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Phenotypic and biochemical characterisation and pathogenicity assessment on *Galleria mellonella* L. (Lepidoptera: Pyralidae) of symbionts of the entomopathogenic nematode *Heterorhabditis amazonensis* Andalo et al., 2006

Caracterização fenotípica, bioquímica e avaliação da patogenicidade à *Galleria mellonella* L. (Lepidoptera: Pyralidae) de bactérias simbiontes do nematoide entomopatogênico *Heterorhabditis amazonensis* Andalo et al., 2006

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Highlights

The bacteria strains were positive for all biochemical tests except for lecithinase.
Characterization indicates that the strains are *Photorhabdus* sp.
The bacterial strains were highly pathogenic to *Galleria mellonella.*

Abstract

The objective of this study was to describe phenotypically and biochemically the symbiotic bacteria associated with three populations of *Heterorhabditis amazonensis* Andalo et al., 2006 (isolates: UEL-n 01, UEL-n 07, and UEL-n 08) and evaluate their pathogenicity on *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae. Bacteria were isolated by maceration of infective juveniles (IJs) and grown in culture medium (NBTA and MacConkey). The characterization of the bacteria was evaluated by employing

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motility test and biochemical tests like Gram staining, lipase activity, protease, and lecithinase. The production of antibiotics and bioluminescence was also evaluated. The pathogenicity was evaluated on the last instar larvae of *G. mellonella* at a concentration of 10^4 cells/mL. The bacteria from the three entomopathogenic nematodes isolates were positive for all biochemical tests except for lecithinase, and have presented bioluminescence when subjected to ultraviolet light, indicating that they belong to the genus *Photorhabdus* sp. Both were pathogenic to *G. mellonella* larvae causing 93.3 to 100.0% mortality.
Key words: Microbial control. *Photorhabdus.* Symbiotic association. Virulence.

Resumo

O objetivo desse trabalho foi descrever fenotípica e bioquimicamente as bactérias simbiontes associadas a três isolados do nematoide *Heterorhabditis amazonensis* Andalo et al., 2006 (isolates: UEL-n 01, UEL-n 07, and UEL-n 08) e avaliar sua patogenicidade sobre lagartas de *Galleria mellonella* L. (Lepidoptera: Pyralidae). As bactérias foram isoladas por meio de macerado de juvenis infectantes (Jls) e cultivadas em meio de cultura (NBTA e MacConkey). A caracterização das bactérias foi realizada por meio de testes de motilidade e bioquímicos de coloração de Gram, atividade de lipase, protease e lecitinase. Avaliou-se também a produção de antibióticos e de bioluminescência. A patogenicidade foi avaliada em lagartas de *G. mellonella* na concentração de 10^4 células/mL. Observou-se que as bactérias dos três isolados de nematoides entomopatogênicos foram positivas para todos os testes bioquímicos, com exceção da lecitinase, e apresentaram bioluminescência quando submetidas a luz ultravioleta, indicando que estas cepas pertencem ao gênero *Photorhabdus* sp. Ambas foram patogênicas às lagartas de *G. mellonella* causando mortalidade entre 93,3 e 100,0%.
Palavras-chave: Associação simbiótica. Controle microbiano. *Photorhabdus.* Virulência.

Introduction

Entomopathogenic nematodes (EPNs) have a symbiotic interaction with bacteria of the genus *Xenorhabdus* and *Photorhabdus* (Enterobacteriaceae) (Adams et al., 2006). EPNs of the genus *Steinernema* are associated with bacteria of the genus *Xenorhabdus,* while EPNs of the genus *Heterorhabditis* (Rhabditida: Heterorhabditidae) are associated with bacteria of the genus *Photorhabdus.* Both bacteria are maintained in the intestinal lumen of the third juvenile stage of nematodes, also known as infective juveniles (IJs) (Orozco et al., 2013), but whereas in *Steinernema* IJs the bacteria *Xenorhabdus* are maintained in a special intestinal vesicle (Bird & Akhurst, 1983), *Photorhabdus* is mainly located in the anterior part of *Heterorhabditis* infective juvenile guts (Boemare et al., 1996).

