

Biological characterization of *Photorhabdus* spp. and evaluation of the insecticidal and antibiotic action of cell suspension and extracts

Caracterização biológica de *Photorhabdus* spp. e avaliação da ação inseticida e antibiótica de suspensões celulares e extratos.

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Abstract: This study identified and investigated the insecticidal and antimicrobial properties of symbiotic bacterial strains isolated from entomopathogenic nematode (EPN). The bacterial strains were identified by 16S rDNA amplification confirmed three strains as *Photorhabdus luminescens* (P05, P06 and L08) and one as *Photorhabdus asymbiotica* (P04). The insecticidal activity of the strains was assessed by direct inoculation into the hemolymph of three species of lepidopteran insects, and then the two most effective strains were inoculated at different concentrations over time against *Galleria mellonella* (Lepidoptera: Noctuidae) larvae. All strains were highly pathogenic and virulent to the insect species, resulting in mortality rates ranging from 85% to 100. About inoculum concentrations P04 and L08 exhibited different virulence patterns, with P04 causing more rapid mortality in *G. mellonella* larvae. All the *Photorhabdus* spp. strains in cell suspension showed positive antibiotic activity against bacterial targets and have an antifungal effect. The antibiotic and insecticidal activity of extracts (secondary metabolites) has also been the subject of investigation, but insecticidal properties of the extracts were not observed. Interestingly, the L08 extracts exhibited antimicrobial activity against *Staphylococcus aureus*, while P04 extracts showed fungicidal properties. These results highlight the biotechnological potential of these strains for future studies.

Keywords: Entomopathogenic Nematodes; Enterobacteriaceae; Heterorhabditidae; Symbiosis.

Resumo: Este trabalho teve como objetivo identificar cepas bacterianas simbióticas isoladas de nematoides entomopatogênicos (EPN) e estudar propriedades inseticidas e antimicrobianas de suspensões celulares e de extratos. As cepas bacterianas foram identificadas pela amplificação do rDNA 16S, confirmando três cepas como *Photorhabdus luminescens* (P05, P06 e L08) e uma como *Photorhabdus asymbiotica* (P04). A atividade inseticida das cepas foi avaliada pela inoculação direta na hemolinfa de três espécies de insetos lepidópteros e, em seguida, as duas cepas mais eficazes foram inoculadas em diferentes concentrações ao longo do tempo contra larvas de *Galleria mellonella* (Lepidoptera: Noctuidae). Todas as cepas foram altamente patogênicas e virulentas para as espécies de insetos, resultando em taxas de mortalidade que variaram de 85% a 100. Sobre as concentrações de inóculo, P04 e L08 exibiram diferentes padrões de virulência, com P04 causando mortalidade mais rápida nas larvas de *G. mellonella*. Todas as cepas de *Photorhabdus* spp. em suspensão celular apresentaram atividade antibiótica positiva contra alvos bacterianos e uma têm efeito antifúngico. A atividade antibiótica e inseticida dos extratos (metabólitos secundários) também foi objeto de investigação, mas não foram observadas propriedades inseticidas dos extratos. No entanto, o extrato L08 apresentou atividade antimicrobiana contra *Staphylococcus aureus*, enquanto o extrato P04 apresentou propriedades fungicidas. Esses resultados destacam o potencial biotecnológico dessas cepas para estudos futuros.

Palavras-Chave: Nematoides entomopatogênicos; Enterobacteriaceae; Heterorhabditidae; Simbiose. another common beer contaminant. LAMP emerged as a promising alternative to PCR,

demonstrating high specificity and sensitivity, detecting as little as 100 fg/ μ L of gDNA. This study highlights the potential of molecular methodologies, particularly LAMP, and underscores the need for continuous improvement of these techniques to ensure faster, more accurate, and effective quality control solutions for the brewing industry.

Keywords: Beer, Diagnosis, DNA, Brewing Industry, *Levilactobacillus brevis*.

1 INTRODUCTION

The Entomobacteria *Xenorhabdus* and *Photorhabdus* are characterized by symbiotic association with entomopathogenic nematode (EPN) of the Steinernematidae and Heterorhabditidae families, respectively. In this association, bacteria kill insect hosts by toxicity and by causing septicemia, while the nematodes help bacterial mobility and dissemination and provide nutrients for bacterial growth (Bode, 2009). Although they have similarities regarding life habits and symbiosis, these bacteria have many differences among them, both phenotypically and biochemically (Stock, 2015).

Once inoculated in the host hemolymph, the host internal tissues are metabolized, which makes them assimilable by the nematodes, and produces many secondary metabolites inside the corpse, e.g. crystalline inclusion proteins, proteases, lipases, lipopolysaccharides, and other active compounds that are toxic to insect pests (Bowen & Ensign, 1998; French-Constant et al., 2007). Many of these compounds have antibiotic property on other microbial agents (Almenara et al., 2012) and prevent infection in the nematodes.

Bacterial suspensions were evaluated against *Culex pipiens* L. (Diptera: Culicidae), the principal vector of west Nile Virus and lymphatic filariasis, and were able to kill all the development stages with varying levels of mortality, exhibited the highest susceptibility to the cell-free supernatants and cell suspensions of symbiotic bacteria and the efficacy of the cell-free (Yüksel et al., 2023).

