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Taxonomy of Non-sporulating Bacteria Tested in Biological Control Against *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae)

Mariana Davanzo Miranda[1], Higor de Oliveira Alves[1], Ricardo Antônio Polanczyk[2], Joacir do Nascimento[2] & Jackson Antônio Marcondes de Souza[3]

[1] Postgraduate Program in Agricultural and Livestock Microbiology, Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil

[2] Department of Plant Health, Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil

[3] Department of Biology, Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil

Correspondence: Jackson Antônio Marcondes de Souza, Department of Biology, Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Rodovia Paulo Donato Castellane, Km 5, 14884-900, Jaboticabal, São Paulo, Brazil. E-mail: jackson.marcondes@unesp.br

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Abstract

The complete analysis of the *16S rRNA* gene of some non-sporulating bacterial isolates from the soil led us to a reliable taxonomic classification, allowing us to explore its entomopathogenic potential against insect-pest *Spodoptera frugiperda*. Many studies have shown the rapid development of resistance of *S. frugiperda* in relation to the methods that have been used in recent decades. Therefore, new research exploring the potential of new soil isolates is important. Thus, a comparative study between the complete and partial taxonomic classification based on *16S rRNA* gene was realized as the basis for biological studies. Non-sporulating bacterial isolates were used in lethality bioassays against *S. frugiperda* larvae to compare bioassays efficacy using *Bacillus thuringiensis* (Berliner) (Bt) strains and the commercial product Dipel®. The results confirmed the strong resistance of this pest-larvae once it developed until the adulthood phase in all bioassays, applying sporulating or non-sporulating bacteria. Furthermore, an accurate phylogenetic position of the non-sporulating bacterial isolates become possible showing basically three species: *Brevibacillus nitrificans*, *Curtobacterium* sp. and *Arthrobacter echigonensis*. Besides the new biotechnological options for those bacterial isolates, according to the results further research should be done with new bacterial isolates in order to discover its potential to control *S. frugiperda*, thus assisting Bt in pest control.

Keywords: *16S rRNA* gene, lethality bioassay, Fall armyworm-cartridge larva.

1. Introduction

The maize (*Zea mays*) has become an important worldwide crop representing around 13% of the total agricultural area (Didoné, Silva, Ceccon, & Teixeira, 2018). Apart from being one of the most important exporters of maize grain, Brazil became self-sufficient in the domestic market for this crop (Trojan, Dalla Pria, & Castro, 2018). Maize plants are cultivated in all Brazilian states while the Midwest, Southeast and South regions are the ones that stand out in agricultural production. The states of Mato Grosso, Mato Grosso do Sul, Goiás, Minas Gerais, Paraná and Rio Grande do Sul contribute to 82% of the total Brazilian production (USDA, 2023). The *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) larva is a polyphagic pest that preferentially attacks crops of the Poaceae family, and consequently is the main maize pest in North and South America (Blanco et al., 2010). The larva in the first instars attack the leaves and during their development start to scrape the leaf blade, causing damage at all stages of maize development, infesting ears and feeding directly on growing grains (Siebert et al., 2012; Baudron et al., 2019). Fall armyworm or cartridge larva (*S. frugiperda*) has quickly developed resistance to several *Bacillus thurigiensis* (Bt)-based biological controls due the strong selection pressure imposed by the extensive and disseminated use of transgenic plants, that contain Bt genes in

their genome, and biopesticides containing Bt proteins (Sisay et al., 2019). The growth of resistance against Bt is one of the major challenges that has been faced in recent years in agricultural crops (James, 2015; Yang, Wang, & Kerns, 2022). Bt biological control has been used for more than two decades and at least four resistant species of pests against maize hybrids have already been found, in this short period of time (Tabashnik, Brévault, & Carrière, 2013). One of those pests is the fall armyworm (Storer et al., 2010). The first observed case of *S. frugiperda* resistance to Bt maize was first detected in TC1507 maize fields in Puerto Rico in 2006 (Storer et al., 2010). Resistance of *S. frugiperda* in the corn field has been observed in several regions of Brazil (Farias et al., 2014) and in the United States (Huang et al., 2014). The study of soils becomes an opportunity to pick up new microorganisms that can be prospected for biological control against insect-pests since those environments are potentially rich in microorganisms' diversity, mostly bacteria (Wagg, Bender, & Widmer, 2014). The search for alternatives to biological control of insect-pests leads to the prospecting of non-sporulating entomopathogenic bacteria. The discovery and taxonomic classification of new bacterial isolates from soil, presenting the same or similar function as Bt, would have great potential to reduce pests acquired resistance. The *16s rRNA* molecular marker is spread in all bacterial species and can be used for taxonomic purposes due its conserved and hypervariable regions developed during evolution. Applying this widely used marker it is possible to characterize a bacterium at the level of genus and species, allowing the prospection of its biotechnological potentials (Menna et al., 2006; Alves et al., 2020). Thus, this work aimed to taxonomic characterization and bioprospecting of non-sporulating bacterial isolates from soil vewing the entomopathogenic potential against *S. frugiperda*, compared to *B. thuringiensis* strains.

2. Methods

2.1 Isolation and Maintenance of Non-sporulating Bacteria

Non-sporulating bacteria named LGA-V0513, LGA-V056, LGA-V20C, LGA-V20F, LGA-V0522, LGA-V05D, LGA-EV05, LGA-EV08, LGA-V20B and LGA-V20G were isolated from soil cultivated with sugarcane (*Saccharum* spp.) (Omori et al., 2016), located in Nova Europa/SP, Brazil (21°49′35.17″ and 48°36′41.06″), and maintained at the Laboratory of Applied Genetics (Faculdade de Ciências Agrárias e Veterinárias (FCAV), Jaboticabal/SP, Brazil). Nova Europa has an annual median temperature of 29.3 °C and precipitation of 1,341.4 mm. Bacterial cells were grown in Tryptone-Yeast (TY) medium (Tryptone 5.0 g/L; Yeast extract 3.0 g/L; NaCl 0.87 g/L), pH 7.0 for 48 hours (h). After TY broth, the bacterial isolates were maintained in Petri dishes and in -80 °C stocks. Non-sporulating isolates were previously and partially characterized by Almeida (2017) (Figure 1).

