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In-Silco and In-Vitro Characterization of a Symbiotic Association Bacteria Isolated from Entomopathogenic Nematodes and Producers for Biological Control Non-Ribosomal Peptides

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ABSTRACT

Simple Summary: Bacteria from the genus *Photorhabdus* and *Xenorhabdus* are found in symbiotic association with entomopathogenic nematodes of the family *Heterorhabditidae* and *Steinernematidae*, respectively. These nematodes are used with limited success as an effective biological control against insect pests of the order *Coleoptera* and *Lepidoptera*, including white grubs, weevils, and codling moths, the typical workflow of (*Photorhabdus* and *Xenorhabdus*) form a promising bacterial source of non-ribosomal peptides and it is a great fortune for natural products scientists and microbiologists that bacteria can also be cultivated without their host in the laboratory which could be a perfect bio-control agent against important agricultural insects.

Two symbiotic association bacteria isolated from the *Galleria mellonella* larval hemolymph were identified by 16s rDNA as *Photorhabdus* PMG05 and *Xenorhabdus* XMG01 with accessions numbers of MZ733679 and MZ733678, respectively. The GC-MS analysis for the purified culture of *Photorhabdus luminescens* PMG05 and *Xenorhabdus nematophila* XMG01 bacteria revealed several PKS-NRPs syntheses molecules. The main constituents were Piperidinone (44.09%) and Pyrrolidinone (35.04%) for *Photorhabdus* and *Xenorhabdus*, respectively. The presence of NRPS genes in identified bacteria was detected in four sets of degenerate primers. The degenerate primers of the first set amplified fragments with both strains, while those degenerate primers of the second and third sets amplified only fragments of *Photorhabdus* strain, whereas no fragments were detected with the fourth set. predicted bioactivity step for either antioxidant or antimicrobial for the generated peptides by the NRPSs of the two studied organisms, two antimicrobial predictors show high scores for the abovementioned peptides of the *Photorhabdus luminescens.* However, the Peptide Ranker alone scores a high antimicrobial activity for the same peptide with the moderate antioxidant activity of *Xenorhabdus nematophila*.

INTRODUCTION

Xenorhabdus and *Photorhabdus* and are symbiotic bacteria with entomopathogenic nematodes of the family *Steinernematidae* and *Heterorhabditidae*, respectively. With limited success, these nematodes are used as active biological control agents as opposed to important agricultural insect pests of the order *Lepidoptera* and *Coleoptera* (Lacey & Georgis, 2012).

It is well-known that live entomopathogenic nematodes as any insect bioagents need special requirements such as a limited range of soil moisture, suitable temperature, handling carefully, and refrigerated storage requirements. Due to these specifications, using entomopathogenic nematodes as biological agents against insects became uncommon, subsequently limiting their commercial applications (Lacey & Georgis, 2012).

The nematode-bacteria complex is common as a model system for the exploration of mutualistic and pathogenic symbiosis because it has high pathogenicity against a broad range of insects. In organic farming, this method has been used more than synthetic insecticides due to its timely and precise application (Lacey & Georgis, 2012). The rapid kill, as well as the production of additional virulence factors, encourage nematodes to overcome the insect's innate immunological defenses, as a result, the nematodes can survive and reproduce within the insect remains. After the insect dies, the symbiotic bacteria produce multiple compounds to secure the prey cadaver from the competitors' organisms and other natural predators (Lacey & Georgis, 2012).

Entomopathogenic nematodes employ symbiotic bacteria of *Photorhabdus* and *Xenorhabdus* that live within their guts to destroy the hosts after invading susceptible larvae either through anal, oral, or tracheal pathways. The associated bacteria are released directly into the insect hemocoel after the nematodes enter their host, subsequently, the bacteria replicate rapidly (Bennett & Clarke, 2005). The bacteria produce various secondary metabolites that guarantee either their viability or nematode host. These metabolites conquer the insect's immune system, as a result, the prey usually dies within two days at maximum (Lacey & Georgis, 2012).

However, overcoming the insect's immune system is not an easy task, because this system has cellular and humoral responses that comprise nodulation, phagocytosis, and encapsulation which led to the production of antimicrobial peptides and the proteolytic cascade of prophenoloxidase. The produced compounds significantly tackle the insect's ability to show a considerable antimicrobial peptide-based immune response (Casanova-Torres & Goodrich-Blair, 2013).

It is worth noting here that, *Photorhabdus* and *Xenorhabdus* bring out various bioactive compounds that led to insect prey pathogenicity. The majority of these natural products are synthesized by nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), or hybrids thereof (Casanova-Torres & Goodrich-Blair, 2013).

The NRPS compounds inhibit multiple pathways of the insect immune system such as (4-acetylamino-4-deoxyglycosyl suppresses the phenoloxidase activity, and subsequently protects the bacterial cell surface (Hirschmann *et al.*, 2017). The indole derivatives xenocyloins showed activity towards insect hemocytes. Moreover, the endogenously α-pyrone moiety that contains small molecules (photopyrones) takes part in a new cell-cell communication system leading to the expression of the *Photorhabdus* clumping factor operon (*pcfABCDEF*) that results in insect toxicity due to clumping of insect's cells (Kresovic *et al.*, 2015).