The role played by these bacteria and their compounds have been the focus of many studies on the potential for biocontrol of metabolites produced by the symbiont bacteria *Photorhabdus*. In recent studies, bacterial broth prepared

from *P. luminescens* showed high levels of toxicity equivalent to the pyrethroid insecticide bifenthrin and caused higher insect mortality against *Monellia caryella* (Hemiptera: Aphididae) and *Melanocallis caryaefoliae* (Davis) (Hemiptera: Aphididae) (Wu et al., 2022).

Compounds produced by symbiont bacteria of EPN might also have a fungicide – and especially antibiotic – property, affecting the growth of bacteria. Extracts obtained from two strains of *Photorhabdus l. sonorensis* (Enterobacteriaceae) had a strong antibiotic effect on *Pseudomonas syringae* (Pseudomonadales: Pseudomonadaceae) at 40 μ g/ml, inhibited the growth of *Bacillus subtilis* (Bacillales: Bacillaceae) at 40 μ g/ml, and strongly inhibited the growth of the fungus *Fusarium oxysporum* (Hypocreales: Nectriaceae) (Orozco et al., 2016).

The aim of this study was to identify and characterize biologically and chemically symbiont bacteria from four isolates of entomopathogenic nematodes, as well as screen and determine the virulence of extracts of *Photorhabdus* based on their inhibitory activities against insects, fungi, and bacteria.

2 MATERIALS AND METHODS

2.1. Bacterial isolation, culture, growth conditions and molecular identification

The symbiont bacteria were isolated according to Akhurst (1980), from hemolymph of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae infected with nematodes inoculated in a Petri dish with Nutrient Bromothymol Blue Agar medium (NBTA) (15 g nutrient agar; 0.025 g Bromothymol blue; 0.04 g triphenyl tetrazolium chloride; 1000 mL distilled water) and incubated for 48h at 28°C (Table 1).

Genomic DNA (gDNA) was extracted from cultures grown overnight in Luria-Bertani medium (LB) at 28°C using the commercial kit “Pure Link” Invitrogen® (for Gram-negative bacteria). The quality and quantity of the gDNA were determined by spectrophotometry using Qubit Fluorometer (Invitrogen). Species of the gDNA were identified using Polymerase Chain Reaction (PCR) with

analysis of 16S rDNA using specific primers (16S rDNA fd1 5'AGAGTTTGATCCTGGCTCAG 3' and 16S rRNA rP1 5'ACGGTTACCTTGTACGACTT 3') (Weisburg, 1991). PCR (Polymerase Chain Reaction) reactions were conducted according to protocols established (Orozco, 2014).

PCR (Polymerase Chain Reaction) was conducted in a final volume of 15 μ L, including 1X GoTaq Master Mix Color Less (Promega), 0.67 μ M of each primer, and 15ng DNA. The PCR program consisted of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute, and a final extension step of 72 °C for 10 minutes.

Table 1. Bacterial strains isolated from entomopathogenic nematodes (EPN).

EPN Isolates	EPN Species	Bacterial strain
UEL 08	<i>Heterorhabdus amazonensis</i>	L08
UENP 05	<i>Heterorhabdus amazonensis</i>	P05
UENP 06	<i>Heterorhabdus amazonensis</i>	P06
UENP 04	<i>Heterorhabdus</i> sp.	P04

Subsequently, PCR products were viewed through electrophoresis in 1% agarose gel, and after that, they were purified using a commercial IllustraExoStar IT® (GE) enzyme according to the manufacturer.

Sequencing was conducted with ABI-PRISM 3500 XL thermal cycler (Applied Biosystems), and the sequencing reactions followed Orozco (2014) protocols.

The sequencing reactions were conducted in a final 10 μ L volume with 5.75 μ L of ultra-pure water, 1 μ L sequencing buffer, 0.25 μ L of 20 μ M primer, 2 μ L of Big Dye Terminator (Applied Biosystems), and 1 μ L of purified PCR. The cycles of PCR sequencing reactions consisted of initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 20 seconds, 50°C for 15 seconds, and 60°C for 2 minutes, and a final extension step of 72 °C for 2 minutes.

A BLASTN search was performed to identify bacterial isolates to the species level by finding the similarity of partial 16S sequences with known sequences in the GenBank - NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul, 1990). Multiple sequences were aligned using BioEdit (Sequence Alignment Editor) (Hall, 1999), and then, the alignment was submitted to a phylogenetic analysis using

MEGA version 5.0 (Tamura et al., 2011), with the Maximum Parsimony method and the Mini heuristic parameters with search level 1. The initial trees were obtained by the random addition of sequences (10 replicates) and all analyzes were conducted with 1000 bootstrap replicates.

