Integral Characterization of the *16S rRNA* Gene of Non-sporulating Bacteria and Its Action Against *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae)

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Abstract

Brazil is the world's largest producer of soybean (*Glycine max*), an extremely important legume due to its source of proteins and essential oils for humans and animals, besides to its applications in the various branches of industry. The velvetbean caterpillar [*Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae)] is a great pest that affects this crop and has been controlled by chemical and biological pesticides based on *Bacillus thuringiensis*. The objectives of this work were to prospect soil microorganisms, to characterize them using the *16S rRNA* gene and to perform bioassays to analyze the lethality or subletality of these isolates against *A. gemmatalis* larvae. The DNA sequencing of the marker gene was complete, covering all conserved regions of it to determine the phylogenetic position of the isolates. Regarding to bioassays, subletality efficacy were low both for sporulant and for the non-sporulant bacterial strains tested. However, based on the signature by complete *16S rRNA* analyses of the non-sporulating bacterial isolates, new characteristics worth of studying and prospecting biotechnologically became available.

Keywords: lethality bioassay, biological control, velvetbean caterpillar

1. Introduction

The soybean (*Glycine max*) is one of the most important legumes in the world, providing an abundant source of protein and oil for humans and animals. It also provides a significant raw material for pharmaceutical and industrial processes. However, soybean crop is severely infected by the action of agricultural pests. The most important are *Chrysodexis includens*, *Anticarsia gemmatalis*, *Phakopsora pachyrhizi*, *Helicoverpa armigera*, and *Spofoptera frugiperda*. Worldwide the impact of pests on soybean yield concerns to losses of 11.0-32.4% (Savary, Willocquet, Pethybridge, Esker, McRoberts, & Nelson, 2019). The velvetbean caterpillar [*A. gemmatalis* Hübner (Lepidoptera: Erebidae)] is one of the main pests in soybean culture, causing large losses and a drop in grain productivity. It is distributed since the USA-Central region to Argentina, and in some indigenous islands. It is inserted throughout Brazil, but it is more frequent in the South. The immature form, a caterpillar, is the most important type concerning danger to plants, as it feeds on the leaves of soybeans and causes destruction of the plant's limbus and ribs. Thus, the final damage is related to the defoliation of the crop when the caterpillar is present in large populations (Gallo et al., 2002; Hoffmann-Campo et al., 2003; Fiaz et al., 2018). Biological control applied to soybean culture has already proven effective by decades (Moscardi, Souza, Castro, Moscardi,

& Szewczyk, 2011). This method of control prevents insect pests from reaching levels capable of causing great economic damage, in addition to having some advantages that override the use of chemical pesticides. Among advantages, could be mentioned its non-toxicity to man and the environment and its harmful specificity to pest insects (Simonato, Grigolli, & Oliveira, 2014; Dong et al., 2018). The major challenge for the use of entomopathogenic microorganisms in the management of pests, such as *A. gemmatalis*, is the selection of resistance due to the continuous use of biological formulations. These formulations in turn are basically composed by *Cry* and *Vip* proteins from *Bacillus thuringiensis*. Currently, there are several proteins that are synthesized through the different and abundant *Cry* and *Vip* gene classes. Therefore, due to the growing resistance of pest populations to a range of those proteins, more research in this area is necessary including the prospection of new bio-insecticidal molecules capable of combating or assisting the management of *A. gemmatalis* (Sosa-Gómez & Miranda, 2012). The molecular marker *16s rRNA* is a gene spread in bacteria species that can be used for taxonomic purposes, due its conserved and hypervariable regions occurring during evolution. Applying this widely used marker, it is possible to characterize a bacterium at the level of genus and species and to associate whether they have biotechnological potentials to be tested (Menna et al., 2006). Thus, predicting the acquired resistance to traditional control methods, the prospection of new microorganisms including non-sporulating bacteria with entomopathogenic potential becomes an attractive alternative for the biological control of insect pests. Hence, this work aimed to the taxonomic characterization and bioprospecting of bacterial isolates from the soil regarding the entomopathogenic potential against *A. gemmatalis*.