The IJs and the bacteria together act as a complex that kills the insect in a short period of time (48-72 h) (Koppenhöfer, 2007). Upon finding a host, the juvenile penetrates through natural openings (mouth, anulus, or spiracles) and releases the bacteria into the host's hemocele, where they multiply and cause lethal sepsis. The bacteria are the responsible for the death of the insect (Han & Ehlers, 2000), but they are aided by the EPN, which also produce lethal toxins (Burman, 1982).

The genus *Photorhabdus* is the only species of terrestrial bacteria capable of producing bioluminescence; however, this feature is not mandatory. Bacteria of the genus *Photorhabdus* are characterized as being Gram negative and having facultative anaerobic respiration, locomotion by peritrichous-type flagella, and absence of spores (Brenner, 1999; Adams et al., 2006).

In the host insect, bacteria produce a wide variety of toxins and hydrolytic enzymes including lipases, phospholipases, proteases, and antibiotics (Forst et al., 1997; Orozco et al., 2013), important in the infection process. These products are secreted into the hemolymph and are involved in colonizing and decomposing host tissues to be used as food by the nematodes (Adams et al., 2006).

The nematode-bacteria complex is very virulent for insects, which makes these organisms a nonchemical alternative for the biological control of insect pests worldwide (Orozco et al., 2013). Specific knowledge about nematodes species and their symbiont bacteria are very important in the use of these as agents for pest control.

In Brazil, isolation of EPNs has increased; however, studies on the characteristics of symbiont bacteria are still rare. In recent research conducted in a pasture area cultivated with brachiaria, in the District of Guairacá - Londrina, Paraná, a new population of *Heterorhabditis amazonensis* Andalo et al., 2006 was collected (Guide et al., 2022) and is being studied for its characteristics as a possible biological control agent.

Thus, considering the control potential of the nematode-bacteria complex, the objective of this study was to describe phenotypically and biochemically the symbiont bacteria associated with these nematode isolates, and the pathogenicity of the bacteria on larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae).

Material and Methods

Entomopathogenic nematodes

The three isolates of EPNs (UEL-n 01, UEL-n 07, and UEL-n 08), all *H. amazonensis* were multiplied in vivo in last instar larvae of *G. mellonella* according to the methodology of Molina and Lópes (2001). Each isolate was inoculated in ten larvae at a concentration of 100 IJ/cm² in Petri dishes with 9 cm diameter, with two filter papers, kept in a climate-controlled chamber at 23±1 °C and without photoperiod, for 10 days. Afterwards, the larvae were placed in White's traps (White, 1927) for emergence of IJs, which were collected, suspended in distilled water, and stored in cell culture flasks at 18 °C for three days until used in the bioassays.

Bacterial isolation

The bacteria were isolated using the IJs maceration method following the methodology of Akhurst (1980) with modifications. For this, 1 mL of the suspension of each isolate was placed in a 1.5 mL microtube (Eppendorf ®) and then centrifuged for 1 min at 11000 rpm. Using a micropipette, the supernatant was removed leaving only the precipitate. In each microtube, 50 µL of 2% sodium hypochlorite was added, which acted for five minutes for external asepsis of the IJs. Then, the supernatant (approximately

50 µL) was removed and 100 µL of distilled water was added. The microtubes were taken to a laminar flow chamber and the suspension was poured into a sterile crucible to macerate the IJs. After maceration, the bacterial cells were inoculated by streaking with a platinum loop on a nutrient agar plate supplemented with bromothymol blue and 2,3,5-Triphenyl tetrazolium chloride (NBTA medium) (15 g Nutrient Agar; 0.025 g Bromothymol Blue; 0.04 g Triphenyl Tetrazolium Chloride; 1000 mL distilled water) and kept in a climate-controlled chamber at 28±1 °C in the dark. (without photoperiod).

All evaluations in this study were performed from bacterial colonies isolated on NBTA medium that were 48 h old and had expressed green coloration.

Phenotypic and biochemical characterization of bacteria

For bacterial characterization, morphotinctorial analysis (Gram stain), motility evaluation, as well as biochemical tests for lipase, protease, and lecithinase activity were employed. The production of antibiotics and bioluminescence was also evaluated according to Boemare and Akhurst (1988) and Akhurst (1980). In addition, the ability of neutral red absorption in MacConkey medium, Bromothymol Blue absorption, and Triphenyl-Tetrazolium reduction in NBTA medium was observed (Guerra et al., 2014).