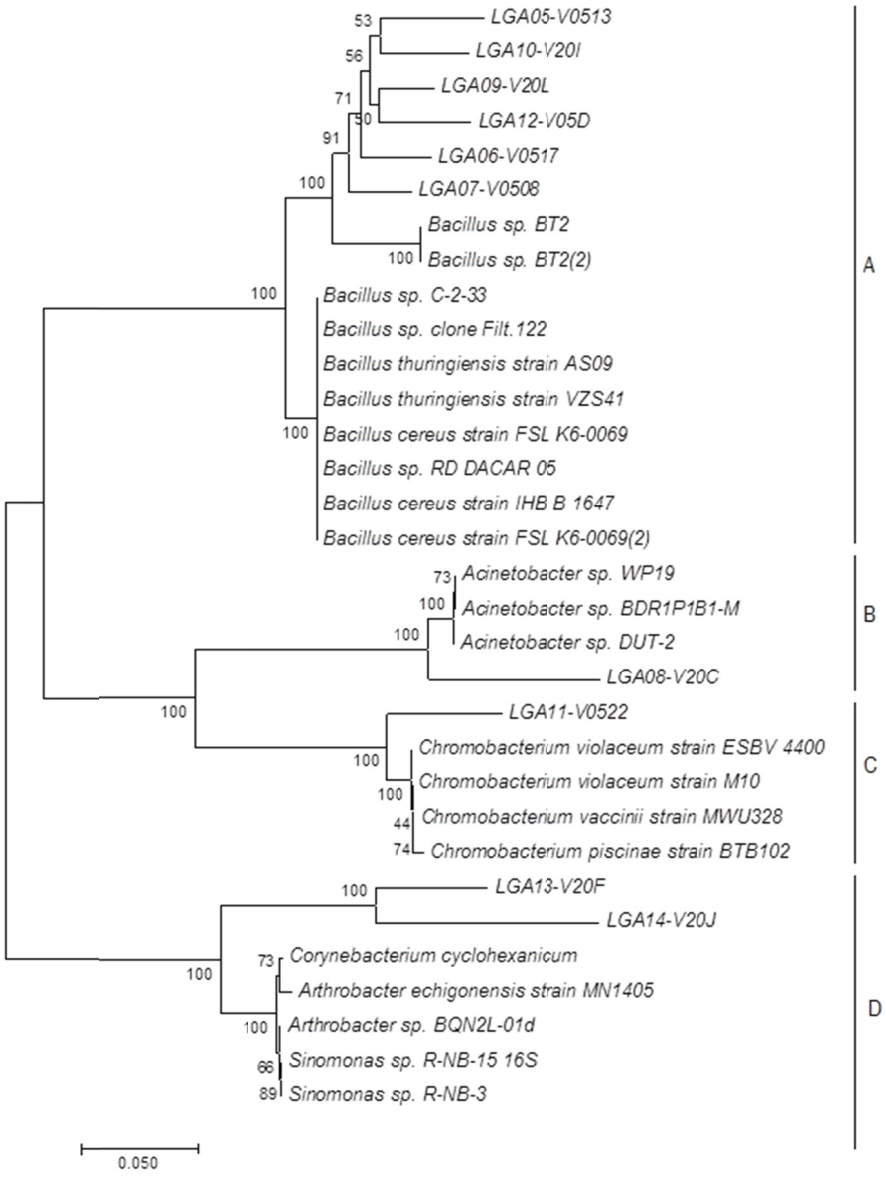

Figure 1. Dendrogram showing non-sporulating soil bacterial isolates classified by Almeida (2017) using *16S rRNA* marker partially sequenced. Branch A: represents the genus *Bacillus*; Branch B: *Acinetobaceter*; Branch C: *Chromobacterium* and branch D: *Arthrobacter*, *Corynebacterium* and *Sinomonas* genera

2.2 Maintenance of Sporulating Bacteria

The wild strains of *B. thuringiensis* named as LGBBA-1355 and LGBBA-1321 were taken from the Laboratory of Bacterial Genetics (FCAV, Jaboticabal/SP, Brazil). Bacterial cells were grown in Luria-Bertani (LB) medium added with salts (Glucose 1.0 g/L; Nutrient broth 8.0 g/L; Yeast extract 5.0 g/L; Tryptone 10.0 g/L, NaCl 5.0 g/L; $MgSO_4$ 0.3 g/L; $FeSO_4$ 0.02 g/L; $ZnSO_4$ 0.02 g/L; $MnSO_4$ 0.02 g/L) (Valicente & Mourão 2008), pH 7.5, for 48 h. After LB broth, the bacterial strains were maintained in Petri dishes and in -80 °C stocks.

2.3 DNA Extraction and Amplification of 16S rRNA Gene

For non-sporulating bacterial isolates characterization, DNA extraction was performed from 1 mL of each culture obtained in TY medium, corresponding to 50 mg of cells. Cells were pelleted and washed in saline [NaCl 0.85% (w/v)] to proceed DNA extraction, based on the adaptation of the method developed by Marmur (1961). Cell lysis was performed by Lysozyme enzyme (20 mg/mL) in the presence of RNAse (50 µg/mL). Dissociation of the DNA/protein complex was carried out by denaturing with 2.0% (w/v) of the Sodium Dodecyl Sulfate (SDS) and increasing the ionic strength with 1 M Sodium Acetate, pH 5.0. The separation of DNA from other

macromolecules happened by organic extraction with one volume of Chloroform:Isoamyl Alcohol [24:1 (v/v)] and DNA precipitation with 2 volumes of Ethanol. Excess of ethanol evaporated in a Concentrator Plus (Eppendorf) in the D-AL mode. The DNA was resuspended in 100 µL of TE (10:1) (Tris-HCl 10 mM, EDTA 1 mM) pH 8.0, overnight at 4 °C. The quality of genomic DNA was expressed by electrophoretic characterization [0.8% Agarose (w/v)] and DNA quantification in a Nanodrop 1000 Spectrophotometer device (ThermoScientific-Uniscience). DNA samples were stored at -20 °C until utilization. The complete 1.5 Kb amplicons for *16S rRNA* gene to determine the molecular signature of each isolate was obtained through PCR. Universal oligonucleotide primers 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 used for amplification in a reaction of 20 µL containing: template DNA (40.0 ng); 7.5 pmol of each universal primer; 1.75 mM $MgCl_2$; 0.2 mM Deoxyribonucleotide Triphosphates (dNTPs); 10X Buffer (2.0 µl); 1U of Taq DNA Polymerase enzyme (Invitrogen). The amplification program followed 94 °C/5 minutes (min); 35 cycles at 94 °C/30 sec (s), 56 °C/40 s, 72 °C/90 s; 72 °C/7 min, performed in a PTC-100™ Programmable Thermal Controller thermocycler (MJ Research, Inc.). The size and purity of the generated amplicons were verified by electrophoresis [1.5% agarose (w/v)].