2.2 Biochemical and biological characterization

a. Gram staining and Motility Test

Characteristics as pink or red pigmentation and bacilli shape of *Photorhabdus* strains were evaluated by standardized Gram stain procedure. Motility was evaluated in Semi-solid Nutrient Agar (SNA) medium (3g yeast extract; 5g peptone; 8g NaCl; 5g nutrient agar; 1000 mL 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 performed 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 obtained diffuse spreading growth.

b. Biochemical tests

Biochemical tests were evaluated in Petri dishes containing a specific culture medium that was inoculated with proportional aliquots of cell suspensions prepared by diluting a spot colony of each bacterial strain in 200 μ L of sterile distilled water. Standardized incubation parameters were used, at 28°C for 48 h with 50% of relative humidity. The results were considered positive to produce the enzymes if an inhibition zone was detected around the bacterial inoculum, corresponding to hydrolysed substrate after 24 hours of incubation.

Lipase: Activity was determined in Peptone Agar (PA) medium (10g bacteriological peptone; 5g NaCl; 0.1g CaCl₂.1H₂O; 15g Agar; 1000 mL distilled water) supplemented with 10% Tween 80 (Sierra, 1957). An aliquot of 10 μ L of the cell suspension was inoculated on PA plates for each bacterial isolate and was after incubated.

Protease: Activity was evaluated using Nutrient Agar (NA) medium (Kasvi®) supplemented with 4% gelatin (w/v) (Whaley et al., 1982). After solidification, agar well diffusion

method (WDM) was performed according to Valgas et al. (2007). In the center of the plates, 0.5 cm wells were made, 50µL bacterial cell suspension was inoculated, and plates were incubated.

Lecithinase: Activity was evaluated in NA (Kasvi®) supplemented with 10% liquid pasteurized egg yolk. The egg yolk was added after the culture medium was autoclaved at a temperature close to 50°C. An aliquot of 10µL cell suspension of each bacterium was inoculated and the results in NA plates were evaluated after the incubation period.

c. Bioluminescence detection

Bioluminescence was evaluated according to protocols established (Akhurst, 1980; Boemare & Akhurst, 1988). A cell suspension was prepared for each bacterial isolate by diluting a spot colony in 200µL of sterile distilled water. An aliquot of 10µL of bacterial suspension was then inoculated in triplicates on the last pair of legs of the *G. mellonella* larvae with a Hamilton microsyringe. After 48 h, the larvae were subjected to ultraviolet light to observe the presence/absence of luminescence.

d. Absorption and reduction of pigments in differential media

The differential medium MacConkey and NBTA was used for *Photorhabdus* characterization (Guerra et al., 2014). *Photorhabdus* strains were confirmed according to the capacity to absorb neutral red in MacConkey medium, the absorption of Bromothymol Blue, and the reduction of Triphenyl-Tetrazolium in NBTA medium.

2.3 Cell Suspension Activity

a. Insecticidal activity

The insecticidal activity of *Photorhabdus* spp. cell suspension was evaluated against different agronomic targets [*G. mellonella*, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), and *Anticarsia gemmatilis* (Lepidoptera: Noctuidae)]. *Photorhabdus* strains were first cultured in LB at 28°C for 48 h. An aliquot of 10µL of each bacterial suspension was inoculated in the last pair of legs of the lepidopteran larvae using a Hamilton micro syringe (Salazar-Gutierrez et al., 2017), previously disinfested with sequential washes in

1% hypochlorite solution, distilled water, 70% alcohol, and saline solution. Negative controls were also performed with 10µL of sterile distilled water.

The assays were conducted in 12-well plates containing a filter paper soaked with 250 µL of distilled water and one larva treated per well, without larvae feeding during the experiment. The mortality was evaluated after 48 h by observing typical symptoms of infection on larvae by *Photorhabdus* sp. Each treatment was conducted in triplicates and the data obtained were submitted to an analysis of variance, and the means were compared using Tukey's test at 5%, using the statistical program Sisvar 5.4 (Ferreira, 2011).

Two isolates, P04 (*P. asymbiotica*) and L08 (*P. luminescens*), were selected for virulence assays in *G. mellonella* at different concentrations and in different periods. The aliquots of each cell suspension strain were inoculated in ten *G. mellonella* larvae at concentrations 0.25, 0.5, and 1.0 (at McFarland standards) as described above (Salazar-Gutierrez et al., 2017). The larvae for each treatment were placed in a 9 cm diameter Petri dish with a filter paper soaked with 2mL of distilled water and incubated at 28°C, without feeding during the experiment. The mortality was evaluated every 6 hours for 48 h. Each treatment was conducted in triplicates and the data obtained were also submitted to statistical analysis using the statistical program Sisvar 5.4 (Ferreira, 2011).

b. Antimicrobial activity

The antimicrobial activity of *Photorhabdus* spp. was previously evaluated in assays using cell suspension from bacterial cultures using the Disc Diffusion Method (DDM). *Photorhabdus* isolates were first cultivated in NBTA media for 72 h at 25 °C. A single colony from each isolate was transferred into a LB medium at 25 °C for 48 h. The entire *Photorhabdus* spp. cell suspension was considered for all the assays. After sterile filter paper disks of 0.3 cm were soaked in *Photorhabdus* spp., cell suspensions were arranged in the center of Petri dishes containing the test culture media. A clear zone from the edge of the growth colony of *Photorhabdus* spp. was considered as positive against the target strain.

