Morphological and biochemical characterization of bacterial species of *Bacillus*, *Lysinibacillus* and *Brevibacillus*[1]

Gabriela Teodoro Rocha[2*], *Sandro Coelho Linhares Montalvão*[3], *Paulo Roberto Martins Queiroz*[3], *Marcelo Rodrigues Berçot*[4], *Ana Cristina Meneses Mendes Gomes*[3], *Rose Gomes Monnerat*[3]

10.1590/0034-737X202370030010

ABSTRACT

The objective of this work was to characterize reference bacteria strains, belonging to the genus *Bacillus* and species of correlated genera, by simplified morphological and biochemical methods. The morphological characterization is based on the aspects of the colonies, as well as cytomorphology of the species, by optical and scanning microscopy. For biochemical characterization, the sensitivity test to antimicrobials by disk-diffusion is performed. Moreover, the strains were characterized by extracting intracellular proteins. Characteristics such as shape, color, and consistency of the colonies, in addition to the type of spore and production of protein crystals were determinants for the morphological characterization of these species. The antibiogram revealed high resistance to β-lactam group antibiotics, in species of *Bacillus cereus s.l* group. In *Bacillus subtilis s.l.* group there was high susceptibility to antibiograms, mainly for species of *B. subtilis*. The protein profile provided specific protein patterns for some species, mainly bands of 130 e 65 kDa for *B. thuringiensis*, 140 e 130 kDa for *Lysinibacillus sphaericus*, and 115 kDa for *Brevibacillus laterosporus*. Our results showed that the morphological and biochemical characterizations, provided a simple identification, with easy interpretation, and low cost.

Keywords: home production; *Bacillus thuringiensis*; protein profile; antibiogram.

INTRODUCTION

Spore-forming bacteria are currently grouped into 40 genera and seven families, with *Bacillaceae, Alicyclobacillaceae, Paenibacillaceae, Planococcaceae, Pasteuriaceae, Sporolactobacillaceae,* and *Thermoactinomycetace*, all belonging to the *Bacillalles* order, *Bacilli* class. *Bacillus* is the genus that integrates the largest number of endospore-forming species, with 318 cataloged species (Galperin, 2013; Logan & Halket, 2011; De Vos *et al.*, 2009).

Many of these species have high biotechnological potential. Their biodiversity is used for many purposes, especially in the agricultural sector, such as biological control of pests and diseases vectors; solubilization of phosphorus; promotion of plant growth; and production of chemical substances (Lanna-Filho *et al.*, 2010; Ramírez *et al.*, 2014).

Due to the functional multiplicity of these bacteria, in recent years, interest in growth and home production (fermentation) of bacterial species of this genus has increased, mainly for direct application in crops (Monnerat *et al.*, 2018; Lana *et al.*, 2019). However, this fermentation process is often done inappropriately, generating undesirable contaminants at the end of production. Thus, it is necessary to know the material to be grown, and correctly identify it, to avoid possible pathogens and/or losses in organic and agricultural production (Valicente *et al.*, 2020; ABCbio,

Submitted on March 24th, 2021 and accepted on September 08th, 2022.
[1] Manuscript extracted from academic thesis.
[2] Universidade de Brasília, Faculdade de Agronomia e Veterinária, Campus Universitário Darcy Ribeiro, Brasília, DF, Brazil. gabriela_teodoro.rocha@yahoo.com.br
[3] Embrapa Recursos Genéticos e Biotecnologia - CENARGEN, Brasília, DF, Brazil. sandro.coelho@yahoo.com.br; esteryoshie@gmail.com; prqzueiroz@gmail.com.br; rose.monnerat@embrapa.br
[4] Universidade de Brasília, Campus Universitário Darcy Ribeiro, Brasília, DF, Brazil. marcelobercot@gmail.com
*Corresponding author: gabriela_teodoro.rocha@yahoo.com.br

2019).

Tough most of the correct and precise identification of these bacteria is carried out by molecular techniques, such as gene amplification by Polymerase Chain Reaction - PCR (Erlich, 1989), DNA sequencing and protein profile by spectrometry of mass by MALDI-TOF, these techniques are considered sophisticated tools, which require qualified professionals and a higher investment cost, making them ultimately inaccessible to rural producers (Assis *et al.*, 2011).

Identification methods considered dependent on cultivation, which include the morphological analyzes of the colonies; Gram stain; and microscopic observations, to verify the production of endospores and protein inclusions, mainly in *Bacillus thuringiensis* species. As well as the use of biochemical and physiological tests, became simple and low-cost tools, which can be performed quickly and easily interpreted and accessed by farmers, which can help them establish an efficient quality control plan (Assis *et al.*, 2011; Madigan *et al.*, 2016).

The main objective of this work was to characterize bacteria strains of reference belonging to the genus *Bacillus* and species of related genera, by simple morphological and biochemical methods.

MATERIAL AND METHODS

Bacterial strains

Among the 20 strains analyzed, all belong to Invertebrate Bacteria Collection of Embrapa Genetic Resources and Biotechnology - CERNAGEN, including *Bacillus thuringiensis* (S2566); *Bacillus thuringiensis kurstaki* (S1450); *Bacillus thuringiensis israelenses* (S1989); *Bacillus thuringimensis aizawai* (S1576); *Lysinibacillus sphaericus* (S0002) and (S0127); *Brevibacillus laterosporus* (S1438); *Bacillus cereus* (S0426); *B. atrophaeus* (S2792); *B. subtilis* (S2790); *Bacillus sp.* (S2789); *B. pumilus* (S2795); *B. licheniformis* (S2777); *B. subtilis* (S2776); *B. mycoides* (S2786); *Bacillus amyloliquefaciens* (S2784, S2788, S2785, and S2791), and *Bacillus subtilis* (S2794), the latter came from the biological products Nemix and Nemacontrol (CCT7600).

Morphological characterization

The strains were inoculated in Petri dishes containing EMBRAPA-agar culture medium at 30 ºC for 72 h (Monnerat *et al*., 2007). After this period, the colonies were visualized by microscope transmission, model JEM-2100-Jeol, and classified according to the principles of Bergy's Manual of Systematic Bacteriology (De Vos *et al.*, 2009). For cytomorphological characterization, the strains were inoculated in EMBRAPA-liquid medium for 48 h at 30 °C and subsequently visualized by an electronic scanning microscope (SEM), model Zeiss DSM 962.

Biochemical characterization
Antibiogram

Disk-diffusion method, from Cefar Diagnótica Ltda©, lot D0030, was used to verify the antimicrobial action on *Bacillus* and related species. Twenty types of antibiotics were tested, in their respective minimum inhibitory concentrations (MICs) (µg/mL) defined by their commercial brand, which were Rifampicin - Rif (5 µg); Chloramphenicol - Clo (30 µg); Vanomycin - Van (30 µg); Clindamycin - Cli (2 µg); Erythromycin - Eri (15 µg); Penicillin G - Pen (10 µg); Oxacillin - Oxa (1 µg); Levofloxacin - Lvx (5 µg); Ciprofloxacin - Cip (5 µg); Cotrimoxazole - Sut (25 µg); Tetracycline - Tet (30 µg); Gentamicin - Gen (10 µg). Ceftazidime - Caz (30 µg); Ampicillin - Amp (10 µg); Cefazolin - Cfz (30 µg); Amikacin - Ami (30 µg); Amoxicillin + Clavulanic Acid - Amc (20/10 µg); Cefepime - Com (30 µg); Azithromycin - Atm (30 µg); and Ceftriaxone - Cro (30 µg).