Gram staining and motility test

Characteristics as pink or red pigmentation and bacilli shape of *Photorhabdus* strains were evaluated by standardizing Gram stain. Motility was evaluated in Semi-solid Nutrient Agar medium (SNA) (3g of yeast extract; 5g of peptone; 8g of NaCl; 5g of nutrient agar; 1000 mL of distilled water). Tests were performed in 15 cm high test tubes containing 10 mL of SNA. For the tests, a stab with a sterile platinum needle was done in the middle of the SNA tube and incubated at 28°C for 48 h. After that the presence or absence of turbidity in the culture medium could be observed, whereas motile bacteria 'swarm' and give diffuse spreading growth.

Lipase

The lipase activity was determined on Peptone Agar medium (10 g Bacteriological Peptone; 5 g NaCl; 0.1 g $CaCl_2.1H_2O$; 15 g agar; 1000 mL distilled water) supplemented with 10% Tween 80 according to the methodology of Sierra (1957), in which Tween 80 is added when the autoclaved medium reached a temperature between 50 and 40 °C. The medium was poured into 9-cm diameter Petri dishes, and after it became transparent, the bacteria were inoculated. For each isolate, a cell suspension was prepared by diluting a batch containing the bacteria in 200 µL of sterile distilled water. A 10 µL drop of each cell suspension was then applied to the medium. The plates were stored in a climate-controlled chamber at 28 °C, without photoperiod for 48 h. After this period, we observed if there was any formation of translucent halos around the colonies, which correspond to the zones of the hydrolyzed substrate.

Protease

Protease production was evaluated using Nutrient Agar medium (Kasvi®) containing 4% gelatin according to Whaley et al. (1982). The gelatin was added after the culture medium was autoclaved and at a temperature close to 50 °C. After solidification, the culture medium was perforated forming 0.5-cm wells in the center of the plates where 50 µL of the bacterial suspensions were inoculated. The experiment was conducted in duplicate, and plates were incubated in an air-conditioned chamber at 28 °C, without photoperiod. After 48 hours of incubation, the plates were evaluated to verify the presence or absence of a transparent halo around the bacterial growth, which is indicative of gelatin degradation.

Lecithinase activity

Lecithinase activity was evaluated in Nutrient Agar medium (Kasvi®) supplemented with 10% pasteurized egg yolk (15 g Nutrient Agar; 10 g egg yolk, and 1000 mL distilled water). The egg yolk was added after the culture medium was autoclaved and at a temperature close to 50 °C. The medium was poured into Petri dishes, and after solidification, a 10 µL drop of cell suspension of each bacterium was inoculated. The suspension was prepared for each bacterial isolate by diluting a batch containing bacterial cells in 200 µL of sterile distilled water. The plates were stored in a climate-controlled chamber at 28 °C without photoperiod for 48 hours.

Antibiotic production

Antimicrobial activity was evaluated by growth inhibition of *Bacillus thuringiensis* (Bt). After cultivation for 24 h in liquid LB medium, a Bt cell suspension was inoculated in Nutrient Agar (15 g Nutrient Agar; 5 g bacterial peptone; 1000 mL distilled water) (Costa et al., 2010). The inoculation was performed using a sterile swab soaked in the bacterial suspension, in three directions, to obtain confluent growth throughout the medium. After 5 min to dry the plates, the bacteria isolated from the EPN were inoculated. For this, a 0.3 cm diameter sterile filter paper disk was soaked in the cell suspension and placed in the center of the plate. The plates were stored in a climate-controlled chamber at 28 °C, and after 24 hours, the evaluation was performed by observing the presence or not of the halo of Bt growth inhibition.

Bioluminescence

To evaluate bioluminescence, *G. mellonella* larvae were inoculated with Hamilton microsyringe containing 10 µL (10^4 cells/mL) of the cell suspension of each bacterium. For the preparation of the bacterial inoculum, 24-h colonies multiplied in NBTA at 28 °C were started. The cells were washed and suspended in phosphate-buffered saline (PBS), and a bacterial suspension of concentration 10^4 cells/mL was prepared using Mc Farland's turbidimetric scale and serial dilutions. The syringe was previously submitted to the washing protocol that includes serial washing in 1% hypochlorite solution, in distilled water, in 70% alcohol, and

in PBS. The washing process was performed before, during, and after the experiment. We used fifth instar larvae that were light colored and without black spots. The inoculation was done in the last pair of prolegs, injecting the bacteria directly into the hemolymph.