2.4 Sequencing of 16S rRNA Amplicons

The amplicons produced by PCR had their DNA sequences determined to identify the bacterial affiliation. For the DNA sequencing reactions, in addition to the external primers (fD1 and rD1), *16S rRNA* internal region was targeted by primers designed by L. M. Cruz and described 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 sequencing reactions were standardized to a final volume of 10 µL [(0.5 pmol of primer; 1X sequencing buffer; 1.0 µL BigDye enzyme (Thermo Fisher Scientific); 7 ng of bacterial total DNA (for primers fD1 and rD1) or 7 ng of *16S rRNA* amplicon (for primers 362f, 786f and 1203f)]. The amplification program followed 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 washings in cold 70% (v/v) ethanol. The samples were dried in a laminar flow for 1 h, resuspended with 9.0 µL of Hi-Di Formamide (4311320-ABI Prism) and denatured at 95 °C for 5 min. Amplicon sequencing was performed in the ABI 3100 Automated Sequencer model capillary sequencer (PerkinElmer) in FCAV facility.

2.5 Analysis of Molecular Signature of Bacterial Isolates Through Bioinformatics

The *16S rRNA* gene was concatenated at approximately 1.5 Kb by the Phred/Phrap/Consed software package (Gordon, Abajian, & Green, 1998). After assembling of the *16S rRNA* contigs, the sequences were submitted to nucleotide similarity query against the nucleotide database (non-redundant) of GenBank (National Center for Biotechnology Information-NCBI), using the nucleotide tool BLAST (BLASTn-Identity = 99%) (Altschul et al., 1990). For sequence alignment the MAFFT v7.215 program was used (Katoh, 2002). The search for the best nucleotide replacement matrix was performed by the Phangorn package (Schliep, 2011) in R (R Development Core Team, 2011). The alignment matrix and the nucleotide substitution matrix were used by IQTREE program (Trifinopoulos, Nguyen, Von Haeseler, & Minh, 2016) for construction of the phylogenetic tree based on Maximum Likelihood (ML). Bootstrap and SH-aLRT branch tests applied the ML nucleotide frequency optimization, Ultrafast bootstrap analysis and 1,000 replicates options. The taxonomic classification of non-sporulating bacterial isolates LGA-V0513, LGA-V056, LGA-V20C, LGA-V20F, LGA-V0522 were processed by Alves et al. (2020).

2.6 Creation and Maintenance of S. frugiperda

The population of *S. frugiperda* was obtained through EMBRAPA Maize & Sorghum (Sete Lagoas/MG, Brazil). The larvae were kept on an artificial diet (Greene, Leppla, & Dickerson 1976) in 70 mL plastic containers, where one larva per pot was individualized, until the pupal stage. Pupae were removed and placed in 1 L containers with filter paper at the bottom. After hatching, the adults were placed in cylindrical PVC cages (30.0 cm high × 28 cm in diameter), internally lined with white bond paper and covered with nylon tissue to prevent them from escaping. Adults were fed with 10% (w/v) liquid honey solution. Every two days, eggs were collected and placed in 1 L containers with artificial diet on the bottom.

2.7 Lethality Bioassays Against S. frugiperda

Non-sporulating bacteria: Ten bacterial isolates were grown on 25 mL of TY medium at 120 rpm, 28 °C for 12 h in rotary shaker (Shaker Incubator Model G25 New Scientific). Then, the pre-inoculum was poured onto 75 mL of TY medium and incubated at 200 rpm, 28 °C for 48 h in a rotary shaker. An aliquot of 75 µL of TY broth was

removed and pipetted into each 5 mL pot containing the artificial diet (Greene, Leppla, & Dickerson, 1976), and kept in laminar flow for approximately 10 min for drying. After that, a unique *S. fugiperda* neonate was placed into individual pot for mortality testing. For each treatment and the negative control (H_2O), 50 larvae were used, separated in 5 repetitions, accounting for a total of 11 treatments and 550 larvae. The bioassay was maintained under controlled conditions for relative humidity of the air (RH) and temperature (T) (RH: 75±12% and T: 25±2 °C) for 10 days and the effect of treatments was observed weighing the larvae.

Sporulating bacteria: *B. thuringiensis* strains LGBBA-1355 and LGBBA-1321 were grown on 62.5 mL of LB medium at 120 rpm, 28 °C for 24 h in rotary shaker until vegetative growth phase. Then, the pre-inoculum was poured onto 187.5 mL of LB medium and incubated at 200 rpm, 28 °C for 72 h in a rotary shaker, until sporulation. The bioassays were conducted using six concentrations of spores for each strain: 3×10^4, 3×10^5, 3×10^6, 3×10^7, 3×10^8 and 3×10^9 spores/mL. Bioassays were complemented by treatment with the commercial product Dipel® (Abbott Laboratories, North Chicago, IL). (16.80 g in 0.5 L of water) and the control (H_2O), performing eight treatments and 30 repetitions. The diet used in the bioassays was the one standardized by Greene, Leppla, and Dickerson (1976) and processed as described for the non-sporulating bioassays. The experiment was carried out at room temperature (25±2 °C) and the effect over *S. fugiperda* neonate was carried out 7 days after application of suspensions.