Strains were cultured in Embrapa-liquid culture medium, for 72 hours, at 30 °C, by incubation on a rotary shaker at 200 revolutions per minute (rpm). The turbidity of the bacterial solution was adjusted according to the manufacturer to 0.5 on the Mac Farland scale, which corresponds to an amount of 10^8 CFU/mL. 2 mL of the bacterial inoculum was streaked, on each Petri dish (15 cm) with Embrapa-agar culture medium. Sterilized forceps were used to deposited diffusion discs, containing the antibiotics.

The experimental design was entirely random, and three replications were performed per strain. To determine the positive and negative control, 20 strips of autoclaved filter paper were deposited in autoclaved dH_2O Milli Q.

The plates were incubated at 30 °C for 24 h and, the formation of inhibition halos was evaluated. The measurement of halos was performed using a caliper in millimeters. The determinations of the inhibition zone were based and adapted according to Bauer *et al.* (1966); Romeiro (2001), Clinical and Laboratory Standards Institute – CLSI (2019), with modifications, in which samples with values < 13 mm were considered resistant, and sensitive strains with halo > 14 mm.

Protein profile - SDS-PAGE

To obtain cultures in their vegetative state, selected strains were grown in Embrapa-liquid culture medium, for 24 hours at 30 °C, in a rotary shaker at 200 rpm. The bacterial culture was centrifuged for 5 minutes at 12,000 rpm, at 4 °C. The pellets (bacterial mass) were resuspended in 1.0 mL of 10 mM tris-HCl solution, pH 8.0, containing 20% sucrose (w/v) and chilled 0.125% SDS. Immediately after the complete resuspension of the pellet, 20 µl of EDTA 0.5 M, pH 8.0 was added, and the samples were placed on ice for 10 minutes with moderate agitation. The cell solutions were centrifuged at 12,000 rpm for 5 minutes at 4 °C. The pellet was resuspended in 500 µl of 0.5 mM $MgCl_2$ solution, maintained at room temperature for 10 minutes, and then centrifuged for 5 minutes.

The supernatant, coming from the bacterial mass extraction process, was reserved and used for the analysis of the protein profile, since the objective was to analyze the total intracellular proteins of the bacterial isolates.

Samples were boiled together with the protein buffer at 90 °C for 5 minutes, in a 1:1 ratio. The analysis of the protein profile was performed by electrophoresis of proteins in polyacrylamide gel (SDS-PAGE 12%) and, fixed in a silver nitrate solution, and the process started with 40% methanol and 5% acetic acid for 12 h, followed by washing with 50% ethanol. Then, the gels were sensitized with a 0.2% sodium thiosulfate solution, followed by the impregnation solution of 0.2% silver nitrate, 0.028% formaldehyde. Finally, gels were fixed with the protein banding solution, which contained a solution of 6% sodium carbonate, 0.4% sodium thiosulfate, and 0.018% formaldehyde. To stabilize the appearance of protein patterns, a solution containing 45% methanol and 10% acetic acid in 100 mL of dH_2O was used.

RESULTS AND DISCUSSION

The morphological identification of *Bacillus* strains and related genera found that the 20 strains exhibited wide morphological and cytomorphological variety (Figures 1 and 2), between and within species. Nevertheless, the morphological structures observed are typical of these species grown in an Embrapa culture medium. Thus, the composition of the culture medium and the incubation conditions can strongly influence the morphological variety of bacteria and define details of diameter, shape, elevation, surface texture, color, and consistency of bacterial colonies (Rabinovitch *et al.*, 2015).

The most common characteristics among the species were the form and elevation of the colonies, being 85% circular and 95% flat, except for strain S2785, *B. amyloliquefaciens*, which had a convex elevation. Furthermore, strain S2786, *B. mycoides*, had a rhizoid colony format, differing from the other strains. *B. licheniformis* also showed important morphological variations, such as irregular and small colonies, with a filamentous appearance and cream color (Figure 1).

The analysis of the surface of the colonies showed that 75% of the bacterial strains had a smooth and opaque surface, mainly for the species of the *Bacillus cereus s.l.* group. Furthermore, 25% of the strains had colonies with a rough surface, the isolate being *B. licheniformis*; *B. cereus*; *B. subtilis* (S2794), *B. subtilis* (S2790) and *B. mycoides*. Surfaces with shiny characteristics were observed in the species of *Lysinibacillus sphaericus*, *Bacillus sp*. and *Bacillus atrophaeus*, with a mucous consistency. De Vos *et al.* (2009) describe that the colonies of *B. cereus* can be variable, ranging from circular to irregular shapes. *B. subtilis* is considered a diffusion-reaction model, in which the colony patterns are influenced by the type of substrate and the concentration of nutrients present in the medium.

Also, most species had a dry mucoid consistency and a white to cream color. However, some characteristics such as margin, consistency and color showed a high level of discriminatory factors for the species, for example, it was found that differences in consistency were found between strains of *B. amyloliquefaciens*, since initially the colonies of strain S2785 grew with a watery and viscous appearance, changing to a dry and rough appearance, due to the incubation period, however the other strains of the *B. amyloliquefaciens* species showed colonies with a dry and rough appearance. Furthermore, species of the group *B. subtilis s.l.* were variable among themselves, with lobed margins in the species of *B. amyloliquefaciens* and *B. licheniformis*, and smooth to wavy in the species of *B. subtilis* and *B. pumilus*.

The same heterogeneity was observed in the species of the group *B. cereus s.l.*, in which *B. thuringiensis* isolates exhibited a serrated margin and, while *B. cereus* had a wavy margin, *B. mycoides* had filamentous edges, which are determining characteristic to differentiate such species.