Light colored fifth instar larvae were selected, and for each bacterial isolate, three larvae were inoculated. After 48 h, the larvae were subjected to U.V. light to observe the presence/absence of luminescence.

Lipase, protease, lecithinase activity, antibiotic production and bioluminescence tests had three replicates per test.

Pathogenicity evaluation in G. mellonella larvae

The pathogenicity of three strains of bacteria (L 01, L 07 and L 08) isolated from EPNs was evaluated against *G. mellonella,* and was used the Hamilton microsyringe to inoculate the bacteria into *G. mellonella* larvae like described above in the bioluminescence test. Each larva was inoculated with 10 µL of bacterial suspension. Each treatment had three repetitions consisting of a 9 cm diameter Petri dish, a filter paper moistened with 1 mL of distilled water, and ten larvae inoculated with the bacteria. In the control treatment, the larvae were inoculated with 10 µL of PBS. The plates were covered and kept in a climate-controlled chamber at 28 °C, without photoperiod and with 50% relative humidity. The larvae were kept without feeding during the whole experiment. After 48 h, the evaluation was done by counting the number of dead larvae and observing the infection symptoms by the bacteria.

The data were analyzed for normality and homoscedasticity and the means were compared using the Tukey test at 5% using the statistical program Sisvar 5.4 (D. F. Ferreira, 2011).

Results and Discussion

Phenotypic and biochemical characterization of the bacteria

The bacteria from the three EPN isolates exhibited phenotypic characteristics common to bacteria of the family Enterobacteriaceae and of the genus *Photorhabdus* (Boemare et al., 1993). The main features employed to identify the genus were the coloration presented by the *G. mellonella* larvae (reddish dark brown) and the presence of bioluminescence when subjected to ultraviolet light.

As for colony morphology, the three bacterial isolates presented round, convex, shiny, viscous consistency, colonies with a raised center (umbonate), and dark green coloration in NBTA medium after 48 h of growth.

Bromothymol blue uptake was also observed by the colonies on NBTA medium with a change in the coloration of the medium around the growth from green to blue. On MacConkey agar, the colonies had red coloration after 48 h, indicating neutral red uptake.

The bacteria presented motility in the test performed on semisolid agar, which indicates the presence of flagella in all three isolates observed. In Gram staining, the three isolates were characterized as

Gram negative bacilli, confirming other studies results (Adams et al., 2006; Guerra et al., 2014). Furthermore, the three bacterial isolates demonstrated antibiotic production capacity against *B. thuringiensis,* promoting the presence of a growth inhibition halo of this bacterium.

The evaluation of the lipase activity displayed the presence of a clear halo around the bacterial growth, while the evaluation of the protease activity allowed the observation of two halos, an opaque external one and a translucent internal one, demonstrating that the evaluated bacterial isolates present lipase and protease activity. In the medium supplemented with egg yolk, to evaluate lecithinase activity, an orange halo was observed around the bacterial growth and no transparent halo was visible, indicating that the evaluated bacterial isolates are negative for lecithinase activity (Table 1).

Table 1
Biochemical identification characteristics of *Photorhabdus* spp. isolates

Bacteria Strain	Lipase	Protease	Lecithinase	Absorption B.B.*	Absorption R.**
Photorhabdus sp. L 01	+	+	-	+	+
Photorhabdus sp. L 07	+	+	-	+	+
Photorhabdus sp. L 08	+	+	-	+	+

B.B.* = Bromothymol Blue absorption; R.**= Red (MacConkey medium).

Pathogenicity evaluation on G. mellonella larvae

All three bacterial isolates were pathogenic to *G. mellonella* larvae. Mortality percentages ranged from 93.3 to 100.0% and all differed significantly from the control treatment, but not from each other (Table 2).