2. Method

2.1 Origin of the Bacterial Isolates

The work was carried out with ten non-sporulated bacterial isolates from the Laboratory of Applied Genetics (LGA) and two sporulated isolates from the Laboratory of Genetics of Bacteria and Applied Biotechnology (LGBBA), stored at FCAV/UNESP (Faculdade de Ciências Agrárias e Veterinárias/Universidade Estadual Paulista/Jaboticabal/Brazil). The LGA microorganisms used were isolated from soil samples from the Itaquerê Farm, Santa Fé S.A. Mill (Nova Europa/Brazil) (Figure 1). The samples were collected at a depth of 0-20 cm (Omori, Camargo, Goulart, Lemos, & Souza, 2016). The "LGA-EV" nomenclature refers to the isolates from soil of the vinasse master channel (which transports the vinasse from the industry to the field, under the action of gravity), while the "LGA-V" nomenclature refer to those from soil planted with sugarcane and vinasse irrigation. Non-sporulating isolates were previously and partially characterized by Almeida (2017). The bacterial isolates were obtained after homogenization and serial dilution of 1 g of soil sample in saline solution [NaCl 0.85% (w/v)], at 250 rpm for 30 min at room temperature. Dilutions from 10^5 to 10^8 were distributed in PEG medium [composed of (g/L): K_2HPO_4 (0.6), $MgSO_4$ (0.2), NaCl (0.1), yeast extract (1.0), glucose (10.0) and Agar (9.0); pH 6.9], plus cycloheximide (300 μg/mL), and incubated for 15 days at 28 °C. Different colonies were obtained and cultured in PEG broth for 48 h at 28 °C, 120 rpm, and stored at -80 °C in the presence of glycerol [20% (v/v)].
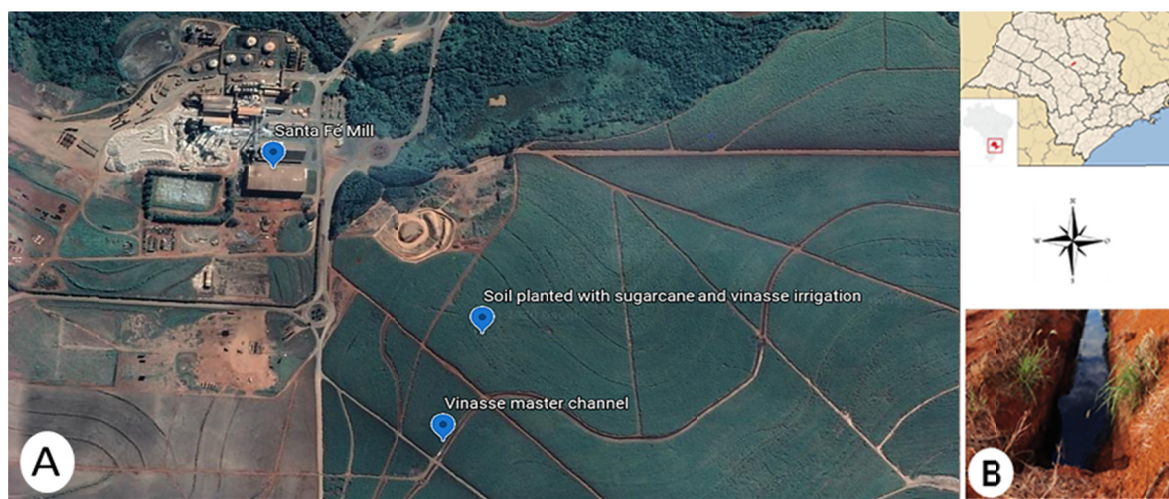

Figure 1. Sites of soil sampling at Fazenda Itaquerê (Nova Europa/Brazil). (A) Santa Fé S.A. Sugarcane Plant, with planting area. (B) Detail of the main vinasse distribution channel
(Images: Google Earth, Wikipedia and Omori, W.P.)

2.2 DNA Extraction and Amplification of the 16S rRNA Gene

About 1 ml of each culture obtained in PEG medium, corresponding to 50 mg of cells, was pelleted and washed in saline [NaCl 0.85% (w/v)] to proceed DNA extraction, based on the adaptation of the method developed to obtaining total DNA from bacteria (Marmur, 1961). Cell lysis was performed by adding lysozyme (20 mg/mL), in the presence of RNAse (50 μg/mL). Then, the DNA/protein complex was dissociated by denaturing with 2.0% (w/v) of the Sodium Dodecyl Sulfate (SDS) and increasing the ionic strength with 1 M Sodium Acetate, pH 5.2. The separation of DNA from other macromolecules happened by organic extraction with one volume of Chloroform:Isoamyl Alcohol [24:1 (v/v)] and the precipitation of DNA with two volumes of Ethanol. Excess of ethanol were evaporated in Concentrator Plus (Eppendorf) in option D-AL and without temperature adjustment. The DNA was resuspended in 100 μL of TE (10:1) pH 8.0, at 4 °C. After electrophoretic characterization [Agarose 0.8% (w/v)] and DNA quantification in a Nanodrop 1000 Spectrophotometer (ThermoScientific-Uniscience), the DNA samples were stored at -20 °C. Following DNA purification, 1.5 Kb amplicons were obtained from the *16S rRNA* gene by PCR, to determine the molecular signature of each isolate. The universal primers (oligonucleotides) fD1 (8-27) <5'-AGA GTT TGA TCC TGG CTC AG-3'> and rD1 (1525-1541) <5'-AAG GAG GTG ATC CAG CC-3'> (Weisburg et al., 1991), described for the target regions of *Escherichia coli* K12, were applied. The amplification reaction consisted of: 40.0 ng of target DNA; 7.5 pmol of each universal primer; 1.75 mM of $MgCl_2$; 0.2 mM of triphosphate deoxyribonucleotides (dNTPs); 2.0 ml of 10 X buffer; 1U of the Taq DNA Polymerase (Invitrogen); in a final volume of 20 μL. The amplification program was: 94 °C/5 min; 35 cycles at 94 °C/30 s, 56 °C/40 s, 72 °C/90 s; 72 °C/7 min, performed on a PTC-100™ programmable thermal controller (MJ Research, Inc.). The size and purity of the amplicons were verified by electrophoresis [1.5% agarose (w/v)]. Amplicons were processed for the isolates LGA-V05_13, LGA-V05_6, LGA-V20C, LGA-V20F e LGA-V05_22.