Rhabdopeptide/xenortide-like peptides (RXP), a unique class of nonribosomal linear peptides, are common with various structures in *Photorhabdus* and *Xenorhabdus* (Cai *et al.*, 2017). During insect infection, the RXP is mainly produced and becomes most abundant

after insect death. Further, it has been confirmed that RXP is active against hemocytes as a result of the involvement of RXP in pathogenesis against insects (Reimer *et al.*, 2013). Nevertheless, the RXP is active against various protozoa (Reimer *et al.*, 2014). So, the biological diversity of RXPs may become a multipotent arsenal that eases *Xenorhabdus* and *Photorhabdus* strains to overcome several insects preys and food competitors (Lacey & Georgis, 2012).

Fortunately, NRPS gene clusters are easy to be identified due to their large multidomain organization by modern genome mining aspects (Süssmuth & Mainz, 2017). The huge and complex outputs of NRPS are also effortless to predict by various bioinformatic predictors, which potentially permit fast evaluation of product novelty (Tietz & Mitchell, 2016). Microbial genome sequencing, in addition to bioinformatics analysis, is considered one of the promising approaches to identifying novel NRP biosynthetic gene clusters that might produce new and bioactive NRPs. Compared to classic screening-based methods, modern achievements in genome sequencing and bioinformatics tools have showed that many microorganisms possess a far greater ability to produce specialized metabolites (Rutledge & Challis, 2015).

Thanks to a thorough understanding of NRPS biosynthetic aspects and significant progress of bioinformatics, structure elucidation potentials to the large and complex NRPs can directly or indirectly advantage from using genomic information that can be now retrieved quickly and cheap, especially for bacteria (Tietz & Mitchell, 2016).

AntiSMASH (antibiotics and secondary metabolite analysis shell) is a state-of-the-art bioinformatic tool for detecting and analyzing gene clusters in genome sequences (Blin *et al.*, 2019). It is worth mentioning that the structural characterization of NRPs from this tool is sometimes imperfect. Even though the specificities of NRPS's substrate modules can be analyzed, the algorithm does not consider the module-skipping, nonlinearity, cyclizations, or modifications. However, the output from antiSMASH and its embedded tools are considered a wealthy resource of structural information (Tietz & Mitchell, 2016).

In the current work, we present a comprehensive characterization either *in-silico* or *in-vitro* for the NRPS synthesized by *Photorhabdus luminescens* PMG05 and *Xenorhabdus nematophila* XMG01 bacteria and their potential biological roles.

MATERIALS AND METHODS

1. Bacterial Isolation and Cultivation Conditions:

The symbiotic association bacteria were isolated from the *Galleria mellonella* larval hemolymph infected with entomopathogenic nematodes juveniles of *Steinernematid riobravis* and *Heterorhabditid bacteriophora*, respectively according to the protocol of Vitta et al (Vitta *et al.*, 2018). The gathered nematodes juveniles were mass-reared in the laboratories of Plant Protection Institute, Agricultural Research Center, Egypt (Kotchofa & Baimey, 2019). The dead larvae exposed to the nematodes juveniles were harvested within white traps and used later for the subsequent experiments (White, 1927).

Briefly, the dead larvae were rinsed with ethanol 70% and washed with distilled water, the collected influx hemolymph of the larvae prolegs was used as a source of symbiotic association bacteria, the looped hemolymph was distributed on the selective (NBT) solid media composite from (peptone 5 $g.L^{-1}$, beef extract 3 $g.L^{-1}$, bromothymol blue 0.25 $g.L^{-1}$ and triphenyl tetrazolium chloride 0.04 $g.L^{-1}$), the pure reddish-blue umbonate colonies were described as the Gram-negative genus of *Xenorhabdus* while the dark green convex colonies were described as the Gram-negative genus of *Photorhabdus* (Fukruksa *et al.*, 2017). The Luria-Bertani (LB) broth was used for bacterial enumeration and biological

control agents' production, the cultivation was conducted at 30°C for 48 h with a shaking frequency of 220 rpm (Elbrense *et al.*, 2021).

2. Identification of Symbiotic Association Bacteria*:*

The protocol of 16S rDNA amplifying was applied (Qihui Teng, 2006), and the genomic DNAs isolation was performed using Wizard ®Genomic DNA Purification Kit from Promega. PCR using the 16S rDNA bacterial universal primers 8F; 5'AGAGTTTGATCCTGGCTCAG'3 and 1492R 5'GGTTACCTTGTTACGACTT'3 was followed. PCR steps were performed at 94°C for 1 min as denaturation step and hybridization at 50°C within 30 sec, elongation at 72°C for 1.5 min, PCR was carried out for 35 cycles and the amplified 16S rDNA genes were separated on 1.2% agarose gel electrophoresis with expected product sizes of 1500 bp (Hussein & Fahim, 2017).

The gel excised bands were purified by Zymoclean TM Gel DNA recovery kit (Epigenetics company). Purified 16S rDNA fragments were ligated to pGEM-T Easy vector (Promega) and then transformed into competent cells *E. coli* JM107 as described in pGEM-T Easy vector manual.

The successful transformants colonies were grown on Ampicillin/IPTG/X-Gal LB media and incubated at 37°C for 24 hours until the appearance of white colonies which were chosen and purified by Mini-Prep Plasmid Purification Kit (Promega). Plasmids were double digested by *EcoRI* restriction enzyme for 16S rDNA genes insertion verification into pGEM-T Easy vector. Cloned genes were then sequenced, and the sequences data were aligned with the GenBank database (Blast) online software, 16S rDNA partial sequenced genes were submitted to GenBank and accession numbers were obtained as listed in Table 1 and 2 (Hussein & Fahim, 2017).