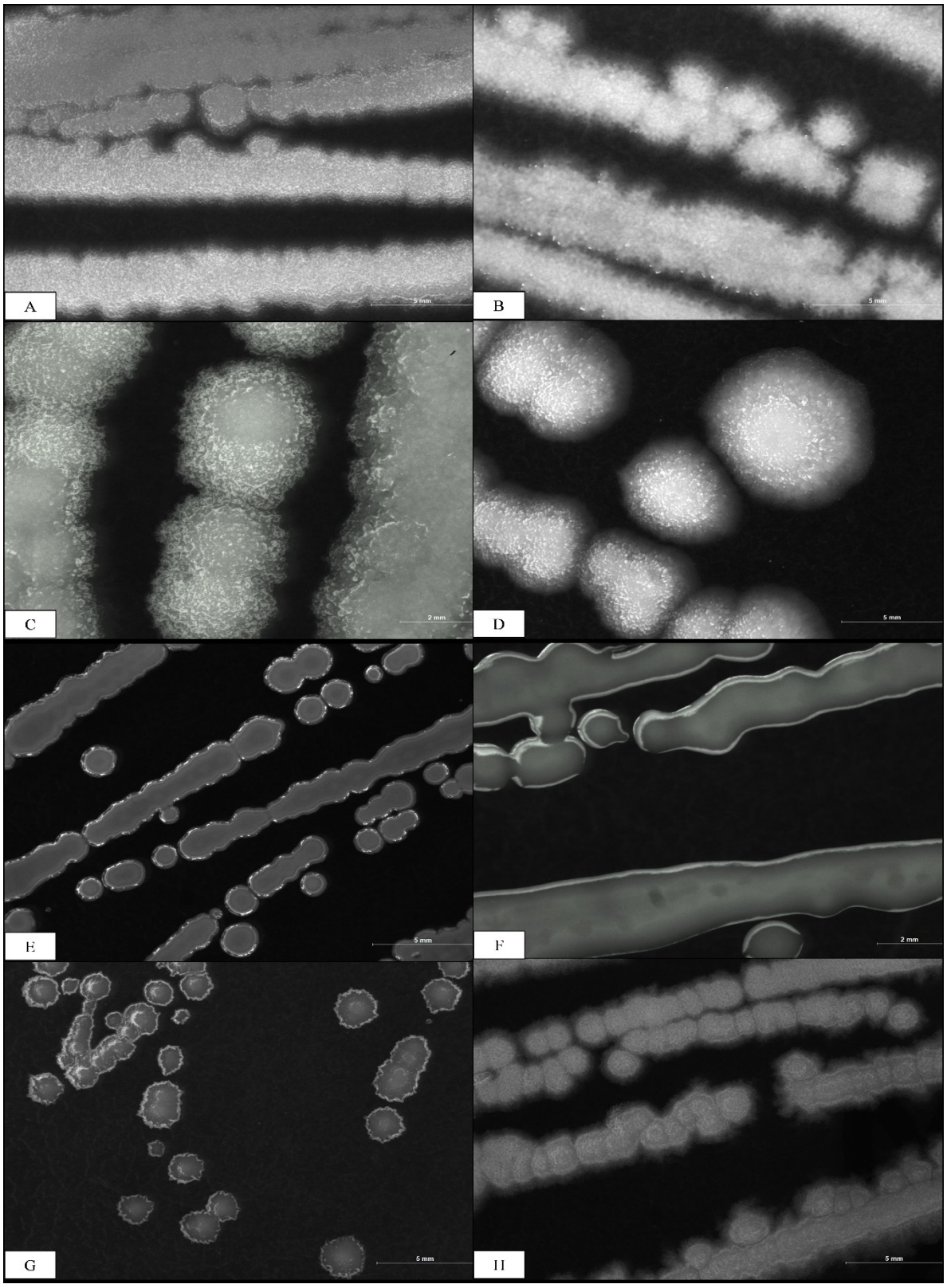

Figure 1: Colonies of endospore-forming bacteria in Embrapa culture medium after 72 hours at 30ºC. These figures illustrate the diversity of colonial appearances within the genus and between species of correlated genera. (**A**) *Bacillus thuringiensis* (S2566); (**B**) *Bacillus thuringiensis kurstaki* (S1450); (**C**) *Bacillus thuringiensis israelensis* (S1989); (**D**) *Bacillus thuringiensis aizawai* (S1576); (**E**) *Lysinibacillus sphaericus* (S0002); (**F**) *Lysinibacillus sphaericus* (S0127); (**G**) *Brevibacillus laterosporus* (S1438); (**H**) *Bacillus cereus* (S0426); (**I**) *B. atrophaeus* (S2792); (**J**) *B. subtilis* (S2790); (**K**) *Bacillus sp.* (S2789); (**L**) *B. pumilus* (S2795); (**M**) *B. licheniformis* (S2777); (**N**) *B. subtilis* (S2776); (**O**) *B. mycoides* (S2786); (**P**) *Bacillus amyloliquefaciens* (S2784); (**Q**) *Bacillus amyloliquefaciens* (S2788); (**R**) *Bacillus amyloliquefaciens* (S2785); (**S**) *Bacillus amyloliquefaciens* (S2791); (**T**) *Bacillus subtilis* (S2794). Scale of 2 mm – 5 mm. **(Continue...)**