The antibacterial activity of *Photorhabdus* spp. was evaluated against *Bacillus thuringiensis* (Bt), *Escherichia coli*, and *Staphylococcus aureus* strains. To evaluate test strains, a sterile swab was soaked in each bacterial suspension

and spread over Nutrient Agar supplemented with 0.5% bacteriological peptone (w/v) (NA) plates. After drying, the NA plate cultures were inoculated with the paper disks containing *Photorhabdus* cell suspension. The plates were incubated at 28 °C and 50% relative humidity for 24 h and the inhibition zone was evaluated.

The antifungal activity of *Photorhabdus* spp. was evaluated against *Beauveria bassiana bassiana* (UNI 44) and *Metharizium anisopliae* (UNI 22) strains that were supplied by the Biotechnology Laboratory of UNIOESTE. Test fungal strains were previously grown in Potato Dextrose Agar (PDA) medium for 15 days, until sporulation. Then, an aliquot of 50 uL of spore suspension (1.106 spores/mL) of each fungal strain was spread in PDA plates. After drying, the PDA plate cultures were inoculated with the paper disks containing *Photorhabdus* cell suspension. The plates were incubated at 25°C for 24 h and the inhibition zone was evaluated.

2.4 Activity of extracts

Extracts production

Each bacterial strain was cultivated previously in Petri dishes with NBTA media for 24 h at 28 °C. A single colony from each isolate was resuspended in 30 mL of TSB medium at 28°C, 200 rpm in the dark (Stock & Goodrich-Blair, 2012). After that, 10 mL of bacterial culture was inoculated in 1 L of TSB for 96 h, at 28°C and 200 rpm in the dark.

Culture was centrifuged at 5 °C and 9,000 rpm. After that, the supernatant was dried at 60 °C for 48 h, until there was a 10% reduction of the initial volume. Supernatant was acidified with HCl 5M to pH 4.0 and treated with ethyl acetate (1:1 v/v) to obtain the semi-purified fraction. The supernatant was treated four times with organic solvent; the extraction was repeated three times to ensure maximum secondary metabolic recovery. A semi-purified fraction of each strain was concentrated in a Buchi R-11 rotary evaporator in vacuum at 37- 40°C until a thick oily residue. The remaining solvent was evaporated to complete the drying process by airflow. Control was a non-inoculated TSB semi-purified fraction obtained through the same procedure. The semi-purified fraction was diluted in solution of 1% DMSO. The extracts were evaluated against *Bacillus thuringiensis*, *Escherichia coli*,

Staphylococcus aureus, *Metarhizium anisopliae*, *Beauveria bassiana*, and *G. mellonella* larvae.

a. Antimicrobial activity

Bacterial strains of *B. thuringiensis*, *E. coli*, and *S. aureus* were prepared and inoculated as described above. After five minutes, four wells were drilled in the middle of each Petri dish and 60µL of semi-purified fraction of each strain was added to individual wells and incubated at 28°C for 24 hours. After that, the inhibition zones formed around the well were measured.

b. Antifungal activity

The isolates of *B. bassiana* (UNI 44) and *M. anisopliae* (UNI 22) were prepared and spread in a Petri dish on the PDA surface as described above, and then, dry discs containing crude extracts were placed at four equidistant points (one per extract) on the plate. Plates were incubated at 25°C for 96 h. Antifungal activity was determined by the inhibition zone formed around the discs. The negative control was treated with a dry disc containing distilled water.

c. Insecticidal activity

The insecticidal effect of extracts against *G. mellonella* larvae occurred with two methods, by applying them in the hemolymph and orally. The hemolymph method was performed by applying 10 µL of semi-purified fraction using a Hamilton microsyringe. The syringe underwent a serial wash as follows: hypochlorite solution 1%, distilled water, 70%alcohol, and saline solution before each application. The larvae chosen had equal size, light color, and no black spots. Inoculation was performed in the last pair of legs, with injection of 10 µL of extracts directly into the hemolymph.

The experiment was performed with four bacterial strains and respective control (sterile saline) with three replicates. The experiment was carried out in a 12-well cell culture plate containing filter paper moistened with 0.25 µL of sterile distilled water and ten larvae inoculated with bacterial strains (one larva per well). The control larvae were inoculated with 10µL distilled water. Plates were covered and incubated in a germination chamber at 28°C in the dark. Larvae were maintained fasting during the experiment. After 48 hours, the number of dead larvae was counted and symptoms of infection, such as activity and color, were observed.

For ingestion, 20µL of crude extract was mixed with food and each larva received 0.25 g of diet. The experimental design was arranged in 12-well cell culture plates with each plot containing 10 larvae arranged with three replicates as described above. The control was larvae treated with food plus 1% DMSO solution to account for any effects of the solvent. Mortality was recorded one week after incubation. The data from both trials were submitted to an analysis of variance and the means were compared using Tukey's test at 5%, and the statistical program Sisvar 5.4 (Ferreira, 2011).