2.8 Analysis of Bioassays

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

3. Results

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

The quality profile in genomic DNA extraction was reported due to the appearance of a single high molecular weight band. The appearance of a single intact band of high molecular weight indicates that the DNA extraction process was successful for all isolates, and that DNA was not degraded (Figure 2). This result confirms the potential of the Marmur's method to extract DNA showing high quality for a wide variety of bacterial species. The quality of the PCR amplicons was also evaluated showing the complete amplification of *16S rRNA* gene by fD1 and rD1 primers, according to the appearance of a unique band with strong intensity at 1,540 bp position, as expected for this gene (Figure 3). By analyzing the complete DNA sequencing of purified amplicons, the concatenated size of the *16S rRNA* sequences involved in the alignment ranged from 957 to 1,436 bp. Nevertheless, the agreement of coverage by aligning with databank reached 99% of similarity for all isolates at the level of genera or species (Table 1).

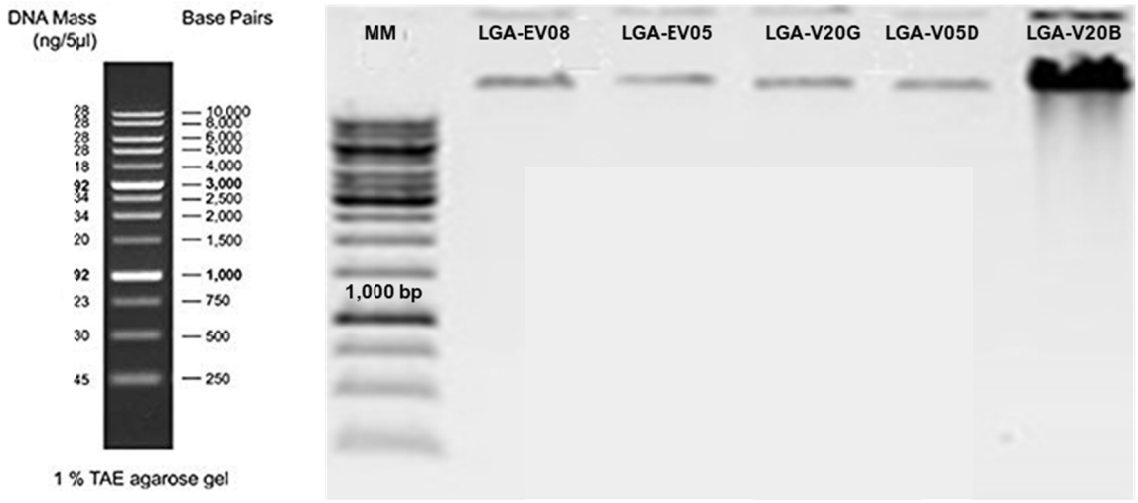

Figure 2. Quality profile of genomic DNA from five different non-sporulating bacterial isolates by the method of extraction described by Marmur (1961), in 0.8% agarose gel electrophoresis.
MM: 1 kb DNA Ladder (Fermentas®)

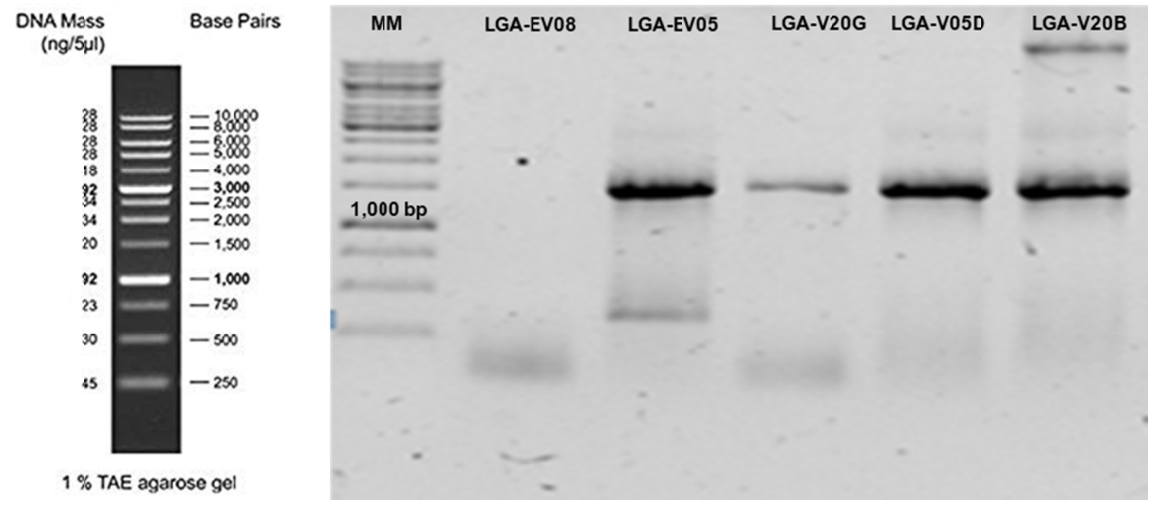

Figure 3. Quality profile of *16S rRNA* amplicons from five different non-sporulating bacterial isolates, in 1.5% agarose gel electrophoresis. MM: 1 kb DNA Ladder (Fermentas®)

The five different non-sporulating bacterial isolates from soil were classified and distributed in three distinct families: Paenibacillaceae, Microbacteriaceae and Micrococcaceae. Those bacterial isolates fallen into three different strains (Table 1). LGA-V20B, LGA-V20G and LGA-V05D were similar in level of genus and species belonging to *Arthrobacter echigonensis* strain MN1405. LGA-EV08 showed identity to *Brevibacillus nitrificans* strain DA2 and LGA-EV05 showed identity to *Curtobacterium* sp. strain BH-2-1-1. The results were highly corroborated by the high level of similarity between the sequences (minimum of 99%). It should also be highlighted the prominence of the phylum Actinobacteria over the phylum Firmicutes.

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**	**Strain**[2]
LGA-EV08	1436	NR_112926.1	Paenibacillaceae	*Brevibacillus nitrificans* strain DA2
LGA-EV05	1142	CP017580.1	Microbacteriaceae	*Curtobacterium* sp. strain BH-2-1-1
LGA-V20B	957	GU326383.1	Micrococcaceae	*Arthrobacter echigonensis* strain MN1405
LGA-V20G	1396	GU326383.1	Micrococcaceae	*Arthrobacter echigonensis* strain MN1405
LGA-V05D	1390	GU326383.1	Micrococcaceae	*Arthrobacter echigonensis* strain MN1405

Note. [1] LGA-EV08: Firmicutes; LGA-EV05, LGA-V20B, LGA-V20G, LGA-V05D: Actinobacteria.