Table 2
Percent of mortality caused by *Photorhabdus* spp. symbiont bacteria in *Galleria mellonella* larvae 48 hours after inoculation

Bacteria Strain	% Mortality (Media ± SD)
Photorhabdus sp. L 01	100.0 ± 0.0 a*
Photorhabdus sp. L 07	100.0 ± 0.0 a
Photorhabdus sp. L 08	93.0 ± 5.7 a
Control	20.0 ± 17.5 b
CV = 11.6	(P-value= 0.0000 and F= 54.8)

* Means followed by the same lower-case letter in the column do not differ by Tukey's test (p≤0.05).

Most *Photorhabdus* species are bioluminescent, due to the presence of all genes of the lux operon, encoding a luciferase, like what occurs in bioluminescent marine microorganisms (Paracer & Ahmadjian, 2000; Almenara et al., 2012), and the bioluminescence that we observed indicates that this is the genus of the evaluated strains.

Similarly, the colony morphology corresponded to characteristics observed for other bacterial isolates of the genus *Photorhabdus* (Orozco et al., 2013; Fukruksa et al., 2017). These characteristics were also observed by T. Ferreira et al. (2013) for the bacterium *P. luminescens* subsp. *noenieputensis,* Orozco et al. (2013) for P. *luminescensens sonorensis,* and Singh et al. (2012) for *P. luminescens.*

According to Dowds and Peters (2002), the pathogenicity of *Photorhabdus* bacteria is related to the inhibition of the defense reactions of the insect immune system. The symbiont bacteria produce substances such as lipases (Thaler et al., 1995) and proteases (Bowen et al., 2000) that are considered virulence factors, because they can destroy the cells responsible for the immune defense. The production of these enzymes identified in the bacterial isolates evaluated in this study may explain the high virulence against *G. mellonella* larvae.

Lipases can present phospholipid activity in microorganisms and are used as a defense mechanism. When secreted, they compete with the microflora, facilitate the digestion of lipids and free fatty acids released, and assist in cell-cell and cell-host tissue adhesion (Stehr et al., 2003).

Proteases act mainly in the hydrolysis of proteins and amino acids and according to Schmidt et al. (1988), proteases may play a role in insect toxicity by analogy with proteases produced by other insect pathogens. On the other hand, Jarosz et al. (1991) suggest that virulence is correlated with the presence of protease activity. However, the role of this enzyme in insect toxicity is conflicting (Bowen et al., 2000).

Bowen and Ensign (1998) reported that inoculation of *P. luminescens* was lethal to *G. mellonella* larvae. The high virulence of bacteria of the genus *Photorhabdus* has also been observed in other studies, in which the inoculation of less than ten cells of the bacteria in the hemocele was sufficient to kill susceptible insects, such as *G. mellonella* or *Manduca sexta* L. (Lepidoptera: Sphingidae) (Jarosz et al., 1991; Liu et al., 2006; Kushwah et al., 2016). Similarly, the mortality data in our study were 100%, although we used a higher concentration than quoted. Concentration studies are needed for a more accurate comparison.

Conclusion

The symbiotic bacteria strains associated with *Heterorhabditis amazonensis* (isolates UEL-n 01, UEL-n 07 and UEL-n 08) belong to the genus *Photorhabdus.* All strains were pathogenic and caused more than 90% mortality of *G. mellonella* larvae.

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Conflicts of Interest

The authors declare no conflict of interest. All authors contributed to the study conception and design and all authors read and approved the final manuscript.

References

Adams, B. J., Fodor, A., Klein, M. G., Smith, H. L., Stackebrandt, E., & Stock, S. P. (2006). Biodiversity and systematics of nematode-bacterium entomopathogens. *Biological Control, 37*(1), 32-49. doi: 10.1016/S1049-9644(06)00126-5

Almenara, D. P., Rossi, C., Neves, C. M. R., & Winter, C. E. (2012). Nematoides entomopatogênicos. In M. A. C. Silva Neto da, C. Winter, & C. Termignoni (Eds.), *Tópicos avançados em entomologia molecular* (pp. 1-40). São Paulo.