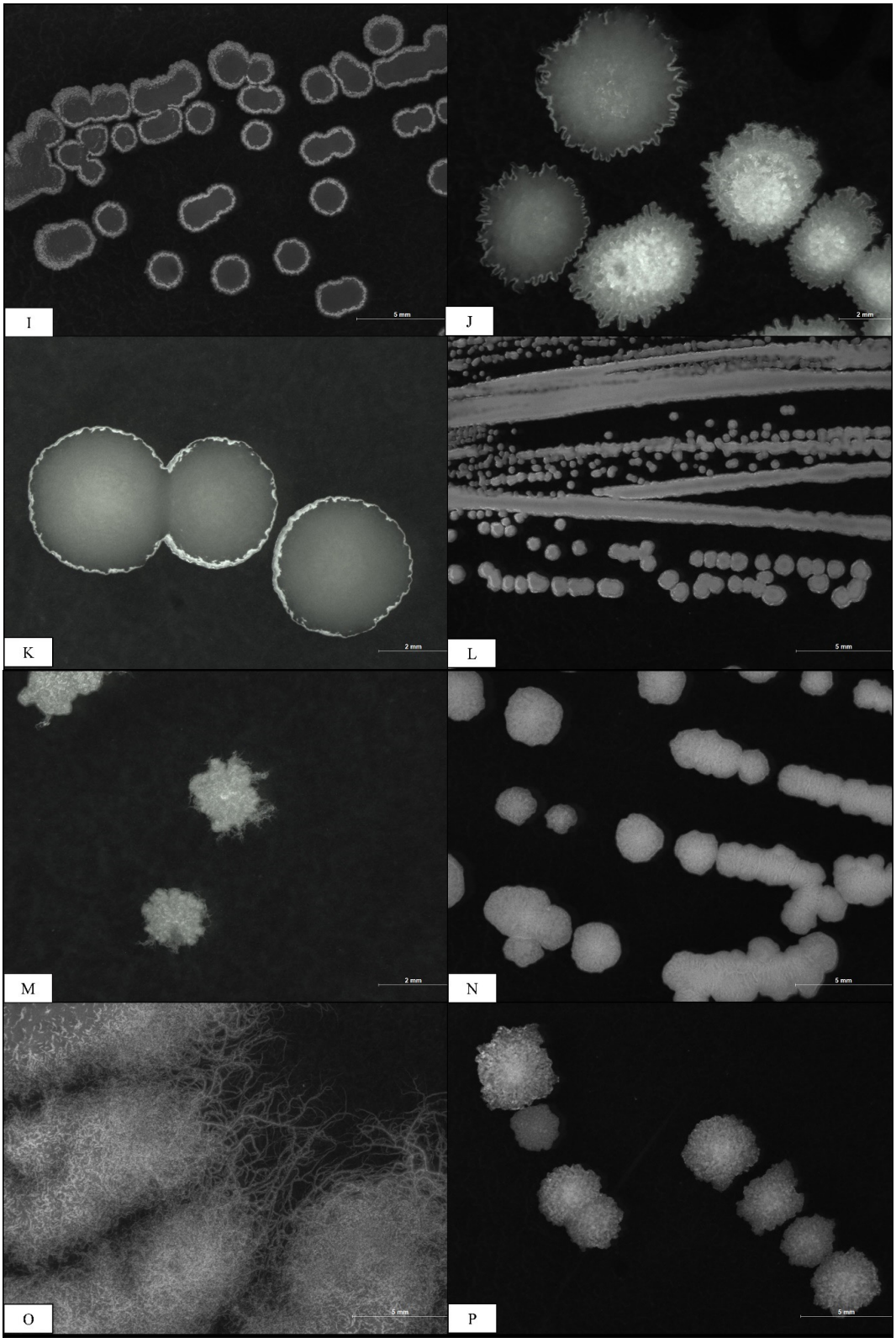

Figure 1: Colonies of endospore-forming bacteria in Embrapa culture medium after 72 hours at 30ºC. These figures illustrate the diversity of colonial appearances within the genus and between species of correlated genera. (**A**) *Bacillus thuringiensis* (S2566); (**B**) *Bacillus thuringiensis kurstaki* (S1450); (**C**) *Bacillus thuringiensis israelensis* (S1989); (**D**) *Bacillus thuringiensis aizawai* (S1576); (**E**) *Lysinibacillus sphaericus* (S0002); (**F**) *Lysinibacillus sphaericus* (S0127); (**G**) *Brevibacillus laterosporus* (S1438); (**H**) *Bacillus cereus* (S0426); (**I**) *B. atrophaeus* (S2792); (**J**) *B. subtilis* (S2790); (**K**) *Bacillus sp.* (S2789); (**L**) *B. pumilus* (S2795); (**M**) *B. licheniformis* (S2777); (**N**) *B. subtilis* (S2776); (**O**) *B. mycoides* (S2786); (**P**) *Bacillus amyloliquefaciens* (S2784); (**Q**) *Bacillus amyloliquefaciens* (S2788); (**R**) *Bacillus amyloliquefaciens* (S2785); (**S**) *Bacillus amyloliquefaciens* (S2791); (**T**) *Bacillus subtilis* (S2794). Scale of 2 mm – 5 mm. **(Continue...)**

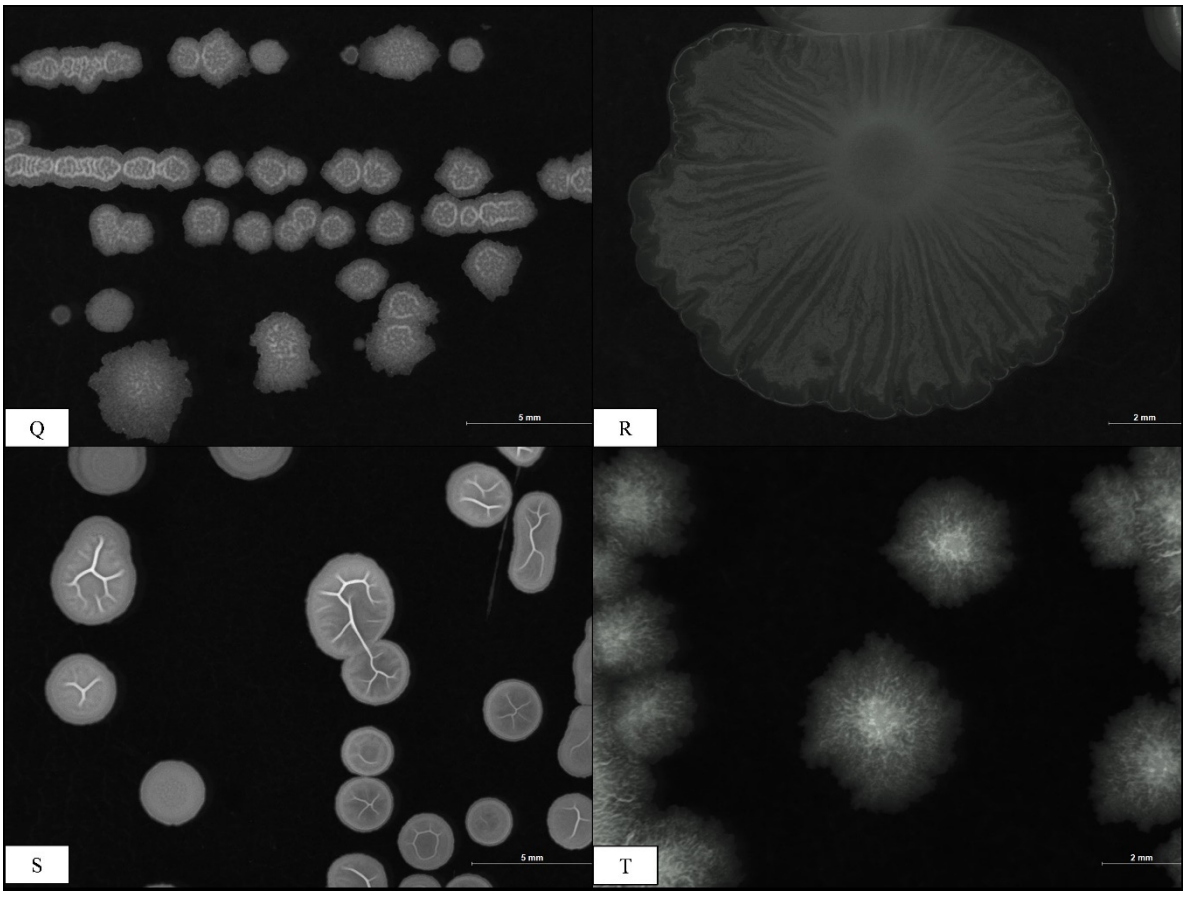

Figure 1: Colonies of endospore-forming bacteria in Embrapa culture medium after 72 hours at 30ºC. These figures illustrate the diversity of colonial appearances within the genus and between species of correlated genera. (**A**) *Bacillus thuringiensis* (S2566); (**B**) *Bacillus thuringiensis kurstaki* (S1450); (**C**) *Bacillus thuringiensis israelensis* (S1989); (**D**) *Bacillus thuringiensis aizawai* (S1576); (**E**) *Lysinibacillus sphaericus* (S0002); (**F**) *Lysinibacillus sphaericus* (S0127); (**G**) *Brevibacillus laterosporus* (S1438); (**H**) *Bacillus cereus* (S0426); (**I**) *B. atrophaeus* (S2792); (**J**) *B. subtilis* (S2790); (**K**) *Bacillus sp.* (S2789); (**L**) *B. pumilus* (S2795); (**M**) *B. licheniformis* (S2777); (**N**) *B. subtilis* (S2776); (**O**) *B. mycoides* (S2786); (**P**) *Bacillus amyloliquefaciens* (S2784); (**Q**) *Bacillus amyloliquefaciens* (S2788); (**R**) *Bacillus amyloliquefaciens* (S2785); (**S**) *Bacillus amyloliquefaciens* (S2791); (**T**) *Bacillus subtilis* (S2794). Scale of 2 mm – 5 mm.

The greatest divergence in color was observed in *B. atrophaeus* with colonies of orange tones. Culture medium with different compositions can cause diversity in stains of the same species, observed for *B. atrophaeus* and *B. licheniformis*. In agar containing glycerol-glutamate, the former can produce black or bluish-black pigment, while the latter can change from white to reddish colonies (De Vos *et al.*, 2009; Logan & Halket, 2011).

The cytomorphological evaluation of the species by, the vegetative cells of the *Bacillus* and related strains showed sizes between 0.5 and 3 mm. Diverse spore shapes were found especially within species (Table 1 and Figure 2).

The ability to produce protein crystals with entomopathogenic action was also verified, mainly in the species *B. thuringiensis* (Figure 2) and *Lysinibacillus sphaericus*. Through scanning microscopy, it was possible to verify the formation of protein inclusions, with bipyramidal and cuboid conformation for *B. thuringiensis* species, and spherical shape for *Ly. sphaericus*. Bipyramidal crystals are related to the presence of Cry1 type proteins, effective against insects of the Lepidoptera and Coleoptera orders, while cuboid crystals are characteristic of Cry2 and Cry3 proteins, which are toxic to Lepidoptera, Coleoptera and Diptera (Silva *et al.*, 2004). Spherical crystals have insecticidal activity to dipterans (Melatti *et al.,* 2005).