3. Detection of Non-Ribosomal Peptides (Nrps) Synthetases Encoding Genes:

NRPs synthetases genes were detected using four sets of primers, degenerate primers Am1-F/ Tm1-R; Ap1-F/ Tp1-R; As1-F/ Ts1-R and AKs-F/ TKs-R were designed previously by Tapi and co-workers (Tapi *et al.*, 2010). On the conserved data of nucleic sequence motifs by the alignment of thiolation and adenylation domains involved in NRPs synthetases genes biosynthesis in bacteria.

The PCR conditions were performed at 95°C for 2 min as the initial denaturation step, followed by 35 cycles of three steps; denaturation at 95°C for 30 s; annealing step for 30 s, at 43°C with (As1-F/ Ts1-R), at 44.4 °C for 45 sec with (AKs-F/ TKs-R) and at 58°C with (Ap1-F/ Tp1-R), an extension step of 45 s at 72°C except with Ap1-F/Tp1-R primers (75 s at 72°C) and (Aks-F/Tks-R), at the end 5 min of extension step was lanced at 72°C (Abderrahmani *et al.*, 2011).

4. Protein Preparation and Nrps Synthetases SDS-PAGE Profile:

One colony of each strain was pre-inoculated in LB medium overnight at 37°C, Landy medium was used for NRPs Synthetase molecules production for 48 hours at 35°C and under agitation rate of 160 rpm in 100 ml flasks (50% filling volume) (Fahim *et al.*, 2012). The total volume of cultures was centrifuged at 10000 rpm for 15 min, then bacterial cells were discarded. Saturated ammonium sulphate was added to supernatants to precipitate proteins and the mixture was incubated on an ice tank for 15 min, then centrifuged at 5000 g for 15 min (Fahim, 2017).

Precipitated proteins were then eluted in 200 µl of phosphate buffer pH 7.00 and stored at minus 20°C for separation on SDS-PAGE performed. Acrylamide gel of 12% was prepared and migrated in TBE buffer at 60 volts. The gel was Coomassie blue-stained for 24 h, followed by destaining several times. The gel was photographed and bands were analyzed using a gel analyzer system as described by Laemmeli and co-workers (Laemmli, 1970).

5. Gas Chromatography-Mass Spectrophotometry of Synthetases NRPs*:*

The purified culture of isolated *Photorhabdus* and *Xenorhabdus* bacterial strains was conducted using a Trace GC-ISQ mass spectrometric protocol (Thermo Fisher Scientific, Autosampler AS1300, USA) with a direct capillary TG-5MS column, column oven temperature was initially adjusted at 50°C, then increased gradually to 300°C with injector and MS transfer line temperatures were 260 and 270°C, respectively. The carrier gas was helium, with a flow rate of one $mL.min^{-1}$, and a solvent delay of 4 min. Full scan mode of EI mass spectra was also taken at 70 eV under ionization voltages spanning ranging from 50-650 $m.z^{-1}$. Finally, the main components were detected by comparing their mass spectral and their retention times to the NIST14 and WILEY9 databases, respectively (Elbrense *et al.*, 2021).

6. In Silico Analysis of Photorhabdus Luminescens and Xenorhabdus Nematophila:

In this work, we carried out bioinformatic analysis of the sequence/features of some NRPs synthetases of *Photorhabdus luminescens* and *Xenorhabdus nematophila*. Firstly, we retrieved the respective sequences from NCBI, then detected their known domains by Pfam (v. 33.1) (Mistry *et al.*, 2021). The profile Hidden Markov Model library using hmmscan of HMMER (v. 3.3) (Eddy, 1998). Secondly, we located the NRPs synthetases Adenylation (A)-domains through the NRPS predictor-2 (Röttig *et al.*, 2011).

In addition, a 3D structure prediction has been performed for all the predicted (A)-domains using the SWISS-MODEL server (Biasini *et al.*, 2014). Moreover, for all the produced peptides, a bioactivity analysis has been carried out including the antimicrobial and antioxidant activities.

An OxPePred, a web server for predicting the antioxidant properties based on deep learning methods, was used for determining the free radical scavenger (FRS), and chelation (CHEL) scores (Olsen *et al.*, 2020). For the antimicrobial activity prediction, we used two state-of-the-art freely accessible antimicrobial peptide methods, Peptide Ranker (Mooney *et al.*, 2012), and CAMP3 (Waghu *et al.*, 2016).

RESULTS

1. Identification of Bacterial Strains by 16S rDNA:

The two bacterial isolates have amplified fragments of 1500 bp in length with the 16S rDNA gene primers 8F and 1492R (Fig. 1). The BlastN database alignment of sequenced fragments of the bacterial isolates 1 and 2 have shown a high similarity to *Photorhabdus* and *Xenorhabdus*.