2.3 Sequencing of 16S rRNA Amplicons

The DNA sequences of the amplicons were determined to identify the bacterial affiliations. DNA sequencing reactions were performed without the need for further amplicon purification. For DNA sequencing, in addition to the external primers (fD1 and rD1), primers targeted to the internal regions of *16S rRNA* were added, designed by L. M. Cruz and released by Menna et al. (2006): 362f (339–362) <5′-CTC CTA CGG GAG GCA GCA GTG GGG-3′>, 786f (764-786) <5′-CGA AAG CGT GGG GAG CAA ACA GG-3′> and 1203f (1179-1203) <5′-GAG GTG GGG ATG ACG TCA AGT CCT C-3′>. The coverage of concatenated amplicons averages up to 1.3 Kb. The sequencing reactions were standardized to a final volume of 10 μl, using the following reagents: 0.5 pmol of primer; 1X sequencing buffer; 1.0 μl of BigDye enzyme (Thermo Fisher Scientific); 7 ng of total bacterial DNA (for primers fD1 and rD1) or 7 ng of *16S rRNA* amplicon (for primers 362f, 786f and1203f). The amplification program was: 96 °C/2 min; 40 cycles at 96 °C/10 s, 52 °C/20 s, 60 °C/4 min. Each sequencing reaction was precipitated with 80 μl of 75% (v/v) isopropanol and subjected to successive washes in cooled 70% (v/v) ethanol to remove the labeled Dideoxynucleotide triphosphates (ddNTPs). The samples were dried in laminar flow for 1 h, resuspended with 9.0 mL of Hi-Di Formamide (4311320-ABI Prism) and denatured at 95 °C for 5 min. The DNA sequencing was performed on the ABI 3100 Automated Capillary Sequencer (PerkinElmer).

2.4 Analysis of the Molecular Signatures of Bacterial Isolates Using Bioinformatics

The electropherograms generated by the capillary sequencer were evaluated for Phred ≥ 20 quality. The *16S rRNA* gene was concatenated at approximately 1.5 Kb by the Phred/Phrap/Consed software package (Gordon, Abajian, & Green, 1998). After assembling the *16S rRNA* contigs, the sequences were subjected to nucleotide similarity query against the GenBank (non-redundant) nucleotide database (National Center for Biotechnology Information-NCBI), using the BLASTn tool (Altschul, Guish, Miller, Myers, & Lipman, 1990). The MAFFT v7.215 program (Katoh, 2002) was used for sequences alignment. The Phangorn package (Schliep, 2011) in R (R Development Core Team, 2011) was carried out to search the best nucleotide replacement matrix, to correct mutation rates in the alignment data. The alignment matrix and the nucleotide substitution matrix were used in the online IQTREE program (Trifinopoulos, Nguyen, Von Haeseler, & Minh, 2016) to build the phylogenetic tree based on Maximum Likelihood (ML). The optimization of ML nucleotide frequencies, Ultrafast bootstrap analysis and 1,000 repetitions options were used for the bootstrap and SH-aLRT branch tests.

2.5 Creation and Maintenance of A. gemmatalis

The larvae were maintained with an artificial diet adapted by Greene, Leppla and Dickerson (1976), in which two caterpillars were deposited for each 70 ml flask, remaining until a pupal phase. Then, 20 to 25 pupae were deposited in 1 liter pots with paper lining. After that, the pots were closed and the pupae remain until they reach the adult stage. The adults were placed in cages made of PVC tubes (30 cm high × 28 cm in diameter), lined

internally with white sulfite paper on the sides and maintained with a liquid diet based on 2.5% beer and 10% honey.

2.6 Bioassays With Non-sporulating and Sporulating Isolates

Non-sporulating isolates: 10 non-sporulating isolates were used. In addition to isolates LGA-V05_13, LGA-V05_6, LGA-V20C, LGA-V20F e LGA-V05_22, the ones processed by Miranda (2020) were used, namely LGA-V05D, LGA-EV05, LGA-EV08, LGA-V20B and LGA-V20G. Each of the isolates were grown in TY broth (content for 1 L: 5.0 g of Tryptone, 3.0 g of yeast extract and 0.87 g of NaCl; pH 7.0) in a pre-inoculum of 25 ml under agitation of 120 rpm at 27 °C, for 24 h (Shaker Incubator Model G25 New Scientific). After this period, the pre-inoculum was poured into a 75 ml inoculum, where it was stirred at 200 rpm, 27 °C for 48 h. The diet used in the bioassays was the one standardized by Greene et al. (1976) with modifications, without adding Nipagin and Tetracycline (Fungicide and Bactericide, respectively). The diet was placed up to 1/3 of the acrylic pots (29 mm high × 20 mm in diameter) together 75 μl of the bacterial culture broth, after diet drying, were added. A caterpillar was deposited in each pot after broth drying. Each treatment consisted of 50 repetitions, followed by the control (H_2O), totaling 11 treatments. The bioassay was evaluated after 10 days of setting up the experiment.

Sporulating isolates: Each of the *B. thuringiensis* isolates, designated LGBBA-1304 and LGBBA-1355, were grown in LB broth containing salts (content for 1 L: Glucose 1.0 g; Nutritional broth 8.0 g; Yeast extract 5.0 g; $MgSO_4$ 0.3 g; Tryptone 10.0 g; $FeSO_4$ 0.02 g; $ZnSO_4$ 0.02 g; $MnSO_4$ 0.02 g; NaCl 5.0 g; pH 7.5) in a pre-inoculum of 62.5 ml under agitation of 120 rpm at 27 °C for 24 h. After this period, the pre-inoculum was poured into an inoculum of 187.5 ml, where it was stirred at 200 rpm at 27 °C, for 48 h. The diet used in the bioassays was the one standardized by Greene et al. (1976) and processed as described for the non-sporulating bioassays. For the culture broth containing spores, six concentrations were defined for each isolate: 3×10^4, 3×10^5, 3×10^6, 3×10^7, 3×10^8 and 3×10^9. Each concentration consisted of 30 repetitions, followed by the control (H_2O) and a treatment with the commercial product Dipel$^®$ (16.80 g in 0.5 L of water), totaling 8 treatments. The bioassay was evaluated after 7 days of setting up the experiment.

2.7 Analysis of the Bioassays

The results of the bioassays were obtained through the analysis of the R software with the Scott-Knott test at 5% probability.