It was found that in the same strain there was the formation of two protein inclusions, this being present in the S2566 isolate, which presented bipyramidal and cuboid crystals. The same result was observed by Praça *et al.* (2004), on the S997 (*B. thuringiensis*) strain, to which he found the three types of conformations.

Table 1: Cytomorphology of species of the genus *Bacillus* and related genera. + = Distended (swollen) sporangium; - = Sporangium not distended; ELP = Ellipsoid; ELI = elliptical; Cyl = Cylindrical; S = Spherical; C = Central; T = Terminal; ST = Subterminal. * Some species of *Brevibacillus* spp. present protein crystals

Bacterium	**Length of the vegetative cell**	**Distended sporangium - swollen**	**Form of the spore**	**Position of the spore**	**Parasporal bodies (Crystals)**	**Motility**
B. mycoides	2–3 µm	-	ELP	ST	-	-
Bacillus sp. (S2789)	1,5–3 µm	+	S	T	-	-
Bacillus thuringiensis	2–3 µm	-	ELI	ST	+	-
Brevibacillus laterosporus	1–2 µm	+	S	C	-*	+
Lysinibacillus sphaericus	2–3 µm	+	S	T	+	+
Bacillus cereus	2–3 µm	-	ELP	ST	-	-
Bacillus pumilus	1–2 µm	-	ELP	ST	-	+
B. subtilis	1–2 µm	-	CIL	ST	-	+
Bacillus licheniformis	1–2 µm	+	CIL	ST	-	+
Bacillus atrophaeus	1–2 µm	-	ELP	ST	-	+
B. amyloliquefaciens	1–2 µm	-	CIL	ST	-	+

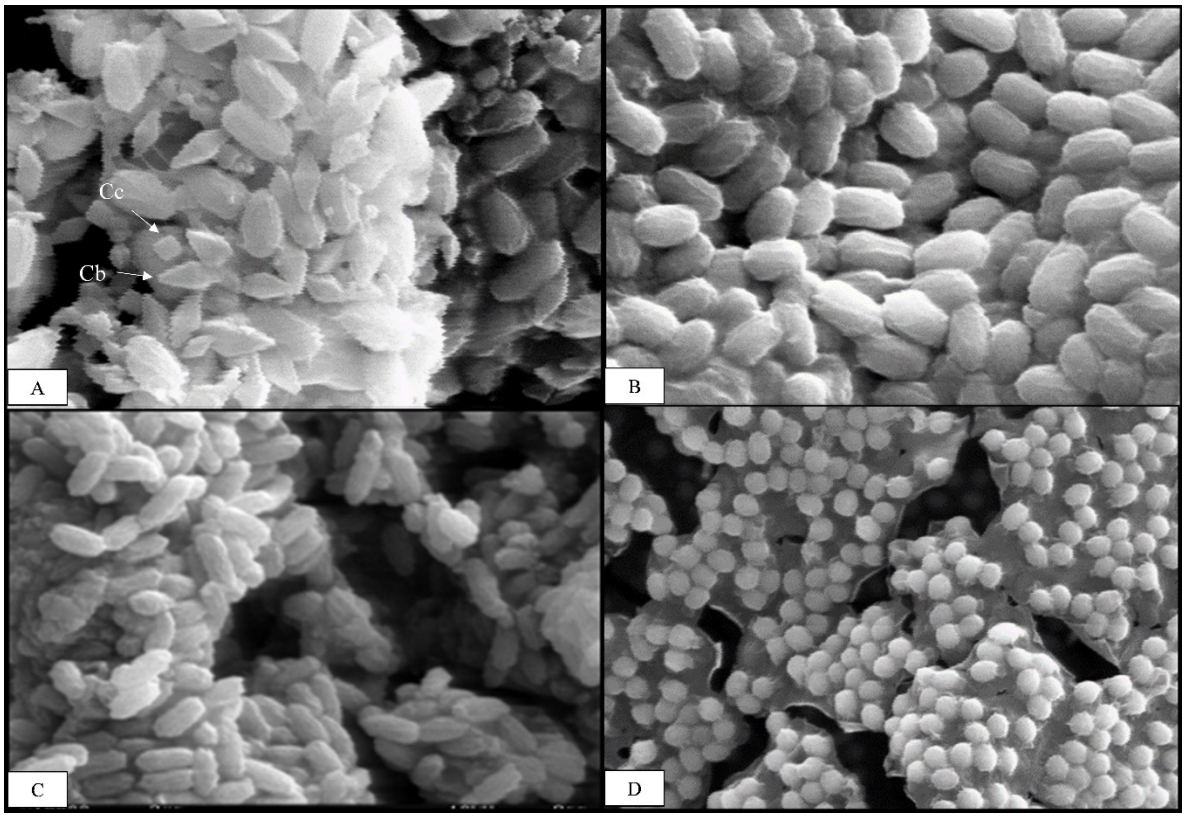

Figure 2: Phase contrast microscopy and scanning microscopy of species: (**A**) *Bacillus thuringiensis* (S2566); (**B**) *Bacillus cereus*; (**C**) *Bacillus pumilus*; (**D**) *Lysinibacillus sphaericus* (S0002); (**E**) *Bacillus atrophaeus*; (**F**) *Bacillus licheniformis*; (**G**) *Bacillus subtilis*; (**H**) *Bacillus amyloliquefaciens*; (**I**): *Bacillus sp.*; (**J**) *Brevibacillus laterosporus*; (**K**) *Bacillus mycoides*; (**L**) *Bacillus thuringiensis israelensis* (S1989) (10,000 x magnification). Scale between 2 and 5 µm. Sp = spore, C = crystal, Cs = spherical crystal, Cb = bipyramidal crystal, Cc = cuboidal crystal. **(Continue...)**