3. RESULTS AND DISCUSSION

3.1 Isolation, identification, and characterization of *Photorhabdus* strains

Regarding molecular identification, the symbiont bacterial strains isolated from *Heterorhabditis amazonensis* (UEL 08, UENP 05, UENP 06) were identified as *Photorhabdus luminescens*. On the other hand, the isolate associated with *Photorhabdus* sp. (UENP 04) was identified as *Photorhabdus asymbiotica* by sequencing part of the 16S rRNA gene. The query coverage and percent identity of OTUs (Operational taxonomic units) displayed high query coverage and identity compared to the sequences deposited in the GenBank (Tab 2). To confirm this analysis, the sequences with the highest similarity were then submitted to the analysis and generated a phylogenetic tree (Figure 1). The sequence accession numbers have been deposited with the numbers: P04: ON490468, P05: ON490466, P06: ON490467 and L08: ON490465.

The bacterial isolates obtained in this study had all characteristics observed previously for other bacterial isolates of the genus *Photorhabdus* (Orozco et al., 2013; Furkruksa et al., 2017), and according to our molecular identification, all bacterial symbiont isolates associated with *H. amazonensis* nematodes belonged to the *Photorhabdus luminescens* species and the isolate associated with *Heterorhabditis* sp. belonged to *Photorhabdus asymbiotica*. In Brazil, the occurrence of this species has not yet been reported, and the present study is the first record of *P. asymbiotica* in our country (Fernandes, 2020; Macedo, 2020).

The species *P. luminescens* is found exclusively in association with nematodes of the genus *Heterorhabditis*. In an analysis of the complete genome of *P. luminescens*, researchers observed several genes with expression exclusive to the mutualistic phase associated with the nematode, indicating a strong regulation of symbiosis between these nematodes and bacterial species (Park, 2013; Clarke, 2014).

On the other hand, *P. asymbiotica* was initially a bacterial species of clinical importance, which was described in 1989 in the United States after isolation from a leg ulcer in a hospitalized patient (Farmer et al., 1989; Hapeshi & Waterfield, 2017). Eventually, *P. asymbiotica* was found to be symbiotically associated with EPN of the genus *Heterorhabditis* (Gerrard et al., 2006). Recent studies have indicated that this species occurs associated with EPN more frequently than previously thought, and new reports of this association are arising, from studies carried out in countries such as the USA (Plichta et al., 2009), Japan (Kuwata et al., 2008) and Australia (Gerrard et al., 2011).

Table 2. Bacterial strains identified by comparing sequences through the BLASTn match with the NCBI GenBank database.

EPN species (isolate)	Strain	Top BLASTn*	pb analyzed	Similarity (%)	Proposed taxa
<i>H. amazonensis</i> (UEL08)	L08	<i>Photorhabdus luminescens</i> (NR115332)	643	100%	<i>Photorhabdus luminescens</i>
<i>H. amazonensis</i> (UENP05)	P05	<i>Photorhabdus luminescens</i> (JQ912649)	575	99%	<i>Photorhabdus luminescens</i>
<i>H. amazonensis</i> (UENP06)	P06	<i>Photorhabdus luminescens</i> (JQ912644)	636	100%	<i>Photorhabdus luminescens</i>
<i>Heterorhabditis</i> sp. (UENP04)	P04	<i>Photorhabdus asymbiotica</i> (AY278672)	427	98%	<i>Photorhabdus asymbiotica</i>

*Access number on Genbank.

The genome of *P. asymbiotica* is relatively small compared to *P. luminescens* (the closest analogue) and have a smaller variety of toxins with insecticidal properties (Wilkinson et al., 2010). Furthermore, unlike other species of the genus *Photorhabdus*, *P. asymbiotica* can grow at 37 °C, undergoing metabolic change to adapt their metabolisms to the mammalian host (Mulley et al., 2015).