3. Results

3.1 Analysis of Bacterial Isolates by Complete Sequencing of the 16S rRNA

Five different types of *16S rRNA* sequences were found for the bacteria analyzed. The sequences correspond to practically the complete gene coverage (1540 Kb), determined by the superposition of contiguous sequences that were produced by amplicons of different reactions. The concatenated size of the *16S rRNA* sequences involved in the alignment varied from 777 to 1477, the smallest for the isolate LGA-V05_13 and the largest for LGA-V05_22. Regardless of this variation, the coverage by aligning with databank correspondence reached 100% for all isolates, except LGA-V20C, which reached the 99% mark (Table 1).

In this analysis, the highest percentage of identity, or maximum identity, for the sequences of each alignment corresponded to 99%. The query similarity corresponds to the degree to which the nucleotides are related, expressed in the percentage of identity between the sequences. The identity between the sequences shows a measure in which two nucleotide sequences have the same residues in the same alignment position.

The results were highly corroborated by the high level of similarity between the sequences (minimum of 99%). The BLASTn tool applied is used to search shorter query sequences and pairwise comparison between species. The sequences were subjected to a nucleotide similarity query in the non-redundant nucleotide database [Nucleotide collection (nr/nt)], which is a collection of sequences from various sources, including the entire GenBank (National Biotechnology Information Center-NCBI), EMBL (European Molecular Biology Laboratory), DDBJ (DNA Database of Japan) and PDB (Protein Database).

After similarity screening on sequences available in the database, the bacterial isolates were classified and distributed in two distinct phyla: Proteobateria and Firmicutes. The LGA-V isolates obtained representatives in both phyla, with predominance in Proteobacteria. There was distinction among genera of each of five isolates, without equality. At the species level, the isolates also differed from each other, with no repetition, with the greatest predominance of species in the phylum Proteobacteria.

Table 1. Similarity among *16S rRNA* sequences from bacterial isolates based on GenBank, using the BLAST nucleotide tool (BLASTn)

Isolates[1]	Length (bp)	Acess number	Family	Lineage[2]
LGA-V05_22	1477	CP017707.1	Chromobacteriaceae	*Chromobacterium vaccinii* strain 21-1
LGA-V20F	810	KU248158.1	Burkholderiaceae	*Cupriavidus* sp. strain UYMRaPT14
LGA-V05_6	1240	KX215153.2	Sphingomonadaceae	*Novosphingobium* sp. strain SA925
LGA-V20C	1292	KY426389.1	Rhizobiaceae	*Rhizobium* sp. strain Moz100
LGA-V05_13	77	MF405756.1	Bacillaceae	*Bacillus wiedmannii* strain cl65

Note. [1] LGA-V05_22, LGA-V20F, LGA-V05_6 and LGA-V20C: Proteobacteria; LGA-V05_13: Firmicutes.

[2] Alignment data: Coverage = 100%, except LGA-V20C = 99%; Identity = 99%; e-value = 0.