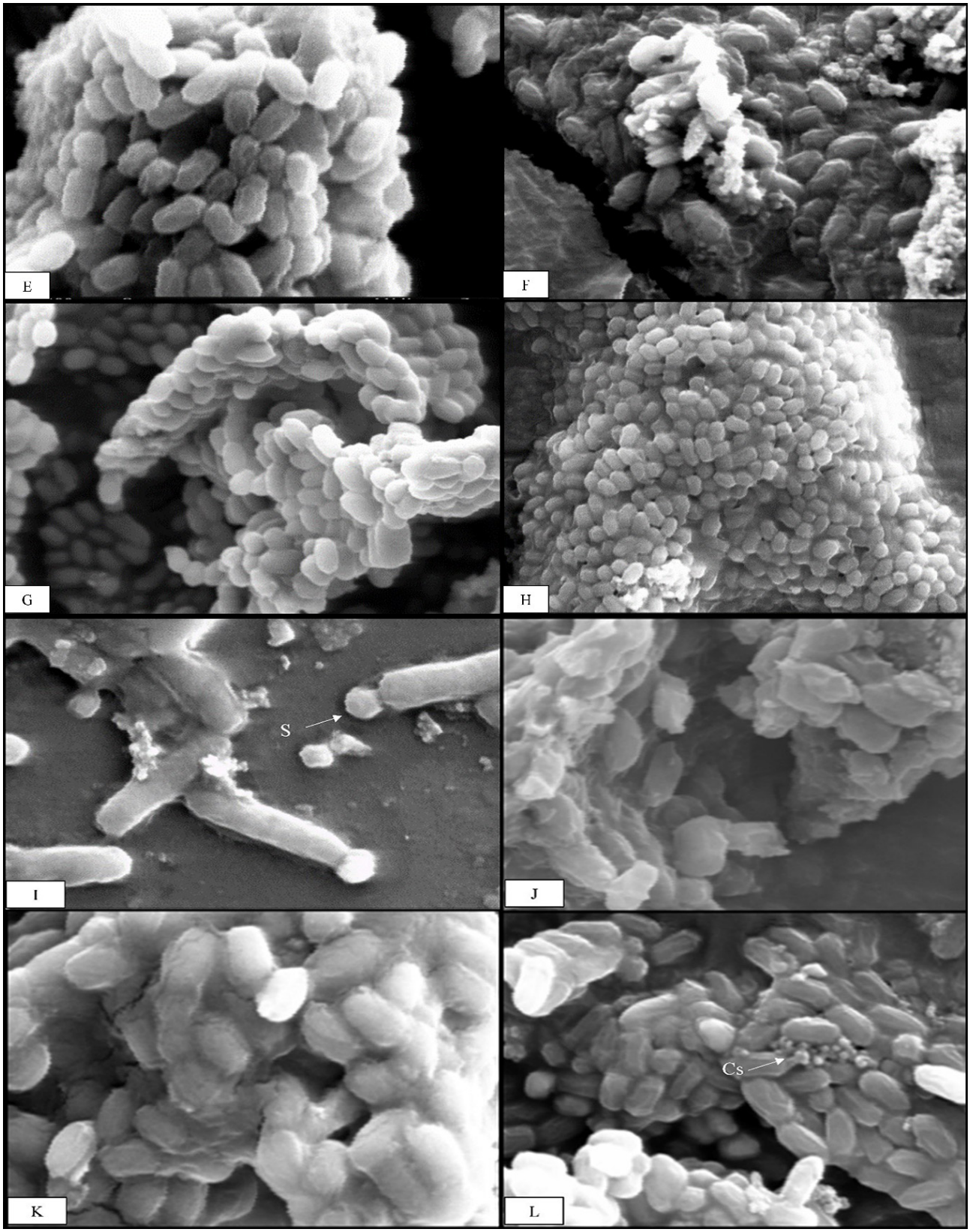

Figure 2: Phase contrast microscopy and scanning microscopy of species: (**A**) *Bacillus thuringiensis* (S2566); (**B**) *Bacillus cereus*; (**C**) *Bacillus pumilus*; (**D**) *Lysinibacillus sphaericus* (S0002); (**E**) *Bacillus atrophaeus*; (**F**) *Bacillus licheniformis*; (**G**) *Bacillus subtilis*; (**H**) *Bacillus amyloliquefaciens*; (**I**): *Bacillus sp*.; (**J**) *Brevibacillus laterosporus*; (**K**) *Bacillus mycoides*; (**L**) *Bacillus thuringiensis israelensis* (S1989) (10,000 x magnification). Scale between 2 and 5 µm. Sp = spore, C = crystal, Cs = spherical crystal, Cb = bipyramidal crystal, Cc = cuboidal crystal.

The production of crystals proteins by *Brevibacillus laterosporus* was not observed; however, Ruiu (2013) states that some strains of the same species form a lamellar parasporal inclusion with oval shape, firm and adjacent to the side of the spore.

However, it is important to note that due to the high phenotypic similarity between some species and subspecies, which can influence their correct characterization, and/or due to the dependence on the metabolic processes of the microorganisms, the result obtained by these techniques often become inconclusive because they may require long periods of evaluation (Assis et al., 2011; Celandroni et al., 2016). Thus, it is necessary to combine different identification techniques to provide a conclusive and accurate identification of these species. In addition to the morphological characterization, it was observed that Bacillus species and related genera were resistant and susceptible to antimicrobials. Thus, it was verified that seven of the 20 antibiotics tested inhibited 100% the bacterial growth of the strains, being amikacin, ciprofloxacin, gentamicin, levofloxacin, erythromycin, tetracycline and vancomycin (Figure 3). The use of these antibiotics can help separate these *Bacillus* and related species from other strains of divergent genera that exhibit resistance to these antimicrobials. A similar pattern observed by Celandroni *et al.* (2016), in which strains of *Bacillus spp*. and *Paenibacillus spp*. were susceptible to ciprofloxacin and tetracycline, in addition to tigecycline and vancomycin.

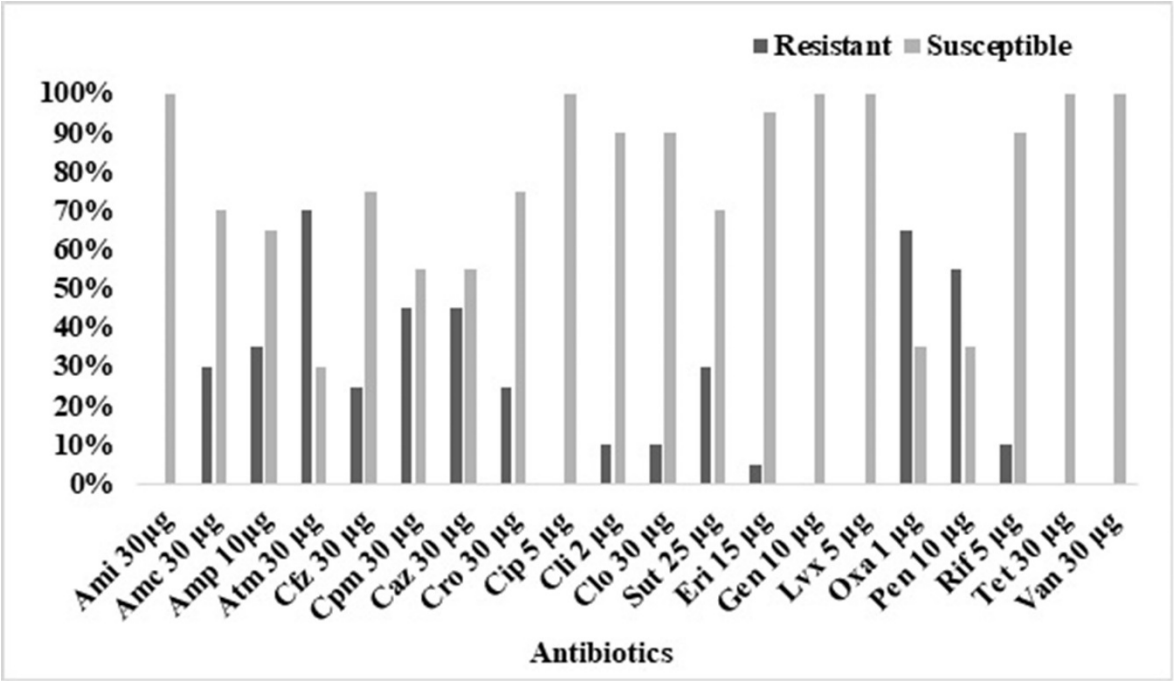

Figure 3: Resistance and susceptibility profile of strains belonging to the genera *Bacillus*, *Lysinibacillus* and *Brevibacillus*.