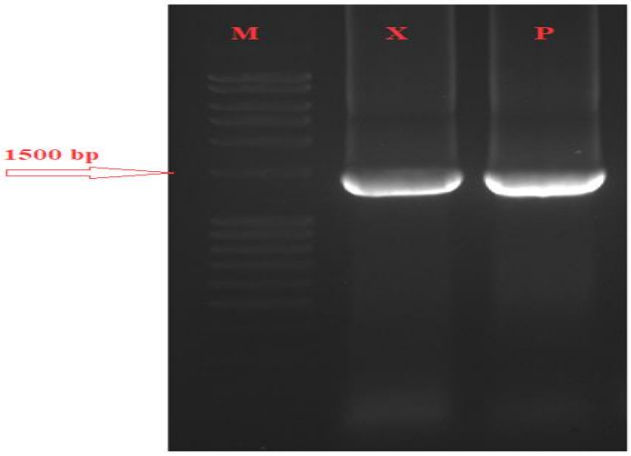


Fig.1. PCR for 16S rDNA partial gene amplification with *Xenorhabdus* (X) and *Photorhabdus* (P) bacterial strains.

The bacterial isolate 1 showed an identity of 98.71% with both *Photorhabdus luminescens* strains BMT19 and BMT17, 98.57% with *Photorhabdus luminescens* strain BMK10, 98.29% with Uncultured *Photorhabdus* sp. clone 9, *Photorhabdus luminescens* subsp. luminescens strain Hb and *Photorhabdus luminescens* strain ATCC 29999 (Table 1).

The bacterial isolate 2 showed an identity of 99.45% with *Xenorhabdus nematophila*, 96.92% with *Xenorhabdus boviennii* strain LB24 and 96.81% with *Xenorhabdus boviennii* strains XbZ1Z, XbZ10Z, XbZ82, XbZ15B, CS03 and SK_BU (Table 2).

Table 1: The highest hits of Blast search using 16S rDNA partial sequences of *Photorhabdus lumeniscens* PMG05

Description	Identity %	Accession
Photorhabdus luminescens strain BMT 19 16S rRNA gene	98.71%	MT574332.1
Photorhabdus luminescens strain BMT 17 16S rRNA gene	98.71%	MT574331.1
Photorhabdus luminescens strain BMK 10 16S rRNA gene	98.57%	MT574330.1
Photorhabdus luminescens subsp. luminescens strain Hb 16S rRNA gene	98.29%	NR_115190.1
Uncultured *Photorhabdus* sp. clone 9 16S rRNA gene	98.29%	DQ314767.1
Photorhabdus luminescens strain ATCC 29999 16S rRNA gene	98.29%	NR_115332.1
Photorhabdus luminescens strain K22 16S rRNA gene	98.14%	KT899933.1
Photorhabdus luminescens strain MN7 16S rRNA gene	97.86%	KY851578.1
Photorhabdus sp. OnKn2 gene for 16S rRNA gene	97.29%	AB355865.1
Photorhabdus sp. Strain R-66822 16S rRNA gene	97. 15%	NR_119151.1

Table 2: The highest hits of blast search using 16S rDNA partial sequences of *Xenorhabdus nematophila* XMG01.

Description	Identity %	Accession
Xenorhabdus nematophila 16S ribosomal RNA gene	99.45%	AY286478.1
Xenorhabdus sp. MY7 gene for 16S rRNAgene	96.92%	AB243434.1
Xenorhabdus boviennii strain LB24 16Sribosomal RNA gene	96.92%	HM140699.1
Xenorhabdus boviennii strain XbZ1Z 16Sribosomal RNA gene	96.81%	KU312061.1
Xenorhabdus boviennii strain XbZ10Z 16Sribosomal RNA gene	96.81%	KU312060.1
Xenorhabdus boviennii strain XbZ82 16Sribosomal RNA gene	96.81%	KU312059.1
Xenorhabdus boviennii strain XbZ15B 16Sribosomal RNA gene	96.81%	KU312058.1
Xenorhabdus boviennii str.CS03 chromosome, (complete genom)	96.81%	FO818637.1
Xenorhabdus boviennii strain SK_BU16S ribosomal RNA gene	96.81%	KF437824.1
Xenorhabdus boviennii strain DSM 476616S ribosomal RNA gene	96.71%	NR_119151.1

2. Phylogenetic Tree Based on the 16S rDNA Partial Sequence:

The phylogenetic tree was designed using Mega version 6.0 based on the 16S rDNA partial genes of the two bacterial isolates under study and the other closely related sequences revealed from BlastN alignment. The phylogenetic tree indicated that all the strains belong to one major phyla of proteobacteria (family of Enterobacteriaceae) including the two different genera of *Photorhabdus* sp. and *Xenorhabdus* sp. (Fig. 2).