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

3.2 Phylogeny of Non-sporulating Bacterial Isolates Based on Complete Sequencing of the 16S rRNA

Phylogenetic analysis based on complete *16S rRNA* sequences allowed a more coherent and reliable grouping among all genera, distributed in the phylogenetic tree (Figures 4 and 5). These data corroborate the evaluation based on sequence alignment (Table 1). LG-V20B, LG-V20G and LG-V05D isolates clustered together in the branch that grouped *Arthrobacter* and *Sinomonas* genera. Dendrogram showed that LG-V20G and LG-V05D isolates are very similar (99%) and includes LG-V20B isolate (96%) in the same branch of *A. echigonensis* strain MN1405 as a monophyletic group (Figure 4). There is a node linking those three isolates together with another branch that presents *Sinomonas echigonensis* strain LC10. Finally, this whole branch forms a node with *S. notoginsengisoli* strain SYP-B575. There is some discussion about the nomenclature of *A. echigonensis* (Ding, Hirose, & Yokota, 2009) since Zhou et al. (2012) proposed the transference of this species to the genus *Sinomonas* as *S. echigonensis* (Ding et al., 2009) Zhou et al. (2012), comb. nov [Combinatio nova, abbreviated comb. nov. (sometimes n. comb.), is Latin for "new combination"]. The LPSN (List of Prokaryotic names with Standing in Nomenclature) presents a note recommending that the name "*Arthrobacter echigonensis*" is correct only if this species is considered as a separate species into *Arthrobacter* genus without any association to another species already described with a valid nomenclature (LPSN, 2023).

LGA-EV05 isolate was associate with *Curtobacterium herbarum* strain SEFSH2 with high similarity (96%) according to the phylogeny obtained by sequencing the *16S rRNA* (Figure 4), clearly expanding the classification possibility of this isolate previously assigned to *Curtobacterium* sp. strain BH-2-1-1 (Table 1). The phylogenetic analysis for LGA-EV08 isolate validates its taxonomic affiliation to *Brevibacillus* genus, clearly *B. nitrificans* strain DA2 (Figure 5).

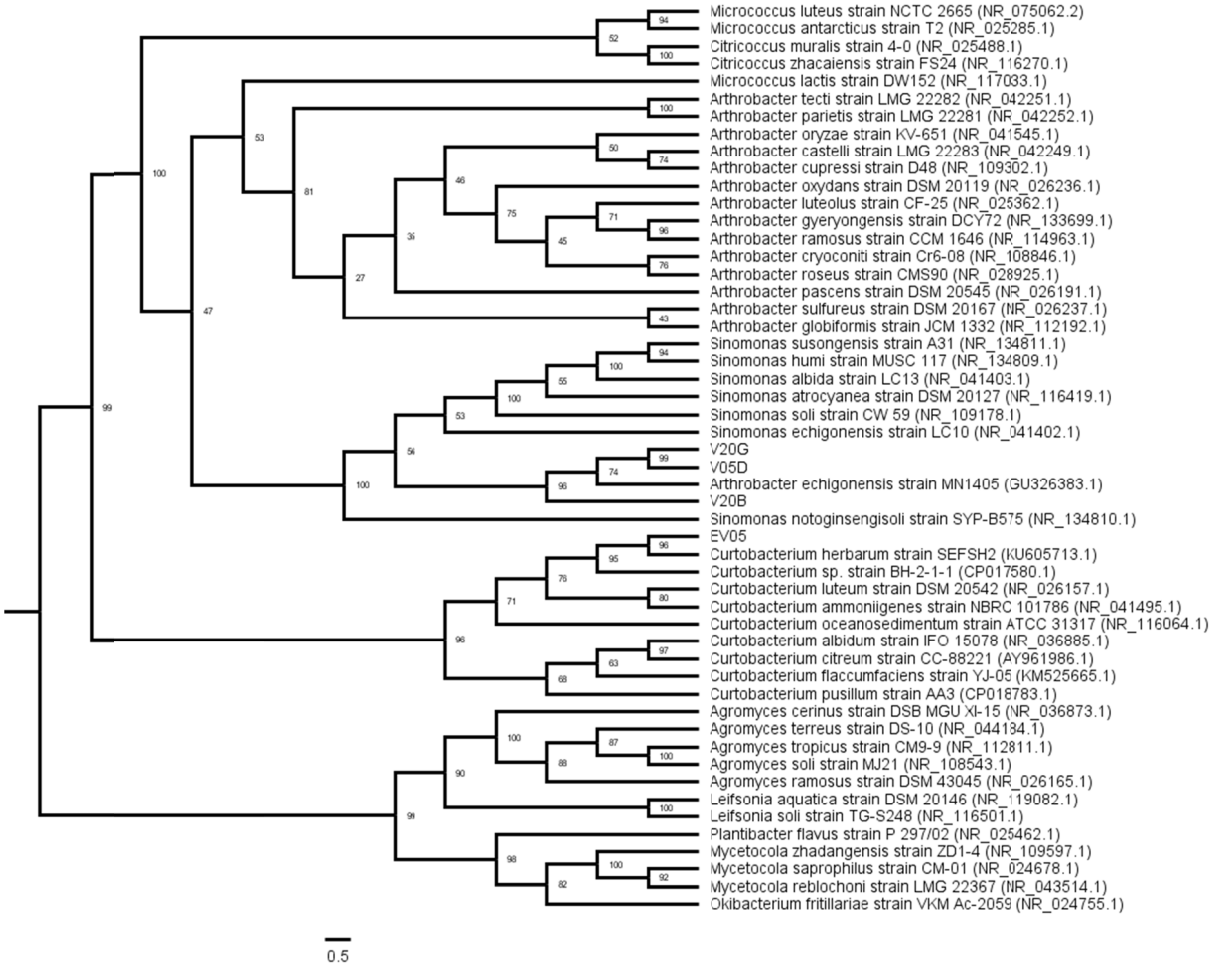

Figure 4. Dendrogram corresponding to isolates LGA-V20G, LGA-V05D, LGA-V20B and LGA-EV05, characterized by the *16S rRNA* genetic marker using the Neighbor-Joining method and 1000 bootstrap