3.2 Phylogeny of Isolates Based on Complete Sequencing of the 16S rRNA

According to the phylogeny obtained by sequencing the *16S rRNA*, all five isolates were grouped into different phyla and species. The LGA-V05_22 isolate was characterized as *Chromobacterium vacinni* (strain type: MWU205 = ATCC BAA-2314 = DSM 25150), which had its first isolation by Soby, Gadagkar, Contreras, & Caruso (2013), from swamp soil cultivated with blackberry in Truro (MA, USA) (Figure 2). The LGA-V20F isolate was similar to *Cupriavidus necator* (strain type: N-1 = ATCC 43291 = CCUG 52238 = CIP 103161 = DSM 13513 = LMG 8453), isolated by Makkar and Cassida (1987) from a soil near the University Park (PA, USA) (Figure 2). The LGA-V05_13 isolate was similar to *Bacillus toyonensis* (strain type: BCT-7112 = CECT 876 = NCIMB 14858), originally *Bacillus cereus* (Frankland & Frankland, 1887) var. toyo, was isolated in 1966 in a laboratory in Japan, during a selection of pure cultures of microorganisms for use as probiotics in animal nutrition (Jiménez et al., 2014) (Figure 3). The LGA-V20C isolate was similar to *Rhizobium vallis* (strain type: CCBAU 65647 = HAMBI 3073 = LMG 25295), originally isolated by Wang et al. (2011) in the red river valley, in Yunnan province, China (Figure 2). The LGA-V05_6 isolate obtained similarity to *Novosphingobium lindaniclasticum* (strain type: LE124 = CCM 7976 = DSM 25409), which was isolated by Saxena et al. (2013) in a landfill contaminated with Hexachlorocyclohexane (HCH) located in Lucknow, India (Figure 2).
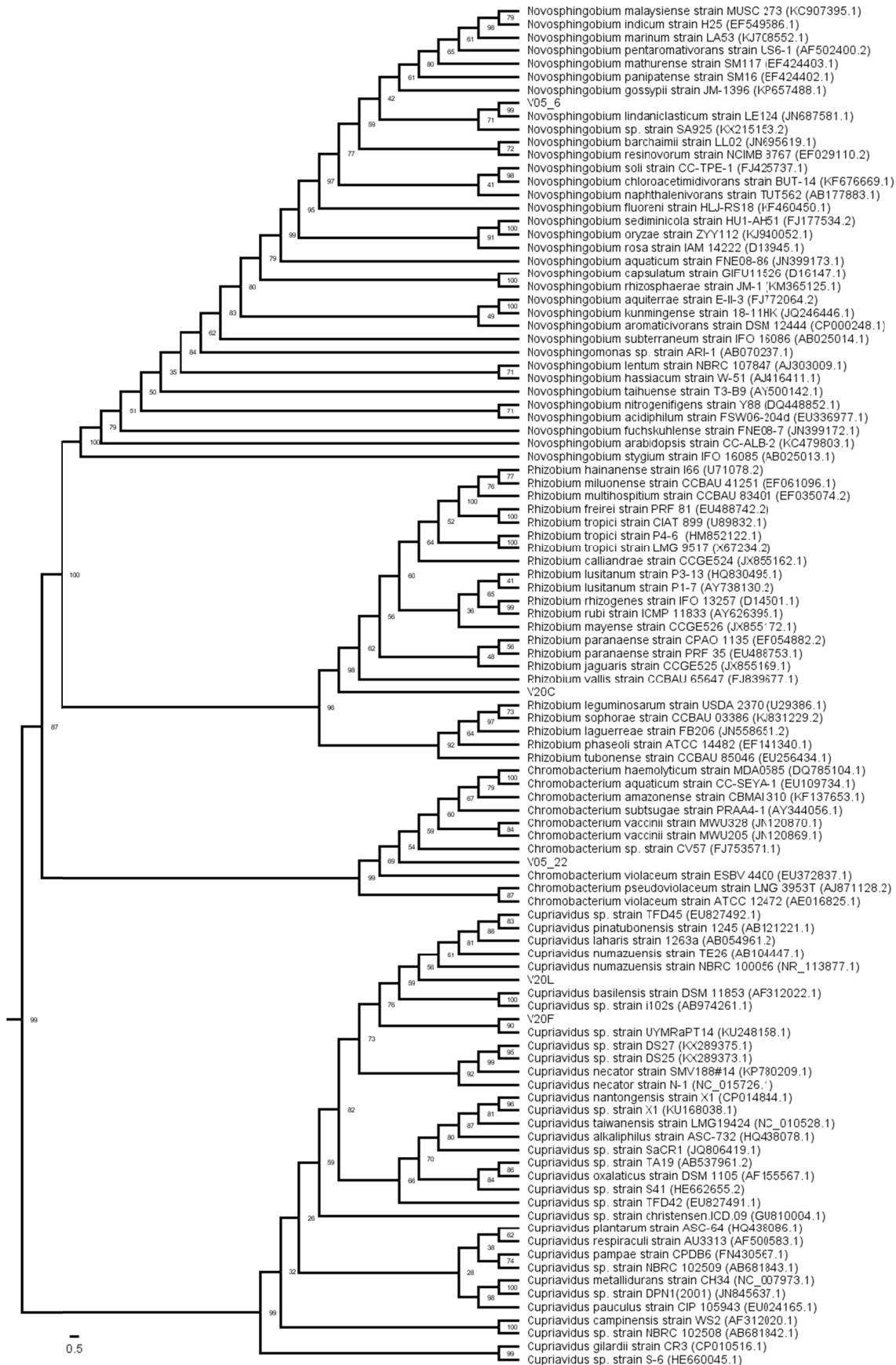
Figure 2. Dendrogram corresponding to isolates LGA-V05_22, LGA-V20F, LGA-V05_6 and LGA-V20C, characterized by the *16S rRNA* genetic marker using the Neighbor-Joining method and 1000 bootstrap
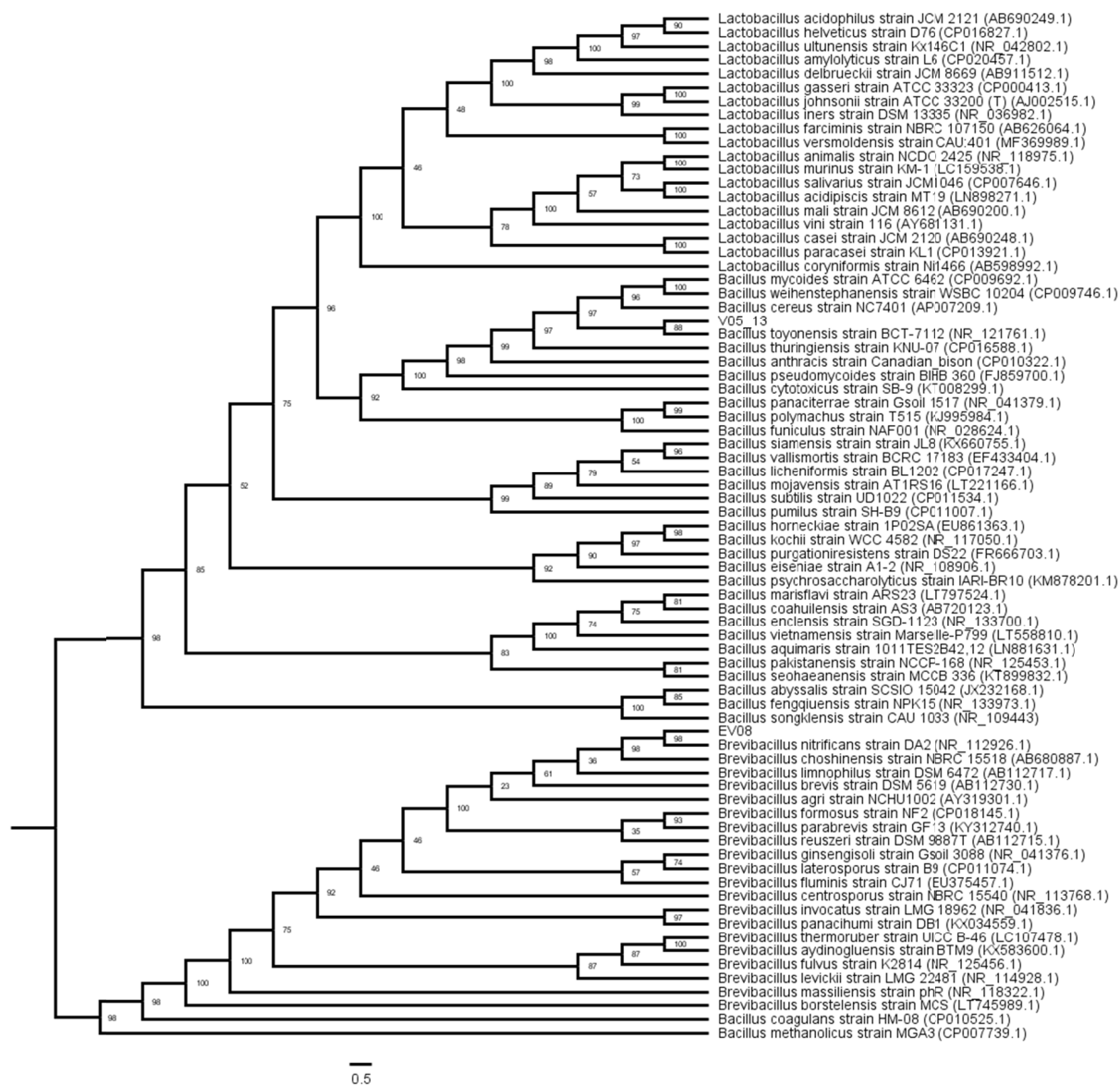
Figure 3. Dendrogram corresponding to isolate LGA-V05_13 characterized by the *16S rRNA* genetic marker using the Neighbor-Joining method and 1000 bootstrap