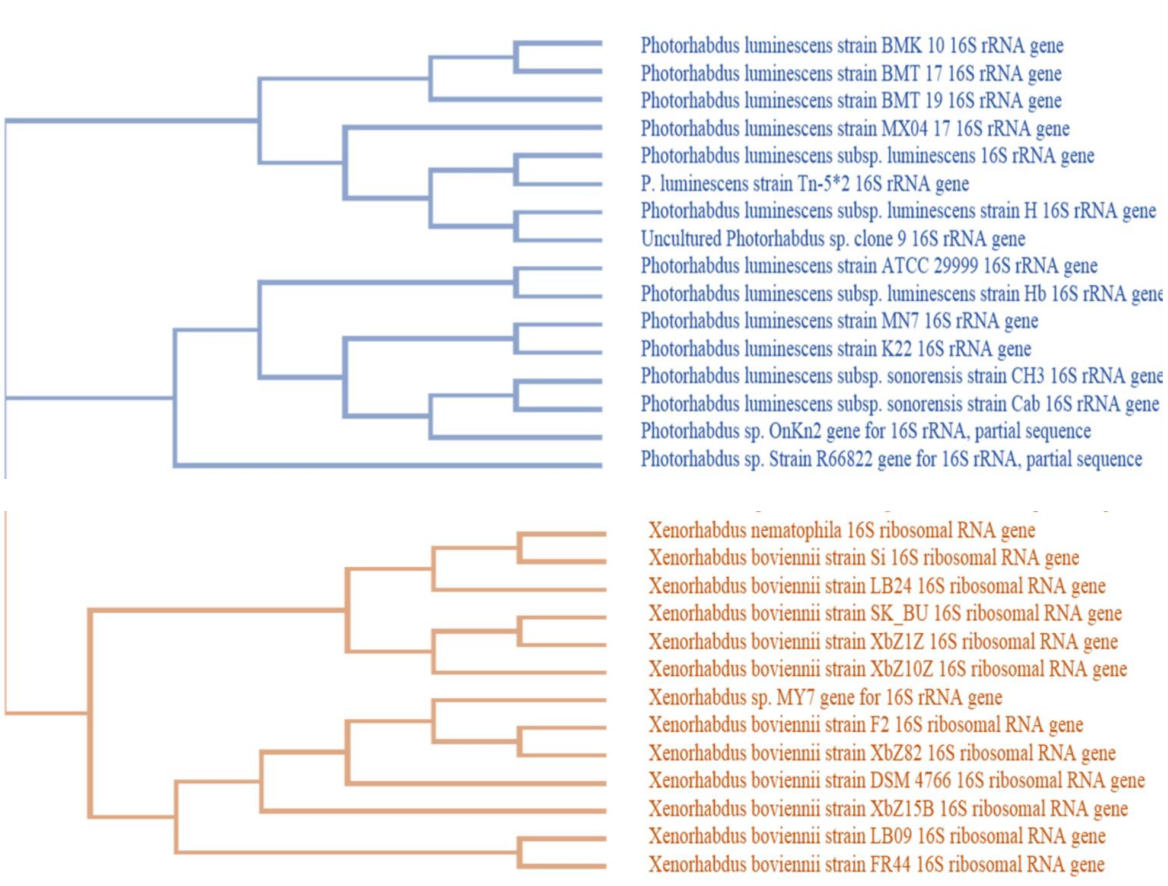


Fig.2: Neighbor-joining phylogenetic tree for alignment of 16S rDNA gene sequences.

According to the latest data, the partial genomes sequences of *Photorhabdus* sp. and *Xenorhabdus* sp. under the names of *Photorhabdus luminescens* PMG05 and *Xenorhabdus nematophila* XMG01 with accessions numbers of MZ733679 and MZ733678, respectively.

3. In Silico Detection of NRPs Synthetases Genes:

The bioinformatics analysis of available complete genomes sequences on GenBank of *Photorhabdus luminescens* and *Xenorhabdus nematophila* revealed 36 and 24 NRPs synthetases genes, respectively. In *Photorhabdus luminescens*, among the 36 NRPs synthetases genes, there are nine operons or genes cluster of 2, 2, 2, 3, 3, 4, 4, 4 and 6 genes, respectively.

Among these operons or genes clusters, there are five of them hybrids of NRPS-PKS and four NRPs synthetases. The genome sequence analysis revealed also unique genes, NRP synthetase (accession: WP_052453826.1) is the most unique super mega gene encoded for a peptide of 15 amino acids called Kollosin with a protein gene length of 16352 a.a (Bode *et al.*, 2015).

The other five genes are encoded for peptides of 6(accession:WP_088375083.1), 5(accession:WP_088371596.1), 3(accessionWP_141096746.1), 3(accession WP_ 088375100.1), and 2(accessionWP_088373156.1) amino acids, respectively (Table 3). The genome sequence analysis of *Xenorhabdus nematophila* revealed 24 NRPs synthetases genes among them, there are seven operons or genes clusters of 2, 2, 3, 3, 4, 4, and 4 genes, respectively.

Among these operons or genes clusters, there are six of them encoding for nonribosomal peptides, while one genes cluster (4 genes) encodes for a hybrid of NRPS-PKS. A study of the genome of *Xenorhabdus nematophila* revealed also three unique genes each one encoded for peptides of 4(accession WP_187007107.1), 3(accession

WP_052453826.1), and 2(WP_038219203.1) mono-modular nonribosomal peptides, respectively.

Moreover, table 3 reveals the produced peptides of the studied strains (*Photorhabdus luminescens* and *Xenorhabdus nematophila*) according to NRPS predictor 2 (Röttig *et al.*, 2011). The graphical models of the produced NRPs synthetases by the studied strains, as well as their domain architectures and the 3D models, are depicted in the (Graphical summary models 1:10).