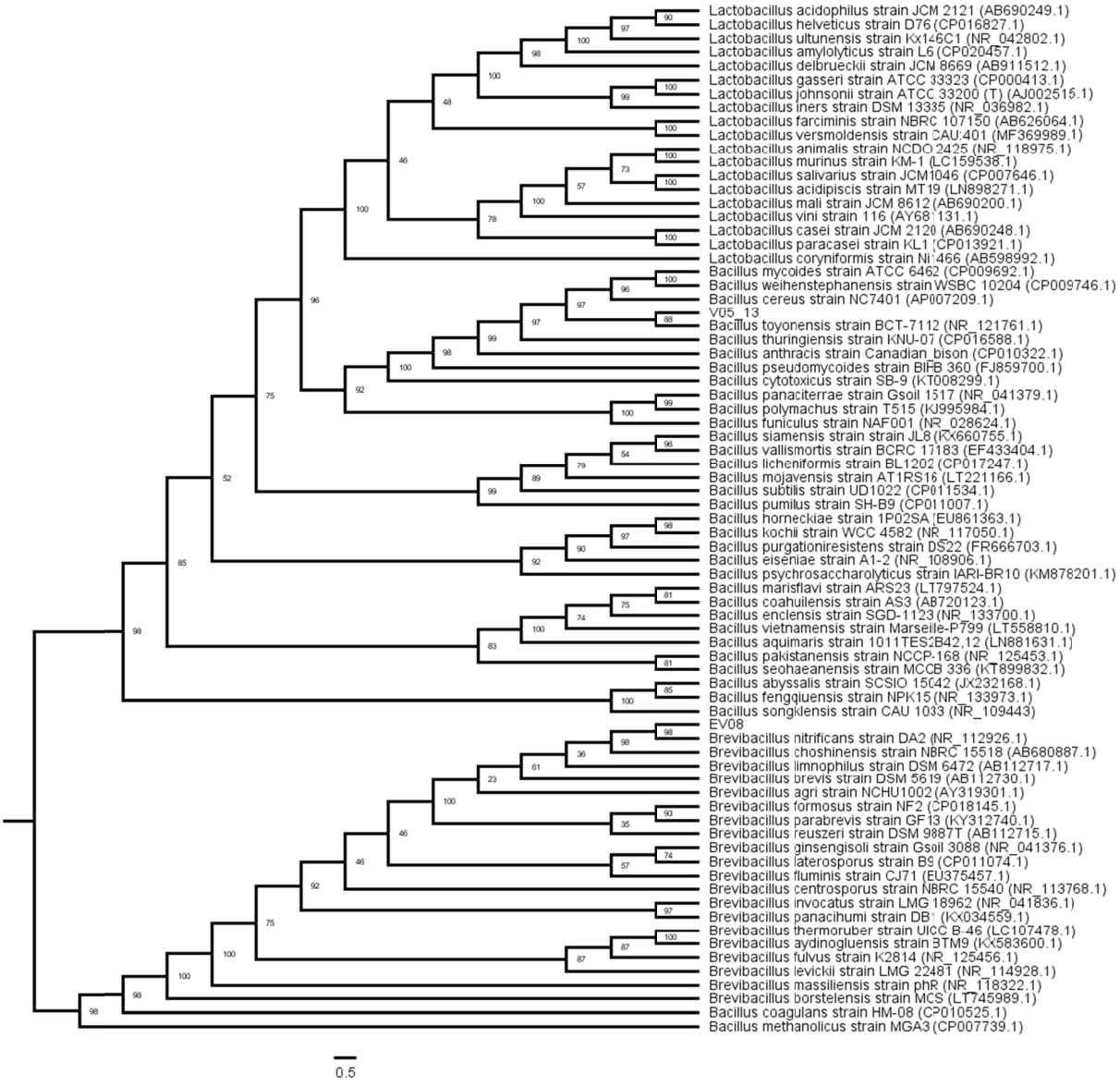

Figure 5. Dendrogram corresponding to isolate LGA-EV08, characterized by the *16S rRNA* genetic marker using the Neighbor-Joining method and 1000 bootstrap

3.3 Lethality Bioassay With Non-sporulating Bacteria

For lethality bioassays against *S. frugiperda* were included the non-sporulating isolates described by Alves et al. (2020): LGA-V0513, LGA-V056, LGA-V20C, LGA-V20F, LGA-V0522. It accounts 10 non-sporulating isolates to be tested. According to the results of bioassays, it was defined to discuss the terms of sub-lethality based on the Anova and Scott-Knott Test at 5% probability. Among all non-sporulating bacterial isolates, there is significative difference ($P > 0.05$) only for LGA-V0513 and LGA-V05D when compared to the control. Analyzing the other eight isolates, it can be observed that there was no difference in the weight values (g) for the larvae (Table 2).

Table 2. Lethality bioassay for *Spodoptera frugiperda* using non-sporulating isolates

Non-sporulating bacteria (R)	Larvae weight (g/lar)
LGA-V05-13	0.5169±0.096 b
LGA-V20F	0.5663±0.089 a
LGA-V05-6	0.5996±0.089 a
LGA-EV05	0.5769±0.081 a
LGA-EV08	0.5796±0.076 a
LGA-V20C	0.5615±0.089 a
LGA-V20G	0.5674±0.091 a
LGA-V20B	0.5569±0.091 a
LGA-V05-22	0.5564±0.083 a
LGA-V05D	0.5432±0.091b
Control (H_20)	0.5572±0.094 a
Anova	p = 0.001882

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

3.4 Lethality Bioassay with B. thuringiensis Strains for Larvae and Pupae Stages of S. frugiperda

Bioassays applying spores from *B. thuringiensis* showed significant differences on larvae sub-lethality by Scott-Knott test at 5% probability for both strains tested. Low concentrations of spores seem to be more efficient in controlling the development of larvae in acquiring weight. LGBBA-1321 strain stood out even more than LGBBA-1355 strain (Table 3).