3.3 Bioassays for Non-sporulating and Sporulating Isolates

Subtle differences on subletality for *A. gemmatalis* were noted according to the Scott-Knott test at 5% probability for the all the non-sporulating isolates used in the experiment in relation to the control (H_2O), exception for LGA-V05D (Table 2). Regarding to sporulating isolates LGBBA-1355 and LGBBA-1304, both presented subtle subletality for *A. gemmatalis* particularly with the greater concentrations of spores until dilution 3×10^7. Subsequent dilutions presented no significant results compared to control (H_2O). The treatment with the commercial product Dipel® was effective and a mortality of 100% of the larvae was obtained (Table 3). The maintenance of experiment and treatments focusing on sporulating isolates showed that LGBBA-1355 was not effective on pupae weight reduction. On the other hand, LGBBA-1304 presented subtle decrease on pupae weight by 3×10^7 and 3×10^7 dilutions (Table4).

Table 2. Sublethal bioassay for *Anticarsia gemmatalis* using non-sporulating isolates.

Non-sporulating bacteria (R)	Larvae weight (g lar^{-1})
LGA-EV05	0.075±0.011 b
LGA-EV08	0.076±0.012 b
LGA-V05_13	0.075±0.009 b
LGA-V05_6	0.075±0.009 b
LGA-V20B	0.075±0.009 b
LGA-V20C	0.076±0.009 b
LGA-V20F	0.074±0.011 b
LGA-V20G	0.072±0.009 b
LGA-V05_22	0.075±0.010 b
LGA-V05D	0.080±0.012 a
Control (H$_2$O)	0.079±0.012 a
Anova	$p = 0.019975$

Note. Means followed by different letters differ by Scott-Knott test at 5% probability.

Table 3. Sublethal bioassay for *Anticarsia gemmatalis* using two strains of *Bacillus thuringiensis*.

Concentrations	Larvae weight (g)	
	LGBBA 1355	LGBBA 1304
3×10^4	0.029±0.013 b	0.014±0.003 c
3×10^5	0.037±0.010 b	0.020±0.008 c
3×10^6	0.048±0.024 a	0.025±0.015 b
3×10^7	0.041±0.017 b	0.028±0.019 b
3×10^8	0.038±0.017 b	0.019±0.011 c
3×10^9	0.052±0.022 a	0.021±0.019 c
Control (H$_2$O)	0.044±0.020 a	0.044±0.020 a
Control (Dipel)	*	*
Anova	$p = 0.0002825$	$p = 4.3 \times 10^{11}$

Note. Means followed by different letters differ by Scott-Knott test at 5% probability.

* Low number of larvae for analysis due to high mortality.

Table 4. Pupae-weight bioassay for *Anticarsia gemmatalis* on effect of two strains of *Bacillus thuringiensis*

Concentrations	Pupae weight (g)	
	Isolate LGBBA-1355*	Isolate LGBBA-1304
3×10^4	0.225±0.038 a	0.226±0.024 a
3×10^5	0.208±0.038 a	0.255±0.030 a
3×10^6	0.199±0.030 a	0.236±0.026 b
3×10^7	0.239±0.030 a	0.242±0.037 b
3×10^8	0.210±0.028 a	0.248±0.034 a
3×10^9	0.240±0.027 a	0.238±0.030 b
Control (H$_2$O)	0.252±0.037 a	0.252±0.037 a
Anova	$p = 0.34944$	$p = 0.038379$

Note. Means followed by different letters differ by Scott-Knott test at 5% probability.

*Anova not significant.

4. Discussion

The use of *16S rRNA* DNA sequencing is potentially feasible, because through this technique, identification at the level of gender and even species is provided for some bacterial strains that do not fit any recognized biochemical profile The gene also has approximately 1500 base pairs, being considered large enough for the

purposes of bioinformatics analysis. The use of *16S rRNA* gene sequences for molecular characterization and study of bacterial phylogeny of microorganisms has been used for several years and is considered by far the most applied technique for this interest. *The 16S rRNA* is the most used marker for several reasons, including the fact that it is universal in the Eubacteria and Archaea domains, in addition to offering regions with conserved and hypervariable domains that evolutionarily distinguish these microorganisms (Janda & Abbott, 2007).