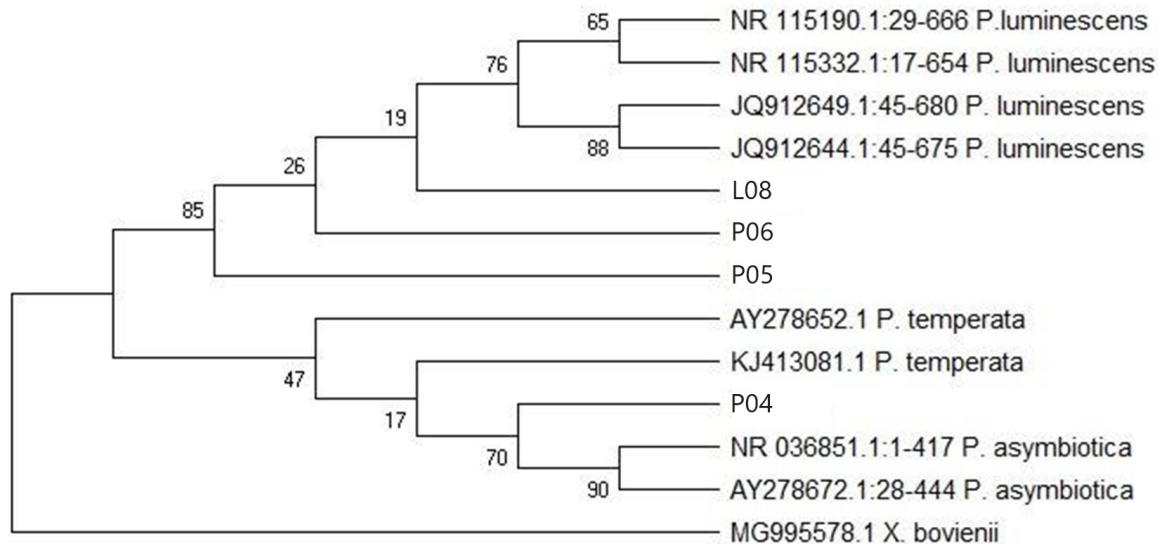


Figure 1. Phylogeny of bacterial symbionts based on the analysis of part of the 16S rRNA gene inferred by Maximum Parsimony (MP) analysis. Outgroup: *Xenorhabdus bovienii*.

G. mellonella larvae inoculated with the isolates of EPNs UENP 04, 05, 06, and UEL 08 showed typical symptoms of infection by the complex nematode + bacteria of the genera *Heterorhabditis* + *Photorhabdus*. After they were infected, the larvae had intact tegument, absence of putrefied odors, and dark red coloration. After bacterial isolation, colonies obtained in NBTA showed phenotypic characteristics of symbiotic bacteria of the genus *Photorhabdus* in phase I. These bacteria had convex, bright, circular, and sticky colonies with dark green coloration due to the absorption of Bromothymol Blue and the reduction of Triphenyl-Tetrazolium Chloride (Figure 2).

Evaluations were performed counting dark green colonies formed by absorption of bromothymol blue dye and reduction of triphenyl-tetrazolium chloride.



Figure 2. Morphology of *Photorhabdus* sp. (L08) after 48h of growth in NBTA.

3.2 Biochemical and Biological Characterization

In terms of biochemical characterization, all strains showed positive reactions to the motility test and were classified as Gram +. All strains were also positive for lipase, protease and bromothymol blue absorption, but negative

for lectinase, which is expected for bacteria of the genus *Photorhabdus*.

The presence of motility in semi-solid agar is expected, since bacteria of the genus *Photorhabdus* are reported to have peritrichial flagella, which give them mobility and the ability to move (Boemare & Akhurst, 1988), corroborating what was observed in this study. The data on the presence of lipase and protease enzymes and the absence of lectinase also contribute to this confirmation and have been reported in other studies (Orozco, 2014; Guerra et al., 2014).

3.3 Cell suspension activity of *Photorhabdus* spp.

a. Antimicrobial activity

All strains of *Photorhabdus* spp., P04, P05, P06, and L08, showed an inhibition zone against bacterial targets *B. thuringiensis*, *E. coli*, and *S. aureus*, except for isolate P04, which did not show activity against *S. aureus*. On the other hand, regarding fungal targets, all *Photorhabdus* isolates inhibited *M. anisopliae* and *B. bassiana*. These results indicate the production of antimicrobial compounds by *Photorhabdus* isolates (Table 3). After that, the bacterial culture-based extracts underwent activity assays to detect potential secondary metabolites.

All cell suspensions of *Photorhabdus* isolates showed potential to produce antimicrobial compounds against different interesting targets in the present study. *Photorhabdus* is known for having antibacterial properties inside the host, as it allows the development of nematodes in a favorable environment, which is the insect cadaver, without the presence of other bacteria, fungi, and other nematode species (Park & Stanley, 2005) side from killing the host in a period of 24 to 48 horas, *Photorhabdus* produces enzymes responsible for the bioconversion of internal host tissues, facilitating nematode feeding (Almenara et al., 2012).

Regarding competition with other microorganisms inside the host, it is known that the inhibition by *Photorhabdus* occurs through the production of antibiotics and secondary metabolites, which in turn shall prevent the parasitized insect corpse from rotting (Almenara et al., 2012; Derzele

et al., 2002). Secondary *Photorhabdus* metabolites have different chemicals and are known in literature for a wide array of activities of medical and agricultural importance, e.g. antibiotics, anti-fungal, insecticides, nematicide, and antiviral properties (Clarke, 2016).

Against pathogenic bacteria and fungi, symbiotic EPN bacteria are known to produce many compounds with antimicrobial activity, such as stilbene derivatives (Bode, 2009), trans-kinnamic acid (TCA) (Bock et al., 2014), xenocoumacins, and hydrolytic enzymes such as chitinases (Chen et al., 1994). Therefore, the compounds produced by bacteria of the genus *Photorhabdus* are potential sources of new molecules (Tobias et al., 2018; Xue et al., 2018). In medical applications, *Photorhabdus* has also been evaluated as an alternative to the need for new antibiotics, with the emergence of cross-resistance of pathogenic bacteria to several known antibiotics.

In our study, the cell suspensions of *Photorhabdus* strains had an inhibitory effect on *Escherichia coli* and/or *Staphylococcus aureus*, which are potential human pathogens. The extract obtained from the L08 strain also showed antibiotic activity against *S. aureus*, indicating this strain as promising in the search for bioactive compounds that may act as antimicrobials of clinical interest. However, more studies need to be carried out to identify the antibiotic molecules.