According to the results of bioassays over pupae weight, no significant differences ($P > 0.05$) on sub-letality were noted according to the Scott-Knott test at 5% probability for any strains (Table 4).

Table 3. Lethality bioassay for *Spodoptera frugiperda* larvae using *Bacillus thuringiensis* strains

Concentrations	Larvae weight (g/lar)	
	LGBBA-1355	**LGBBA-1321**
3×10^4	0.0143±0.005 d	0.0132±0.005 c
3×10^5	0.0119±0.006 d	0.0102±0.009 c
3×10^6	0.0319±0.009 c	0.0128±0.004 c
3×10^7	0.0343±0.013 b	0.0171±0.005 b
3×10^8	0.0270±0.009 c	0.0134±0.002 c
3×10^9	0.0414±0.011 a	0.0181±0.003 b
Control (H_20)	0.0355±0.005 b	0.0355±0.005 a
Anova	$p = 3.3 \times 10^{-28}$	$p = 1.1 \times 10^{-42}$

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

Table 4. Lethality bioassay for *Spodoptera frugiperda* pupae using *Bacillus thuringiensis* strains

Concentrations	Pupae weight (g/lar)	
	LGBBA-1355[1]	**LGBBA-1321[1]**
3×10^4	0.2991±0.027	0.2352±0.005
3×10^5	0.3005±0.026	0.2775±0.006
3×10^6	0.3213±0.034	0.2516±0.009
3×10^7	0.3145±0.025	0.2891±0.013
3×10^8	0.3083±0.028	0.1903±0.009
3×10^9	0.3068±0.027	0.2691±0.011
Control (H_2O)	0.3079±0.027	0.2669±0.005
Anova	p= 0.08985	p= 0.81679

Note. [1] There is no significance by Anova test.

3.5 Lethality Bioassay With Dipel®

The treatment with commercial product Dipel® was also not effective over larvae weight showing no significant differences ($P > 0.05$) according to the Anova and the Scott-Knott test at 5% probability (Table 5).

Table 5. Lethality bioassay for *Spodoptera frugiperda* larvae using Dipel®

	Larvae weight[1] (g/lar)	**Larvae weight[1] (g/lar)**
Control (H_2O)	0.0308±0.005	0.2669±0.021
Dipel®	0.0660±0.011	0.2207±0.027
Anova	$p = 0.1629$	$p = 0.16293$

Note. [1] There is no significance by Anova test.

4. Discussion

The complete DNA sequencing of the *16S rRNA* gene allowed a more reliable and accurate taxonomic classification for each non-sporulating isolate, when compared to the partial DNA sequencing of the *16S rRNA* gene performed by Almeida (2017). Based on data from *16S rRNA* partial sequencing, LGA-V05D isolate was classified as *B. thuringiensis* with an identity of 96%. However, based on *16S rRNA* complete sequencing the LGA-V05D, LGA-V20B and LGA-V20G were taxonomically classified as *Arthrobacter echigonensis* (Micrococcaceae). Advances in taxonomic classification have shown that *A. echigonensis* should be reclassified within the genus *Sinomonas* [*S. echigonensis* (Ding et al., 2009) Zhou et al. (2012), comb. nov.], signed by the similarity in composition of the main fatty acids, polar lipids and cell wall amino acids (Zhou et al., 2009).

Members of the genus *Sinomonas* can synthesize silver nanoparticles with antimicrobial activity (Manikprabhu et al., 2016), hydrolyze starch (Ser et al., 2015), biodesulfurize coal (Mishra et al., 2014), present ability to degrade oil in the sea (Wu et al., 2010) and to degrade the antioxidant sesamine (Kumano et al., 2016). Recently, it was discovered that members from this genus exhibit plant growth-promoting effects and antagonistic activity against many root and leaf pathogens, potentiating their use in sustainable agriculture (Adhikari et al., 2017). These studies also showed this genus may have inhibitory activity against phytopathogenic fungi isolated from a soil sample, such as *Exserohilum turcicum* (Fu et al., 2019). *Sinomonas* also showed antimicrobial activity against the multi-resistant *Staphylococcus aureus* and can be used in future studies to minimize the problems related to the resistance of this bacteria (Manikprabhu et al., 2016).

The complete DNA sequencing of the *16S rRNA* for LGA-EV05 isolate assigned it as *Curtobacterium herbarum* strain SEFSH2 (Microbacteriaceae). Their cells are gram-positive, strictly aerobic, spore-free and mobile, with an optimum average temperature for growth at 25 °C (Behrendt et al., 2002). This genus is well known for composing plant growth-promoting bacteria (Vimal et al., 2019), however, it also presents plant pathogenic species such as *C. flaccumfaciens* (Soares et al., 2013). There are bacteria from this genus able to manage degradation of hydrocarbons, particularly in soils contaminated with oil (Lumactud et al., 2016), and to reduce toxicity caused by aluminum metal in soils (Ma et al., 2016). Strains of *C. herbarum* applied as bioinoculant could improve the production of saffron plants showing multifunctional ability to produce siderophores, plant growth hormones like IAA, and to solubilize phosphate (Díez-Méndez & Rivas, 2017). *C. herbarum* also improved both root and shoot growth for *Arabidopsis* plants as well as root growth for lettuce and basil (Mayer, Dörr de Quadros, & Fulthorpe, 2019).

The non-sporulating isolate LGA-EV08 was taxonomically affiliated with *Brevibacillus nitrificans* strain DA2 by the complete DNA sequencing of the *16S rRNA*. The strain DA2^T (= JCM 15774^T = NCIMB 14531^T) is the type strain of species and was isolated from a microbiological agent for enhancing microbial digestion in sewage treatment tanks. This bacterium presents gram-positive cell, facultatively anaerobic, mobile and capable of growing at pH 5-8. As a nitrifying bacterium, it removes nitrogen through biological nitrification and can be applied in wastewater treatments (Takebe, Hirota, Nodasaka, & Yumoto, 2012).