Andalo, V., Nguyen, K. B. & Moino, A. Jr. (2006). *Heterorhabditis amazonensis* n. sp. (Rhabditida: Heterorhabditidae) from Amazonas, Brazil. *Nematology, 8*(6), 853-867. doi: org/10.1163/156854106779799286

Akhurst, R. (1980). Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis. Journal of Genetic Microbiology, 121*(2), 303-309. doi: 10.1099/00221287-121-2-303

Bird, A. F., & Akhurst, R. J. (1983). The nature of the intestinal vesicle in nematodes of the family *Steinernematidae. International Journal of Parasitology, 13*(6), 599-606. doi: 10.1016/S0020-7519(83)80032-0

Boemare, N. E., Akhurst R. J., & Mourant, R. G. (1993). Deoxyribonucleic acid relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, with a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus. International Journal of Systematic Bacteriology, 43*(2), 249-255. doi: 10.1099/00207713-43-2-249

Boemare, N. E., Laumond, C., & Mauléon, H. (1996). The nematode-bacterium complexes: biology, life cycle and vertebrate safety. *Biocontrol Science and Technology, 6*(1), 333-345. doi: 10.1080/09583159631316

Boemare, N., & Akhurst, R. (1988). Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobactereaceae). *International Journal of Systematic Bacteriology, 134*(3), 751-761. doi: 10.1099/00221287-134-3-751

Bowen, D., & Ensign, J. (1998). Purification and characterization of a highmolecular-weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens. Applied and Environmental Microbiology, 64*(8), 3029-3035. doi: 10.1128/AEM.64.8.3029-3035.1998

Bowen, D., Blackburn, M., Rocheleau, T., Grutzmacher, C., & Ffrench-Constant, R. H. (2000). Secreted proteases from *Photorhabdus luminescens:* separation of the extracellular proteases from the insecticidal Tc toxin complexes. *Insect Biochemistry and Molecular Biology, 30*(2), 69-74. doi: 10.1016/s0965-1748(99)00098-3

Brenner, D. J. (1999). Additional genera of enterobacteriaceae. In M. Dworkin, Falkow, S., Rosenberg, E., Schleifer, K.-H., & Stackebrand, E. (Eds.), *The prokaryotes: an evolving electronic resource for the microbiological community* (3nd ed., release 3.4). New York.

Burman, M. (1982). Neoaplectana carpocapse: toxin production by axenic insect parasitic nematodes. *Nematológica, 78*(1), 62-70. doi: 10.1163/187529282X00510

Costa, J. R. V., Rossi, J. R., Marucci, S. C., Alves, E. C. C., Volpe, H. X. L., Ferraudo, A. S., Lemos, M. V. F., & Desidério, J. A. (2010). Atividade tóxica de isolados de *Bacillus thuringiensis* a larvas de *Aedes aegypti* (L.) (Diptera: Culicidae). *Neotropical Entomology, 39*(5), 757-766. doi: 10.1590/S1519-566X2010000500015

Dowds, B. C. A., & Peters, A. (2002). Virulence mechanisms. In R. Gaugler (Ed.), *Entomopathogenic nematology* (pp. 79-98). New York.

Ferreira, D. F. (2011). Sisvar: a computer statistical analysis system. *Ciência e Agrotecnologia, 35*(6), 1039-1042. doi: 10.1590/S1413-70542011000600001

Ferreira, T., Van Reenen, C., Pagès, S., Tailliez, P., Malan, A. P., & Dicks, L. M. T. (2013). *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov., a symbiotic bacterium associated with a novel Heterorhabditis species related to *Heterorhabditis indica. International Journal of Systematic and Evolutionary Microbiology, 63*(5), 1853-1858. doi: 10.1099/ijs.0.044388-0

Forst, S., Dowds, B., Boemare, N., & Stackebrandt, E. (1997). *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annual Review of Microbiology, 51*(1), 47-72. doi: 10.1146/annurev.micro.51.1.47

Fukruksa, C., Yimthin, T., Suwannaro, J. M., Muangpat, P., Tandhavanant, S., Thanwisai, A., & Vitta, A. (2017). Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against *Aedes aegypti. Parasites & Vectors, 10*(1), 1-10. doi: 10.1186/s13071-017-2383-2

Guerra, B. E., Chacón, J. G., Muñoz, J. E., & Caicedo, M. A. (2014). Evaluación de la patogenicidad de *Xenorhabdus* spp. nativos en Colombia. *Revista Colombiana de Biotecnologia, 16*(1), 111-118. doi: 10.15446/rev.colomb.biote.v16n1.44277