As for the antifungal effect, the cell-free supernatant obtained from seven species of *Xenorhabdus* spp. and *Photorhabdus* spp. showed antifungal activity against *Fusicladium carpophilum*, *F. effusum*, *Monilinia fruticola*, *Glomerella cingulate* and *Armillaria tabescens*. Higher antibiotic activity was observed against *Xenorhabdus szentirmaii*, compared to the other bacteria tested (Hazir et al., 2016). *P. sonorensis* extracts strongly inhibited *Fusarium oxysporum* (Hypocreales: Nectriaceae), and no effect was found in *Alternaria alternata* (Pleosporales: Pleosporaceae).

On the other hand, *B. bassiana* growth was inhibited (Orozco et al., 2016). According to our findings, extracts produced by *P. asymbiotica* and *P. luminescens* are a promising alternative to help with bacteria and fungi control. Although the targets tested in our study, *B. bassiana* and *M. anisopliae*, are beneficial fungi used in biological control, the fungicidal

action of the *Photorhabdus* strains on these fungi suggests that these strains can promote inhibition of phytopathogenic fungi in field applications. Further studies must be conducted on these fungal species.

b. Insecticidal activity and virulence of difference concentrations on *Galleria mellonella*

Photorhabdus isolates were submitted to an evaluation of insecticidal activity in *G. mellonella*, *S. frugiperda*, and *A. gemmatalis*. All bacterial strains showed insecticidal effects on the evaluated hosts, proving to be more active, with more than 85% of mortality. In addition, the level of activity

was also shown to be isolate-dependent, when mortality is compared among different hosts and between strains in the same host (Table 3).

The bacterial isolate L08 was the most active, with 100% of mortality in all hosts. For the other strains, there was variability with differences among the evaluated hosts. Regarding the effect against *G. mellonella*, the P05 isolate was the least virulent, and the other bacterial isolates had no significant difference. In addition, P05 and L08 isolates were more virulent against *A. gemmatalis* than P04 and P06. All strains showed statistically significant differences against *S. frugiperda* (Table 3).

Table 3. Biological activities of *Photorhabdus* symbiont strains against different targets.

Bacterial strains	Biological activities							
	Antibacterial ^a			Antifungal ^b		Insecticidal ^c		
	Ec	Sa	Bt	Ma	Bb	<i>G. mellonella</i>	<i>A. gemmatalis</i> <i>S. frugiperda</i>	
Control						10.0 ± 5.5 Aa	10.0 ± 0.0 Aa	0.0 ± 0.0 Aa
P05	+	-	+	+	+	86.6 ± 5.77 Ca	100.0 ± 0.0 Cc	96.6 ± 5.77 Db
L08	+	+	+	+	+	100.0 ± 0.0 Ba	100.0 ± 0.0 Ca	100.0 ± 0.0 Ea
P04	+	+	+	+	+	100.0 ± 0.0 Ba	93.3 ± 11.5 Bb	90.0 ± 0.0 Cc
P06	+	+	+	+	+	100.0 ± 0.0 Bc	93.3 ± 5.77 Bb	86.7 ± 5.77 Ba

a. Bacterial targets with medical interest *Escherichia coli* (Ec), *Staphylococcus aureus* (As), and of agronomic interest *Bacillus thuringiensis* (Bt). b. Fungal targets with agronomic interest *Metarhizium anisopliae* (Ma) and *Beauveria bassiana* (Bb). c. Host mortality after 48 hours of inoculation in tests carried out with a CV of 1.99%. The same uppercase letters in the columns and lowercase letters in the rows represent means with no significant difference according to Tukey's test ($p \leq 0.05$).

There was significant variation in the parameters evaluated; the bacterial isolate P04 did not show variation between the inoculum concentrations, causing mortality of 90% of *Galleria* larvae after 18h of infection. On the other hand, the isolate L08 showed higher variability; the lowest inoculum concentration (0.25 of Macfarland scale) caused differences in mortality in 24 and 30h of infection. We noticed that isolate P04 takes longer to cause sepsis, but the effect turns out to be more homogeneous, causing mortality above 90% of larvae at all inoculum concentrations evaluated after 18h of infection (Figure 3).

The investigations about virulence mechanisms and secondary metabolites of the bacteria *Xenorhabdus* and *Photorhabdus* have primarily focused on their potential use in the management of agricultural pests (Zhang et al., 2012;

Kumari et al., 2015; Stock et al., 2017; Hinchliffe et al., 2018), in the control of disease-vector dipterans (Yooyangket et al., 2018; Kajka et al., 2019). Guide et al. (2023) observed high mortality for the host *G. mellonella* when inoculated with different symbiont bacteria of nematodes of the species *H. amazonensis*.

The main mechanism of the insecticidal activity is the production of toxins of high molecular weight (Tcs), which are effective against many insect genera and orders (Ffrench-Constant et al., 2003), and many toxins produced by *Photorhabdus* bacteria are lethal both when ingested and when directly injected into the hemocoel (Bowen and Ensing, 1998), thus corroborating the high pathogenicity of the bacteria directly injected in the hemolymph of insects in the present study.