The taxonomic characterization based on complete DNA sequencing of *16S rRNA* for non-sporulating bacteria performed by Alves (2020) were also more accurate than those based on partial DNA sequencing (Almeida, 2017). Apart from genus classification, complete DNA sequencing of ribosomal gene brings classification in the level of species in this work as in the work of Alves (2020). For isolates LGA-V0513, LGA-V056, LGA-V20C, LGA-V20F, LGA-V0522, the reliable classification was as follow, respectively: *Bacillus toyonensis,*

Novosphingobium lindaniclasticum, *Rhizobium vallis*, *Cupriavidus necator* and *Chromobacterium vacinni* (Alves, 2020).

Based on the increasing interest on the search an application of non-sporulating bacteria as biological control against insect-pests, the ten new isolates originated from the agricultural soil (Omori et al., 2016) were tested for their effectiveness against *S. frugiperda*, the fall armyworm-cartridge larva. Entomopathogenic potential could be evaluated only as sub-lethality on larvae weight. LGA-V0513 and LGA-V05D were the two non-sporulating isolates that showed some subtle effect on the weight of *S. frugiperda* larvae. Therefore, this data includes the *Bacillus toyonensis* and *A. echigonensis* species as possible agents against this pest. Future studies should be done improving the conditions of bioassays to acquire a better response against *S. frugiperda*. The resistance of this pest is well known. As noted, *S. frugiperda* presented resistance in several countries and is the only target pest species that has developed field resistance to Bt crops in several areas of the world (Dangal & Huang, 2015).

The two strains of Bt (LGBBA-1321 and LGBBA-1355) showed effective response over larvae but not pupae of *S. frugiperda*. Despite similar results, the LGBBA-1321 strain stood out in terms of spore concentration optimization. This allows to hypothesize that probably this strain will present better average lethal concentration (CL50) rates in improved bioassays. It was also noted for both strains that the lowest concentrations of spores initially seemed more efficient in reducing the weight of the larvae. Again, one can speculate about the potential resistance of this insect-pest against the traditional Bt-based biological control. The biological control practices adopted in recent years against this pest have already shown their evolution with resistance in the control of insect-pests (Yang, Wang, & Kerns, 2022).

S. frugiperda belongs to the order Lepidoptera and family Noctuidae and it has been demonstrating their resistance to the use of *B. thuringiensis* as the protein toxins produced by these bacteria have the same binding sites in larval intestines, thus reducing their mortality effect (Heckel, 2015). These insects belong to the order Lepidoptera (Noctuidae) and demonstrate their resistance to the use of *B. thuringiensis*, as the protein toxins produced by these bacteria have the same binding sites in larvae, thus reducing their mortality effect (Heckel, 2015). It can be observed that there are few studies that show the capacity of non-sporulating bacteria to control this insect-pest. *Serratia* spp., a non-sporulating bacteria, are effective but not immediately lethal when they inhabit the digestive tract of the larva. Some of the strains often become pathogenic upon reaching the hemocoel (Mason et al., 2022). There are new molecules that can act against the pest. Rhabduscin synthesized by *Xenorhabdus nematophila* (non-spore forming) is an isocyanide that acts at nanomolar-level as an inhibitor of phenoloxidase, a key component of the insect innate immune system (Nuñez-Valdez et al., 2019). Thus, this drug is able to kill the insect-pest once it cannot fight against this and others applied in addition a (Crawford et al., 2012).

Bt-derivatives are the main products used in the formulation of biopesticides, with Dipel® being one of the most used and known (Roh et al., 2017). It contains toxins and proteins that lead to insect death (Bravo, Gill, & Soberón, 2007). In this study, it can be observed that with the application of Dipel® there was no effect on the mortality of larvae, as they developed normally gaining weight and reaching the pupal stage. It is possible that population of *S. frugiperda* used in this study should be resistant to this commercial product. Other authors have also written about resistance to Dipel®. The first reports occurred in the early 1990s, as the case of *Plutella xylostella* larvaes (Lepidoptera: Plutellidae), which were resistant to this product. The development of resistance also was observed in *Sesamia nonagroides* (Lepidoptera: Noctuidae), *Ostrinia nubilalis* (Lepidoptera: Pyralidae) and in genetically modified maize fields in Spain (Kranthi et al., 2006). In South Africa, a specific case of resistance by *Busseola fusca* (Lepidoptera: Noctuidae) over GM Cry2Ab maize crops was well studied and the resistance was effect of the evolution and inheritance of a recessive autosomal gene by the pest, conferring the tolerance trait (Berg & Campagne, 2015; Campagne et al., 2017).

Concerning transgenic plants, the Herculex® I Insect Protection (TC1507 event) was launched in Argentina released during the 2005-2006 season as an innovative product. The technology consists in plants expressing the entomopathogenic protein Cry1F and was widely adopted due its high level of effectiveness against fall armyworm. However, resistance against this has already been detected and it seems to be increasing in that county threatening the reliability and durability of this control trait. Resistance against Cry1F was characterized as recessive autosomal and monogenic in Puerto Rico, Brazil and Argentina (Chandrasena et al., 2017), which is very worrying.

Due to the development of resistance by the current insect pests, the increasing production of new chemical and commercial products and the need for more target-specific and environmentally sustainable products for

biological control, the exploration of innovations in biotechnology is extremely necessary. The search for new entomopathogenic organisms, including non-sporulating bacteria, is a matter of fact. In this case, the correct taxonomic classification and entomopathogenic effectiveness of non-sporulating bacteria can reveal promising biological resources to be prospected for many biotechnological properties.

5. Conclusion

The application of primers that cover the entire region of the amplicon for the complete DNA sequencing of *16S rRNA* gene provides greater reliability to the taxonomic classification of non-sporulating bacteria. The nomenclature for the five new classified isolates was in accordance with *Brevibacillus nitrificans* strain DA2 (LGA-EV08), *Curtobacterium herbarum* strain SEFSH2 (LGA-EV05) and *Arthrobacter echigonensis* strain MN1405 (LG-V20B, LG-V20G and LG-V05D). LGA-V0513 and LGA-V05D presented subtle sub-lethality effect on larval weight of *S. frugiperda*. Bt-LGBBA-1321 was slightly more efficient than Bt-LGBBA-1355 in controlling larval weight of *S. frugiperda*. The population of *S. frugiperda* used in this work was resistant to Dipel®. The correct taxonomic positioning of non-sporulating soil isolates reveal new biotechnological possibilities to be prospected in the future.

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