The isolates of the present work had their phylogeny done through the complete DNA sequencing of *16S rRNA* gene. The data obtained were compared to GenBank turning possible the determination of genera of these microorganisms with 100% coverage for all isolates except the LGA-V20C isolate (99% coverage), and 99% identity for all isolates, namely: LGA-V05_22 (*Chromobacterium vaccinni*), LGA-V20F (*Cupriavidus* sp.), LGA-V05_6 (*Novosphingobium* sp.), LGA-V20C (*Rhizobium* sp.) and LGA-V05_13 (*Bacillus wiedmannii*). Note that for some bacteria, the characterization did not reach the species name as GenBank was unable to support the characterization at that level. Therefore, bacteria were related among them according to the GenBank signature and by phylogenetic proximity.

Previously, Almeida (2017) had partially characterized four of these five isolates, namely LGA-V05_22, LGA-V20F, LGA-V20C and LGA-V05_13, and compared them with the GenBank sequences. Previous results allowed characterization on genera and species levels for all tested, except for LGA-V20C which was characterized only at the level of genus. The partial characterization carried out by Almeida (2017) attributed the following genera and species to the isolates: LGA-V05_22 (*Chromobacterium violaceum*), LGA-V20F (*Arthrobacter echigonensis*), LGA-V20C (*Acinetobacter* sp.) and LGA-V05_13 (*Bacillus cereus*). However, this DNA sequencing was incomplete since the author used only the universal primer FD1. The milestone of the present work is the complete DNA sequencing of *16S rRNA* genetic marker through the universal primers FD1 and RD1, besides the internal primers designed by L.M. Cruz and described by Menna et al. (2006) covering internal region (1.5 Kb). These analyses yield greater credibility to the complete characterization of bacteria.

The genus *Chromobacterium* (LGA-V05_22) presents gram-negative bacteria that are usually isolated from fresh water and soil. Tropical and subtropical isolates from this genus have been associated with infections considered rapid and lethal in humans and other mammals (de Siquera et al., 2005; Ajithdoss, Porter, Calise, Libal, & Edwards, 2009; Yang & Li, 2011). This genus has a property that is the composition of purple pigments, such as violacein and deoxiviolacein, although there are also isolates found in nature that do not produce these pigments (X. Y. Han, F. S. Han, & Sigal, 2008; Yang, 2011). These pigments have been extensively investigated due to their antibiological capacity, as inhibition of chytrids, filamentous fungi, viruses, bacteria, protozoa and human tumor cells (Matz et al., 2004; Kodach et al., 2006; Durán et al., 2007; Becker, Brucker, Shwantes, Harris, & Minbiole, 2009).

C. violaceum is a gram-negative bacterium, usually isolated from soil and fresh water. Its occurrence in humans has been reported several times and it is a highly opportunistic pathogen that is related to severe sepsis and mortality. Infection of this bacterium in humans occurs through exposure of open wounds in contact with the waters of streams, rivers and lakes, and also in contaminated soil (Thwe, Ortiz, & Wankewicz, 2020). Studies on violacein, a violet-colored substance, have shown effective effects against several bacteria, especially gram-positive ones, even combating multidrug-resistant strains of *Staphylococcus aureus* (Choi, Lim, Cho, & Mitchell, 2020). Violacein is detected in several environments and its role should be widespread regarding habitats and microorganisms (Choi, Yoon, Lee, & Mitchell, 2015).

C. vacinni is an aerobic, gram-negative and rod-shaped bacterium, having a size between 0.3±0.555 by 1.19±0.0198 with a relatively short flagellum (Soby et al., 2013). Although there are no studies on the role of this bacterium in its natural environment, bacteria of its kind are violacein producers in addition to having a role as biological-control agents against *Aedes* larvae. They also show effectiveness in the control of *Plutella xylostella* moth. This pest causes damage to a variety of crops such as sugar cane, cotton and rice (Vöing, Harrison, & Soby, 2015).

Bacteria from the genus *Cupriavidus* (LGA-V20F) present gram-negative rod-shaped cells peritrically flagellated. They can perform chemoheterotrophic or chemolitotrophic activities. Its metabolism is oxidative and several carbohydrates can be used as the sole source of carbon. Species from this genus can occur both in natural habitats and soil, but also in humans when they are weakened (Vandamme & Coenye, 2004). Poly (3-hydroxybutyrate) [P (3HB)] belongs to the group of polyhydroxyalkanoate (PHA). PHA is a biopolymer synthesized by many Bacteria and Archaea, among them the species *C. necator*. Biodegradability and production from renewable resources are some of the advantages conferred to this compound, which has numerous recent

applications such as tissue engineering and protein purification. PHA is considered a material on the rise for the replacement of petrodegradable plastic (Biglari, Orita, Fukui, & Sudesh, 2020).

Bacillus corresponds to a wide genus in a range of aspects and its name is an epithet to "little rod". This genus is quite representative, comprising 341 cited species, with *B. subtilis* as the type species (Miller et al., 2016). *B. wiedmannii* (LGA-V05_13) has its nomenclature making an apology to Martin Wiedmann ("wiedmannii"), a modern researcher at Cornell University who has added knowledge to *Bacillus* biology. The nomenclature of *B. toyonensis* alludes to the Toyo Jozo Japanese Company ("toyonensis"). A strain has been used for more than 30 years as an active component of the TOYOCERIN® product, a food supplement intended for the nutrition of pigs, poultry, cattle, rabbits and for aquaculture (Williams, Burdock, Jiménez, & Castillo, 2009).