Bacteria of the genus *Photorhabdus* are characterized by producing a wide array of exo-enzymes such as lipases (Thaler et al., 1995), chitinases (Chen et al., 1996), proteases (Ffrench-Constant et al., 2003), and phospholipases that are responsible for acting in tissues and cells of the host, causing high lethality.

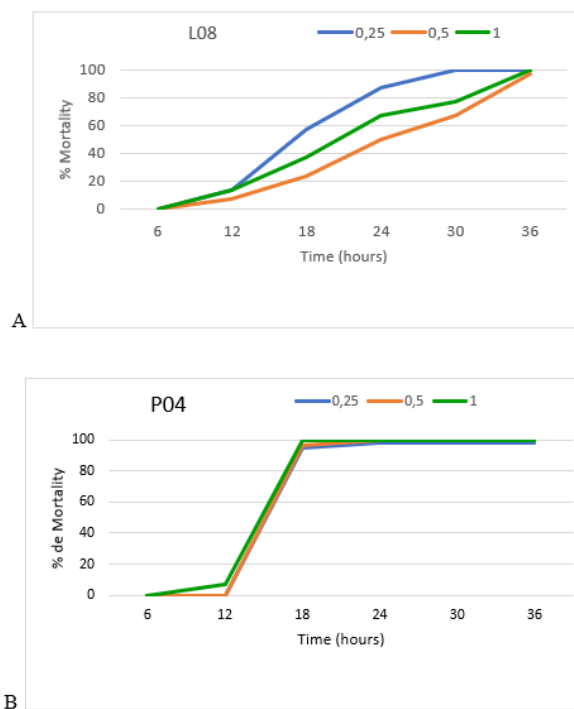
In addition, proteases appear to play an important role in the bioconversion of tissues in the insects into assimilable nutrients for the nematode and in the inhibition of antibacterial factors segregated by the insect, thus explaining the reason for the absence of other microorganisms parasitizing the insect, especially those that have necrophagous activity, which could compromise the cycle of the nematode in the decomposing host (Daborn et al., 2001; Schmidt et al., 2008). Proteases can also play a role of toxicity in insects due to analogy with proteases produced by other pathogens, such as the bacteria *Bacillus thuringiensis* (Schmidt et al., 2008).

According to our results, the bacterial isolates P04, P05, P06, and L08 showed insecticidal activity when the bacterial suspension was inoculated into the insect's hemolymph of the targets *G. mellonella*, *A. gemmatalis* and *S. frugiperda*. The infectivity of *P. asymbiotica* P04 against *G. mellonella* was high, occurring in 93% with 18h of inoculation.

3.4 Bioactivity of *Photorhabdus* spp. extracts containing secondary metabolites

Extracts of P04, P05, and P06 strains did not show antibiotic activity against *B. thuringiensis*, *S. aureus*, and *E. coli*, except for L08 strain, which was observed in the inhibition zone around *S. aureus*. In addition, the extracts produced with P05, P06, and L08 strains were tested against *M. anisopliae* and *B. bassiana* and no antifungal activity was observed, except for *P. asymbiotica*, which inhibited both fungal species tested. In the toxicity assays with *G. mellonella*, the survival was 100% compared to the control, either when injected into the hemolymph or treated orally, incorporated into diet of cell suspensions of bacterial strains P04, P05, P06, and L08.

Figure 3. Percentage of accumulated mortality of *Galleria mellonella* over time, inoculated with different concentrations (0.25, 0.5 and 1 of Macfarland scale) of *Photorhabdus luminescens* L08 (A) and *Photorhabdus asymbiotica* P04 (B).



The assays with extracts of isolate *P. luminescens* (L08) and *P. asymbiotica* (P04), no *G. mellonella* larvae died. Thus, we suggest that the concentration used in the present study is lower than the effective concentration to kill the larvae in both methods tested with *G. mellonella* larvae. The insecticidal activity of *P. asymbiotica* strains is largely known and shows high virulence, yet the factors involved are still unknown. However, when virulence is observed in insects, it may also occur in human infection (Gerrard et al., 2004). Different pathogenic levels were found in *P. luminescens* and *P. asymbiotica* compared to survival cells and metabolism in insect cell cultures. In addition, bacterial growth of each species was different, suggesting that *P. luminescens* and *P. asymbiotica* use different mechanisms to infect insect cells (Maldonado & Eleftherianos, 2012).

CONCLUSIONS

Bacterial strains were identified as being from the species *P. luminescens* and *P. asymbiotica*, and they had phenotypical and biochemical characteristics compatible with other strains of the same species. The strains showed antimicrobial, fungicidal and insecticidal properties, and when the extract obtained from the cell-free culture supernatant was tested, the L08 strain showed the best antimicrobial while P04 strain showed fungicidal effect and no insecticidal effect was observed in the extracts.

Overall, the results obtained in the present study indicate the potential for bacteria of this genus to being a source of bioactive molecules that can be applied in the field for the biological control of pests. These molecules need to be further investigated, so that they are identified and characterized to better elucidate their antimicrobial and insecticide properties on different biological targets of clinical and agronomic interest.

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