Guide, B. A., Andaló, V., Ferreira, D. G., Alves, V. S., Fernandes, T. A. P., & Neves, P. M. O. J. (2022). First report and biological characteristics of Heterorhabditis amazonensis in the state of Paraná, Brazil. *Brazilian Journal of Biology, 84*(1), e262374. doi: 10.1590/1519-6984.262374

Han, R. C., & Ehlers, R. U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. *Journal Invertebrate Pathology, 75*(1), 55-58. doi: 10.1006/jipa.1999.4900

Jarosz, J., Balcerzak, M., & Skrzypek, H. (1991). Involvement of larvicidal toxin in pathogenesis of insect parasitism with the rhabditoid nematodes *Steinernema feltiae* and *Heterorhabditis bacteriophora. Entomophaga, 36*(1), 361-368. doi: 10.1007/BF02377940

Koppenhöfer, H. S. (2007). Bacterial symbionts of *Steinernema* and *Heterorhabditis. Nematology Monographs and Perspectives, 2*(1), 735-808. doi: 10.1163/ej.9789004152939.i-816.43

Kushwah, J., Kumar, P., Garg, V., & Somvanshi, V. S. (2016). Discovery of a highly virulent strain of *Photorhabdus luminescens* ssp. *akhurstii* from Meghalaya, India. *Indian Journal of Microbiology, 57*(1), 125-128. doi: 10.1007/s12088-016-0628-y

Liu, W., Ye, W., Wang, Z., Wang, X., Tian, S., Cao, H., & Lian, J. (2006). *Photorhabdus luminescens* toxin-induced permeability change in *Manduca sexta* and *Tenebrio molitor* midgut brush border membrane and in unilamellar phospholipid vesicle. *Environmental Microbiology, 8*(5), 858-870. doi: 10.1111/j.1462-29 20.2005.00972.x

Molina, J. P. A., & López, N. J. C. (2001). Producción *in vivo* de três entomonematodos con dos sistemas de infección en dos hospedantes. *Revista Colombiana de Entomologia, 27*(1-2), 73-78.

Orozco, R. A., Hill, T., & Stock, P. S. (2013). Characterization and phylogenetic relationships of *Photorhabdus luminescens* subsp. *sonorensis* (γ-Proteobacteria: Enterobacteriaceae), the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae). *Current Microbiology, 66*(1), 30-39. doi: 10.1007/s00284-012-0220-6

Paracer, S., & Ahmadjian, V. (2000). Bacterial associations of bactéria, protozoa and animals. In S. Paracer, & V. Ahmadjian (Eds.), *Symbiosis: an introduction to biological associations* (pp. 33-50). New York.

Schmidt, T. M., Bleakley, B., & Nealson, K. H. (1988). Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens. Apply of Environmental Microbiology, 54*(11), 2793-2797. doi: 10.1128/aem. 54.11.2793-2797.1988

Sierra, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek, 23*(1), 15-22. doi: 10.1007/BF02545855

Singh, S., Eric, M., & Floyd, I. (2012). Characterization of Photorhabdus luminescens growth for the rearing of the beneficial nematode *Heterorhabditis bacteriophora. Indian Journal of Microbiology, 52*(3), 325-331. doi: 10.1007/s12088-011-0238-7

Stehr, F., Kretschmar, M., Kröger, C., Hube, B., & Schäfer, W. (2003). Microbial lipases as virulence factors. *Journal of Molecular Catalysis B: Enzymatic, 22*(5-6), 347-355. doi: 10.1016/S1381-1177(03)00049-3

Thaler, J. O., Baghdiguian, S., & Boemare, N. E. (1995). Puri® cation and characterization of xenorhabdicin, a phage tail-like bacteriocin, from the lysogenic strain F1 of *Xenorhabdus nematophilus. Applied and Environ- mental Microbiology, 61*(5), 2049-2052. doi: 10.1128/aem.61.5.2049-2052.1995

Whaley, D. N., Dowell, V. R., Wanderlinder, L. M., & Lombard, G. L. (1982). Gelatin agar medium for detecting gelatinase production by anaerobic bacteria. *Journal of Clinical Microbiology, 16*(2), 224-229. doi: 10.1128/jcm.16.2.224-229.1982

White, G. F. (1927). A method for obtaining infective nematodes larvae from cultures. *Science, 66*(1709), 302-303. doi: 10.1126/science.66.1709.302-a