Phylogenetic analyzes of the sequences of the *16S rRNA* and *rpoB* genes (β subunit of RNA polymerase) show that *B. toyonensis* and *B. wiedmannii* are closely related and form a monophyletic clade within the *B. cereus* group. The species closest to these lines was *B. anthracis* (Ames strain) and the most distant was *B. cytotoxicus* (NVH 391-98T strain) (Miller et al., 2016). Isolate LGA-V05_13 corroborate this characteristic showing the same pattern, although our data was based only on *16S rRNA* indicating the reliability of these data and technique. However, the group containing LGA-V05_13 was shown to be more closely related *to B. thuringiensis* (KNU-07 strain). A more discriminatory phylogenetic analysis is important for the *B. cereus* group even knowing that species in this group cannot be delineated based only on *16S rRNA* sequences (Liu et al., 2015).

It should be noted that the group *B. cereus* includes pathogenic and non-pathogenic species of wide ecological diversity, which are closely related, being gram positive bacteria, facultative anaerobes and spore-forming (Priest, Barker, Baillie, Holmes, & Maiden, 2004). Pathogenicity and the ability to cause disease and food toxicity had been the main source of interest for the group. However, the ecological diversity of the group has added potential interest in the saprophytic and symbiotic (commensal and mutualistic) lifestyles associated to the roots of plants and intestines of insects and mammals. The diversity of ecological niches consists on the varied and competitive role of toxins produced by the group, affecting the dynamics of the microbial communities involved (Ceuppens, Boon, & Uyttendaele, 2013).

Bacteria of the genus *Rhizobium* (LGA-V20C) are nitrogen fixers. They can reduce N_2 in ammonia after the formation of nodules in the roots and/or stem of leguminous plants (Wang et al., 2011). The *Rhizobium vallis* species show its cells in the form of mobile rods, gram-negative and without spore formation, measuring 1.0-1.5 μm × 2.0-3.5 μm (Wang et al., 2011). The roots of plants grown in the soil not only have the function of absorbing nutrients, but also provide places for the association of several microorganisms that inhabit the rhizosphere soil. The microbiota of vegetables includes fungi, protozoa and bacteria that can negatively influence their crop production. Beneficial relationships can also happen, such as the symbiosis of several species of microorganisms in the root of leguminous plants (Xu et al., 2020).

The genus *Novosphingobium* (LGA-V05_6) was first described by Yabuuchi et al. (1990) and was later reviewed by Takeuchi et al. (2001) based on phylogenetic and chemotaxonomic analyzes. So far this genus has more than 50 species validly described and published. Some microorganisms in this group have the ability to degrade Hexachlorocyclohexane (HCH), a cyclic saturated chlorinated hydrocarbon that has been widely studied and used in the control of agricultural pests since the 1950s. *N. lindaniclasticum* is a gram-negative, aerobic, non-flagellated, mobile, spore-free bacterium (1.3 μm long and 0.7 μm in diameter) primary isolated from HCH dumpsite (Saxena et al., 2013). By the other hand, *N. aromaticivorans* has been associated with diseases once it can initiate primary biliary cirrhosis (Kaplan, 2004).

Regarding the subletality for *A. gemmatalis* inoculated with non-sporulating isolates, it was noted that all bacterial strains tested showed little but significant effect on reduction of larvae weight compared to control, but the isolate LGA-V05D corresponding to *Arthrobacter echigonensis* (Miranda, 2020) showed no effect. As expected for both sporulating bacterial strains the subletality rate was more prominent than those observed for non-sporulating isolates, while LGBBA 1304 was yet more effective on larvae weight reduction. It was noted that for both isolates the treatments with the highest concentration (lowest dilution) showed higher subletality index when compared to the treatments with lower concentrations and control. In fact, data from lower concentrations ($< 3 \times 10^7$ dilution) tested were not coherent due the low quantity of spores present in the bioassay. Absolute mortality of larvae was observed just for Dipel® treatment considering this commercial product is indicated for soybean pest control.

Physiological development of *A. gemmatalis* larvae until pupae stage was not prevented by sporulating strains. However, LGBBA-1304 was able to reduce pupae weight in same way based on 3×10^6 and 3×10^7 dilutions,

which corroborates this strain as the best effective against *A. gemmatalis* in all our treatments. We do not exclude that this fact should be related to physiological conditions of larvae applied in the tests instead of bacterial strains potential. Although Barbosa (2017) testing B. thuringiensis strains against *Helicoverpa armigera* did not obtain significant results, the same strains showed significant and satisfactory levels of mortality for *Spodoptera eridania*.

Even if the strains of *B. thuringiensis* tested against *A. gemmatalis* did not have a prominent effect on subletality and larvae development, they may have toxicity effects on other agricultural pests. It is extremely important to continue research on non-sporulating bacteria and their potential for biological control not only of spotlight pests, but also for those able to control other bacterial, fungal and viral pathogens. Regarding this, bacteria of the genus *Chromobacterium* listed above stand out due their potential for biological control on pests' moths and disease vectors, besides its antitumoral potential in human cells. Thus, the accurate and promising taxonomy of non-sporulating isolates revealed perspectives regarding their biotechnological properties which could be prospected in the future.

5. Conclusions

The complete DNA sequencing and the taxonomy based on *16SrRNA* genetic marker for bacterial isolates from agriculture fields were possible and accurate indicating biotechnological potential for those strains. Though bioassays for non-sporulating and sporulating isolates showed low effects on the subletality of *A. gemmatalis* larvae and pupae, they turn promising for bioprospection of activities when using primers that allow the coverage of the whole region of *16S rRNA*.

Acknowledgements

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