Table 3. Predicted peptides by NRPS predictor2 of the isolated bacterial strains

No.	Protein	Organism	Peptide
1	WP_088371022, WP_088371021	*Photorhabdus luminescens*	TFWWW
2	WP_088371596	*Photorhabdus luminescens*	KVRVP
3	WP_088372009, WP_141096737, WP_088372490	*Photorhabdus luminescens*	TVOO
4	WP_088372912, WP_088372911	*Photorhabdus luminescens*	CC
5	WP_088373156	*Photorhabdus luminescens*	VWWWW
6	WP_088375100	*Photorhabdus luminescens*	WKW
7	WP_187007107.1	*Xenorhabdus nematophila*	VFVV
8	WP_041979315, WP_038219417.1, WP_041979313.1	*Xenorhabdus nematophila*	GEEEEEE
9	WP_119365259.1, WP_113935472.1, WP_187007121.1	*Xenorhabdus nematophila*	VFITVVKFAAPV
10	WP_041978874.1, WP_038219948.1, WP_041978871.1, WP_041978869.1	*Xenorhabdus nematophila*	KSGOPPKOK

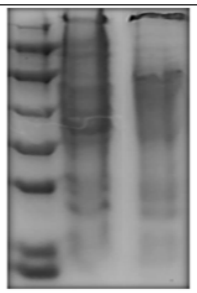
4. Protein Profile of *Photorhabdus luminescens* and *Xenorhabdus nematophila* Cultivation:

The protein profile SDS-PAGE of *Photorhabdus luminescens* and *Xenorhabdus nematophila* strain production of cultivation in Landy medium revealed the presence of eleven bands in *Photorhabdus luminescens* ranging from 135.695 to 16.228 KDa and thirteen bands in *Xenorhabdus nematophila* ranged from 134.184 to 15.173 KDa (Table 4).

Photorhabdus luminescens protein profile showed the presence of four bands of four synthetases genes: condensation domain-containing protein (135.695 KDa), NRP synthetase (98.072 KDa), NRP synthetase (67.5 KDa) and polyketide synthase (54.583 KDa) (protein products no. 14, 15, 22 and 32.

Xenorhabdus nematophila protein profile showed the presence of four bands for four synthetases genes; NRP synthetase (134.184 KDa), NRP synthetase (117.313 KDa), NRP synthetase (98.072 KDa), and NRP synthetase (67.523KDa) (protein products no. 7. 8. 16 and 23.

Table 4. The protein profile of NRPS produced by the isolated bacterial strains.



MW	Lane1	Lane2	Frequency	Polymorphism	MW	Lane1	Lane2	Frequency	Polymorphism
135.695	+	-	0.500	Unique	44.123	-	+	0.500	Unique
134.184	-	+	0.500	Unique	41.721	+	-	0.500	Unique
117.313	-	+	0.500	Unique	40.797	-	+	0.500	Unique
106.067	+	-	0.500	Unique	36.475	+	-	0.500	Unique
103.719	-	+	0.500	Unique	31.889	-	+	0.500	Unique
98.072	+	-	0.500	Unique	27.161	+	-	0.500	Unique
77.233	-	+	0.500	Unique	26.264	-	+	0.500	Unique
67.523	+	+	1.000	Monomorphic	24.284	-	+	0.500	Unique
54.583	+	-	0.500	Unique	20.529	+	+	1.000	Monomorphic
49.351	-	+	0.500	Unique	16.228	+	-	0.500	Unique
47.189	+	-	0.500	Unique	15.173	-	+	0.500	Unique

5. Detection of NRPs Synthetases Genes by PCR Degenerate Primers:

The degenerate four-sets primers were used in PCR detection of NRPS synthetases genes involved in both *Xenorhabdus nematophila* and *Photorhabdus luminescens* strains. Degenerate Primers set 1 amplified two main fragments of 700 and 250bp, with *Xenorhabdus nematophila* and 800, 600 and 300bp with *Photorhabdus luminescens*, respectively (Fig.3).

These degenerated primers were designed previously by (Abderrahmani *et al.*, 2011) on the conserved data nucleic sequence motifs by the alignment of thiolation and adenylation domains involved in NRPs synthetases genes biosynthesis in bacteria. In the absence of these PKS-NRPs (lipopeptides), these degenerated primers could detect other genes containing adenylation and thiolation domains, these results are agreed with (Tapi *et al.*, 2010).

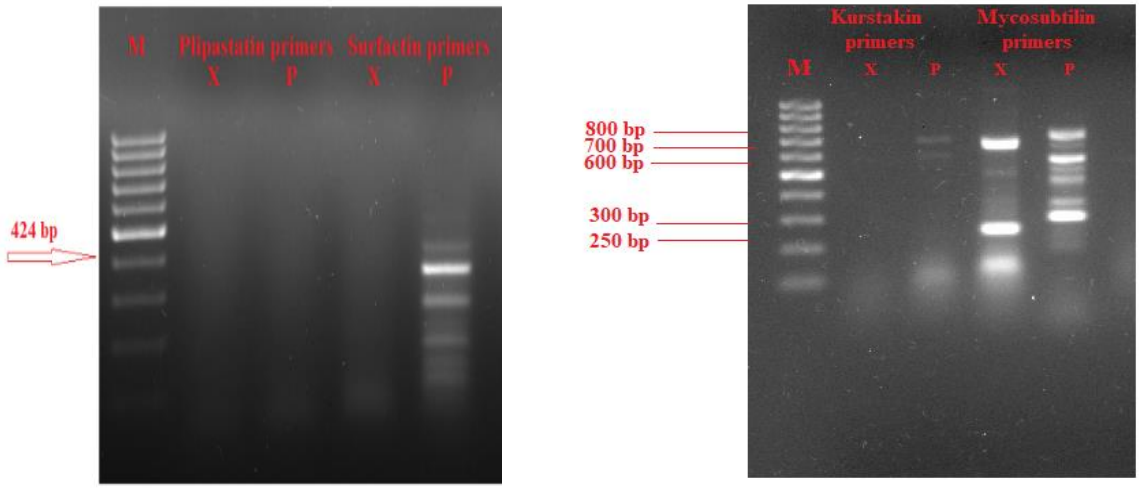


Fig. 3. Degenerate plipastatins, surfactins (left) and kurstakins, mycosubtilin primers (right) amplification with *Xenorhabdus* nematophila (X) and *Photorhabdus luminescens* (P) strains.

