGCAGAACGTGTGCTTTTATTGATGCCGA ACATGCCCTTGATCCAGTTTATGCTAAAAAACTGGGCGTAGATATTGATAACCTGTTATGTTTCGCAGCCTGATACCGGTGAACAGGCGT TGGAAATCTGTGATGCTTTATCCCGTTCCGGCGCAGTGGATGTCATTATTGTTGACTCCGTTGCGGCATTGACACCAAAAGCGGAAATT GAAGGTGAAATCGGTGATTCTCATATGGGGCTGGCAGCGCGTATGATGAGTCAGGCCATGCGTAAGCTGGCAGGTAACCTTAAAAAATT CAAATACCCTGTTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTTTGGTAACCCTGAAACCACGACGGGTGGTAATGCG CTGAAATTCTATGCGTCTGTCCGTTTGGATATCCGCCGTACGGGTTCGTTGAAAAATGGTGATGAAGTTGTTGGTAGCGAAAC	
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