Antimicrobial resistance was also verified, and it was found that 70% of the strains were resistant to aztreonam, followed by the antimicrobial's oxacillin (65%), penicillin G (55%) and ampicillin (35%), all belonging to the chemical group of β -lactam. Furthermore, 45% of the strains were resistant to cefepime and ceftazidime. Most of the species in this study were resistant to antibiotics belonging to the β-lactam group, especially strains in *Bacillus cereus s.l* group. (*B. thuringiensis*, *B. cereus*, *B. mycoides*) (Figure 3). Resistance to these antimicrobials is conditioned to the production of nucleophilic enzymes (β-lactamase) that can promote β-lactam ring opening, present in the bacterial cell wall (Guimarães *et al.*, 2010; Bautista & Teves, 2013; Madigan *et al.*, 2016). Luna *et al.* (2007) verified the sensitivity to 24 antimicrobials in species *B. cereus s. l.* group, finding that strains of *B. cereus* and *B. thuringiensis* were resistant to amoxicillin, ampicillin, ceftriaxone, penicillin, and oxacillin, thus presenting a similar resistant profile; however, some strains of *B. mycoides* and *B. pseudomycoides* were susceptible to β-lactams.

It is important to emphasize that, even if there is a high level of resistance, on the part of species of the *B. cereus*

s.l. group, to antibiotics of the chemical group of β-lactams, the isolation and identification of species that do not belong to this group, makes a viable and promising alternative for their characterization, since these species, namely *Brevibacillus laterosporus*, *L. sphaericus* and, certain species of the *B. subtilis s.l.* group were susceptible to these antibacterial (Table 2).

It is also emphasized that the identification of *B. thuringiensis* species can be performed by observing, under microscopy, the formation of protein crystals, thus separating this species from isolates of the same phylogenetic group.

Table 2: Resistance profile of *Bacillus spp*., *Lysinibacillus spp*. and *Brevibacillus sp*. Wherein: Amikacin (Ami), Amoxicillin + Clavulanic acid (Amc), Ampicillin (Amp), Azithromycin (Atm), Cefazolin (Cfz), Cefepime (Cpm), Ceftazidime (Caz), Ceftriaxone (Cro), Ciprofloxacin (Cip), Clindamycin (Cli), Chloramphenicol (Clo), Cotrimoxazole (Sut), Erythromycin (Eri), Gentamicin (Gen), Levofloxacin (Lvx), Oxacillin (Oxa), Penicillin (Pen), Rifampicin (Rif), Tetracycline (Tet), Vancomycin (Van). **R** = resistant species; **(-)** = susceptible species

	Antibiotic																			
Strain	**Ami 30 µg**	**Amc 30 µg**	**Amp 10µg**	**Atm 30 µg**	**Cfz 30 µg**	**Cpm 30 µg**	**Caz 30 µg**	**Cro 30 µg**	**Cip 5 µg**	**Cli 2 µg**	**Clo 30 µg**	**Sut 25 µg**	**Eri 15 µg**	**Gen 10 µg**	**Lvx 5 µg**	**Oxa 1 µg**	**Pen 10 µg**	**Rif 5 µg**	**Tet 30 µg**	**Van 30 µg**
S0002	-	-	-	R	-	-	R	-	-	-	-	R	-	-	-	R	-	-	-	-
S0127	-	-	-	R	-	-	R	-	-	-	-	-	-	-	-	R	-	-	-	-
S0426	-	R	R	R	R	R	R	R	-	-	-	R	-	-	-	R	R	-	-	-
S1438	-	-	-	R	-	R	R	-	-	-	-	-	-	-	-	R	-	-	-	-
S1450	-	R	R	R	R	R	R	R	-	-	-	R	-	-	-	R	R	-	-	-
S1576	-	R	R	R	R	R	R	R	-	-	-	-	-	-	-	R	R	-	-	-
S1989	-	R	R	R	R	R	R	R	-	-	-	-	-	-	-	R	R	R	-	-
S2566	-	R	R	R	R	R	R	R	-	R	-	R	R	-	-	R	R	-	-	-
S2777	-	-	-	R	-	-	-	-	-	R	-	-	-	-	-	R	-	-	-	-
S2784	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-
S2785	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S2786	-	-	-	R	-	R	R	-	-	-	-	R	-	-	-	R	R	-	-	-
S2788	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R	-	-	-
S2789	-	R	-	R	-	R	-	-	-	-	-	-	-	-	-	R	R	-	-	-
S2790	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S2791	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	R	R	-	-	-
S2792	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
S2794	-	-	R	-	-	-	-	-	-	-	R	-	-	-	-	-	R	-	-	-
S2795	-	-	-	-	-	R	-	-	-	-	R	R	-	-	-	-	-	-	-	-

Strains belonging to the *Bacillus subtilis s.l.* group were highly susceptible to antimicrobials, especially the isolates of *Bacillus subtilis,* for example, strain S2794 was found to be resistant to chloramphenicol, ampicillin and penicillin, in contrast to isolates S2776 and S2790, which were resistant to only aztreonam.

The species of *B. amyloliquefaciens* S2785 was susceptible to all tested antibiotics, while isolates S2784 and S2788 were commonly resistant to penicillin, and S2791 to oxacillin. Within the same phylogenetic group, the species *B. atrophaeus* showed resistance to rifampicin (5 µg), and it is proposed that a better parameter for identification and separation of this specie in relation to others of the same phylogenetic group maybe it's unique orange color colony.

B. licheniformis strain was susceptible to chloramphenicol, ampicillin, and penicillin, nevertheless, it was shown to be resistant to aztreonam and oxacillin and to clindamycin. Unlike these results, Adimpong *et al.* (2012) reported that the 38 strains of *B. licheniformis* tested were susceptible to gentamicin, tetracycline and vancomycin; however, 50% of these strains were resistant to chloramphenicol and clindamycin, thus causing a variation in the resistance profile among several isolates of the same species.

It was found that species belonging to the related genera *Lysinibacillus spp*. and *Brevibacillus sp.* also showed a high profile of susceptibility to the antibiotics tested, mainly under the action of antibiotics from the β-lactam group, such as penicillin and ampicillin. A similar result was observed by Abdel-Salam *et al.* (2018), in which strains of *L. sphaericus* were highly susceptible to ampicillin. However, *L. sphaericus* strains showed small variations in resistance; strain S0002 was resistant to cotrimoxazole, while strain S0127 was resistant to oxacillin. The *Bacillus sp*. was resistant to amoxicillin, aztreonam, cefepime, oxacillin and penicillin, a common resistance profile among almost all strains.

Antibiogram is a method for rapid characterization since, through the level of resistance, different strains showed different resistances between, the bacterial groups observed, and the specificity of resistance of some strains were verified for certain antibiotics.

In addition to the characterization of species by the mechanism of resistance and susceptibility to antimicrobials, the protein profile was also analyzed, and patterns were defined for each strain. Thus, the electrophoretic profile of proteins extracted from whole cells produced heterogeneous patterns of *Bacillus* strains proteins of related genera. Figure 4 shows the distribution of protein bands across the gel.