6. Gas Chromatography-Mass Spectrophotometry of Synthetases NRPS*:*

The GC-MS analysis for the purified culture of *Photorhabdus luminescens* PMG05 and *Xenorhabdus nematophila* XMG01 bacteria revealed several PKS-NRPs syntheses molecules. The main constituents were Piperidinone (44.09%) and Pyrrolidinone (35.04%) for *Photorhabdus* and *Xenorhabdus*, respectively.

Xenorhabdus nematophila XMG01 secreted the most achieved PKS-NRPS molecules, the main constituents were Piperidine, Photoditritide, Tetrone and Paromomycin, and other detected Rhiabdopeptides/Xenortid like peptides (RXPs) molecule by (4.53, 2.91, 2.80, 2.63 and 2.60%), respectively (supplementary material figure group).

7. Bioactivity Prediction of Produced Peptides by NRPs Synthetases of The Two Studied Strains:

Table 5 depicts the predicted bioactivity either antioxidant or antimicrobial for the generated peptides by the NRPSs of the two studied organisms.

Overall, the *Photorhabdus luminescens* show higher bioactivity than the *Xenorhabdus nematophila.* While the former organism contains three peptides: VWWWW; TFWWW; and WKW with high free radical scavenging activity, the latter includes only one peptide, KSGXPPKXK, with a moderate ability of free radical scavenging. Further, the two antimicrobial predictors show high scores for the abovementioned peptides of the *Photorhabdus luminescens.* In contrast, PeptideRanker alone scores a high antimicrobial activity for the same peptide with the moderate antioxidant activity of *Xenorhabdus nematophila.*

Table 5. Bioactivity prediction of the produced peptides by the NRPSs of *Photorhabdus luminescens* (PL) and *Xenorhabdus nematophila* (XN).

Peptide	An OxPe Pred FRS score*	An OxPe Pred CHEL score*	CA3 score*	Peptide Ranker score*	Organism
VWWWW	0,64442825	0,18638554	1	0,995236	PL
TFWWW	0,56553179	0,20245451	0,997	0,99418	PL
WKW	0,509471	0,20905162	0,999	0,978514	PL
KSGXPPKXK	0,47690618	0,28057849	NP	0,765577	XN
CC	0,43554223	0,25790083	0,011	0,952652	PL
GEEEEEE	0,4017294	0,22352321	0	0,0253491	XN
TVXX	0,3833369	0,26011485	NP	0,279368	PL
VFVV	0,33370715	0,21569948	0	0,147852	XN
VFITVVKFAAPV	0,32683617	0,17997791	0,651	0,190853	XN
KVRVP	0,32527718	0,18152958	0,001	0,111629	PL

* A score probability (0 - 1) of antimicrobial or antioxidant activity. NP: Not Performed
As the score exceeds, the possibility of being antimicrobial peptide increases.

DISCUSSION

According to the provided knowledge, the goals of this work are the investigated and characterize the produced NRPs molecules from *Photorhabdus* and *Xenorhabdus* isolates using a combination of chemical and biological and protocols. The nonribosomal peptides (NRPs) were unambiguously comprised of mono modular peptides, linear peptides photohexapeptides and cyclic lipopeptides (phototemtides). However, the C-terminal amine-containing linear peptides Rhiabdopeptide/Xeenortid-like peptides (RXPs) are a type of structurally varied nonribosomal synthetase exclusively found in *Photorhabdus* and *Xenorhabdus* (Cai *et al.*, 2017).

A typical workflow for the genomics-driven discovery of natural products, most useful for NRPs derived from gene clusters expressing NRPS. The identification and

activation of novel NRPS, coupled with the isolation and characterization of their metabolic products, currently represents one of the most promising avenues for the discovery of new NRPS (Tietz & Mitchell, 2016). The Piperidine, Piperidone, Pyrrolidinone endogenously α-pyrone moiety containing small molecules involved in a new cell-cell communication system leads to the expression of the *Photorhabdus* and *Xenorhabdus* clumping factor operon that causes clumping of cells and results in insect toxicity, this foundation agreed with (Kresovic *et al.*, 2015).

Photoditritide X is a new cyclic lipopeptide identified from *Xenorhabdus nematophila* XMG01, the ultimate distinctive structure is that including the uncommon homo nonproteinogenic amino acid, the incorporation of homo nonproteinogenic amino acids by nonribosomal synthetase is a significant contribution to the structural diversity of peptides nonribosomal synthetase by comparing with a ribosomal synthesis which usually including twenty proteinogenic amino acids. So, containing peptides is rare in microbial life, except for the discovery in marine-derived actinomycetes, cyanobacteria and various marine life cells (Saito *et al.*, 2001). Photoditritide revealed strong antimicrobial activity against several Gram-positive and negative bacteria, which may lead to protecting the nutrient-rich of insect cadaver from rival microorganisms in the complicated life cycle of nematode and its associated bacteria (Cha *et al.*, 2012).

The heterologously expressed cyclic lipopeptides are a type of important biochemically varied natural products, the major compound of Phototemtides antiparasitic revealed a selective antiparasitic activity against malaria disease causative agent, including (*Trypanosoma cruzi, Trypanosoma brucei rhodesiense, Leishmania donovani* protozoan parasites), beside (*Micrococcus luteus* G^{+ve}), molds of (*Saccharomyces cerevisiae*) and mammalian L6 cells (Götze *et al.*, 2017). Moreover, the most of Photohexapeptide revealed moderate antiprotozoal activity, despite it did not show any antimicrobial activity, further investigations pursue the other Photohexapeptide activities, especially in the complicated life cycle of nematodes and their associated bacteria (Hacker *et al.*, 2018). On the other side, Rhiabdopeptide/Xeenortid-like peptides (RXPs) are a class of structurally diverse NRPs and widespread in *Photorhabdus* and *Xenorhabdus*, the newly identified RXPs in this work from *Xenorhabdus nematophila* XMG01 further enrich the diversity of RXPs (Cai *et al.*, 2017).

The RXPs show polar putrescine or ammonia as the C-terminal amines and methionine as amino acid building blocks, which are distinctive characteristics in comparison with previously reported RXPs showing non-polar phenylethylamine or tryptamine as the main C-terminal amines and hydrophobic valine or phenylalanine as amino acid building blocks in most strains (Cai *et al.*, 2017). A recent investigation suggested that RXPs derivatives are consistent from two amino acids at least to eight amino acids at maximum, the most frequently incorporate amino acids are valine followed by leucine, tryptamine and *N*-methylated phenylalanine or phenylethylamine as the C-terminal amines (Grundmann *et al.*, 2014). the over-resilience of NRPS- RXP due to the mediating protein-protein of indistinctive docking domains interactions between the various non-ribosomal subunits (Hacker *et al.*, 2018).

The latest *in-Silco* analysis cleared that *Photorhabdus temperata* encodes more than one nonribosomal peptide synthase consisting of five modules termed by *PttBC*, these modules including initiated condensation domain which were expected to produce a PKS-NRPS lipopeptide composting from five amino acids (Cai *et al.*, 2017).

Generally, RXP genetic clusters encode one module of no more than four flexible use NRPS subunits. However, more details about mass spectrum analysis of *Photorhabdus* and *Xenorhabdus* culture extracts referred to the presence of several new RXPs showing another C-terminal amine such as ammonia and putrescine replaced tryptamine and

phenylethylamine, especially those were found in some *Xenorhabdus* wild-type species with antiprotozoal activity (Cai *et al.*, 2017).

Notably, the perfect nonribosomal syntheses determination depended only on the already sequenced genetic clusters closely related encodes. Although, caution must be taken within the bioinformatics predictions since not all Epimersion or Condensation/Epimersion domains should be active. Additionally, D-amino acids may be directly utilized by certain NRPSs, presumably epimerized by enzymes elsewhere in the genome and thus not easily discernable through bioinformatics. Thus, bioinformatic predictions are best trusted when they can be supported by some spectroscopic or synthetic evidence (Tietz & Mitchell, 2016).

Nevertheless, genome-sequencing projects have revealed that more than 6.5% of the overall genome sequence can be assigned to biosynthetic gene clusters of secondary metabolites (Bode *et al.*, 2012). Although in nature, *Photorhabdus* and *Xenorhabdus* have only been isolated from infected insects or their nematode hosts (Poinar & Grewal, 2012). However, the newest detected NRPSs or predicated molecules in this work will also introduce a new data source for present and future biological engineering purposes, particularly for cyclic peptides and cyclic PKS-peptides or for unordinary building blocks containing peptides (Wenzel *et al.*, 2005).

Finally, the introduced data is considered a great fortune for natural products microbiologists, especially since the microorganisms could be laboratory cultivated without their hosts. Moreover, the bacterial/nematodes entomopathogenic turn over *Photorhabdus* and *Xenorhabdus* species a promising fortune of new potentially bioactive molecules that are desperately needed for crops protection technology and agricultural discovery and development.

Conclusion:

The symbiotic association bacteria were isolated from the *Galleria mellonella* larval hemolymph infected with entomopathogenic nematodes juveniles of Steinernematid riobravis and Heterorhabditid bacteriophora. Biosynthetically, most natural molecules produced from *Photorhabdus* and *Xenorhabdus* are derived from polyketide synthases (PKS) or nonribosomal peptide synthetases (NRPS), or hybrids thereof PKS-NRPS. Usually, often, the big genetic clusters so far detected in *Photorhabdus* and *Xenorhabdus* are synthetized nonribosomal. The detection of the non-ribosomal peptides encoding genes in these isolated bacteria was carried out by four sets of degenerate primers designed on the conserved genetic sequence propose by the alignment of thiolation and adenylation domains involved in non-ribosomal synthetases genes biosynthesis in bacteria. These nonribosomal synthetases molecules functionally diverse polyketide peptides or cyclic peptides are essential and crucial to the complex life cycle of both nematode and its associated bacteria. On the other side, all the advanced genome mining confirmed that the bacteria of and *Photorhabdus* and *Xenorhabdus* still have a huge number of unknown non-ribosomal peptides encoding gene clusters that need to be discovered and further achieved investigations for these important molecules in the future.

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