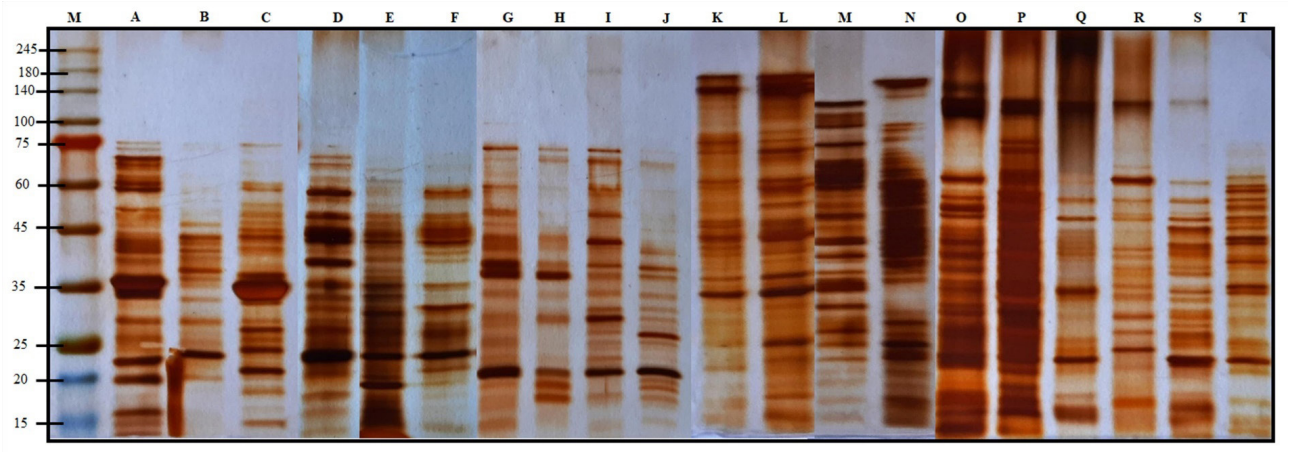

Figure 4: Protein profile (SDS-PAGE) of whole cells of the species: (**A**) *B. licheniformis* (S2777); (**B**) *B. atrophaeus* (S2792); (**C**) *Bacillus pumilus* (S2795); (**D**) *B. subtilis* (S2790); (**E**) *B. subtilis* (S2776); (**F**) *Bacillus subtilis* (S2794); (**G**) *B. amyloliquefaciens* (S2784); (**H**) *B. amyloliquefaciens* (S2788); (**I**) *B. amyloliquefaciens* (S2785); (**J**) *B. amyloliquefaciens* (S2791); (**K**) *Lysinibacillus sphaericus* (S0002); (**L**) *Lysinibacillus sphaericus* (S0127); (**M**) *Brevibacillus laterosporus* (S1438); (**N**) *Bacillus sp.* (S2789); (**O**) *B. thuringiensis kurstaki* (S1450); (**P**) *B. thuringiensis azawai* (S1576); (**Q**) *B. thuringiensis israelenses* (S1989); (**R**) *Bacillus thuringiensis* (S2566); (**S**) *B. cereus* (S426); and (**T**) *B. mycoides* (S2786). **M** = Marker.

The electrophoretic profile of the protein fractions of *Bacillus* species and related ones had a wide range of molecular weights that varied from 140 kDa to 10 kDa. However, the strains shared a similar pattern with protein masses of 25 and 45 kDa, which was determined as a standard protein for the strains in this study, with no reports in other literature.

Bacillus thuringiensis species presented high molecular weight protein bands, with ranges of 130, 75, 70 and 65 kDa. Protein masses of 130 kDa and 65 kDa were common to all *B. thuringiensis* species. However, unlike the other strains, isolates S2566 and S1576 also presented protein masses of 70 and 75 kDa, respectively. According to Constanski *et al.* (2015) protein bands of 130 and 65 kDa correspond to proteins of the Cry1 and Cry2 type, and 70 kDa to proteins Cry4, Cry10, Cry11 and Cyt1. Muniady *et al.* (2011) also emphasizes that proteins with a molecular weight between 135-140 kDa are known markers of *B. thuringiensis*, since this protein is encoded by the cryI and cryIV genes, as observed in six Bt isolates.

The protein profile of *Bacillus cereus* and *Bacillus mycoides* species were similar to *B. thuringiensis* species, all belonging to the same phylogenetic group (*Bacillus cereus sensu lato*). In *B. cereus* there was the presence of a protein band close to 135 kDa, in addition to the more striking presence of the 45 and 20 kDa bands. The Bacillus mycoides strain was distinguished from other strains of the same group, due to the absence of proteins larger than 65 kDa and the presence of protein bands weighing 44 and 36 kDa.

In the group *Bacillus subtilis s.l.* it was observed that the species had similar protein profiles, sharing proteins with weights of 60, 45, 40, 35, 30 and 20 kDa. However, there was also great diversity in protein patterns between species, with the presence of bands with a molecular weight of 75 kDa being observed for the strains of *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens*. This protein fraction was also observed in *B. atrophaeus*, but with the weaker intensity.

There were also proteins exclusive to some species of the group, which enabled their discrimination, so it was observed that strain S2777 was the only one, from the *B. subtilis s.l* group, to present bands of 16, 34, 59, and 73 kDa, while strain S2795 showed proteins weighing 19 and 27 kDa. Among the species of *B. subtilis*, small variations in protein profiles were observed, in which isolate S2790 obtained a fraction of 27 and 72 kDa, while strain S2794 presented a protein band of 47 kDa, and S2776 with two bands of approximately 10 and 34 kDa (Figure 4-1).

Only *L. sphaericus* species showed bands at 140 and 130 kDa, which is standard for this species. Lozano & Dussán (2017) and Hire *et al.* (2014) described molecular-weight bands close to those found in this study, at 42, 51, 110, and 125 kDa in *L. sphaericus* species, mainly due to the presence of the toxic binary protein.

Strains S2789 (*Bacillus sp*.) and S1438 (*Brevibacillus laterosporus*) showed proteins with a wide range of molecular weights, mainly with high molecular weight, ranging from 140 kDa to 20 kDa. However, Bre. laterosporus exhibited two different protein bands, at 115, 100 and 80 kDa, and the other strains analyzed did not obtain protein bands that could differentiate them or determine their exact identification.

Protein profiles obtained by the SDS-PAGE polyacrylamide gel offered an effective and rapid approach to identify bacterial species in the *Bacillus* group. However, this electrophoretic technique has its own level of discrimination and combined with another methodology, can provide a more accurate identification of these species. The aim of this study was to characterize reference species of *Bacillus* and correlates by simplified morphological and biochemical methods and, because of the results presented, validations of the techniques discussed here will be carried out later.

In addition, it was verified that the S2789 strain, considered a *Bacillus sp*., without official species identification, presented characteristics very similar to the species of the genus *Lysinibacillus*, since this species presented colonies with circular, smooth and mucous characteristics; spores with spherical shape and protein bands of 140 kDa. However, a molecular characterization is necessary to confirm the hypothesis that this species belongs to the genus *Lysinibacillus* and not to the genus *Bacillus*.

Hence, the morphological characterization becomes a fast and simplified identification parameter, mainly in species with high heterogeneity. However, this technique becomes useless, when there is a high homogeneity of phenotypic similarities between a large number of species of the same genus. Thus, combining morphological and biochemical characterization methods appear to produce an identification pattern with a higher degree of confidence (Figure 5).

CONCLUSION

Morphological and biochemical characterizations, due to morphological homogeneity between some species and subspecies, provided a simple identification, of easy interpretation and low cost, considering mainly the microscopic observations and the analysis the protein profile.

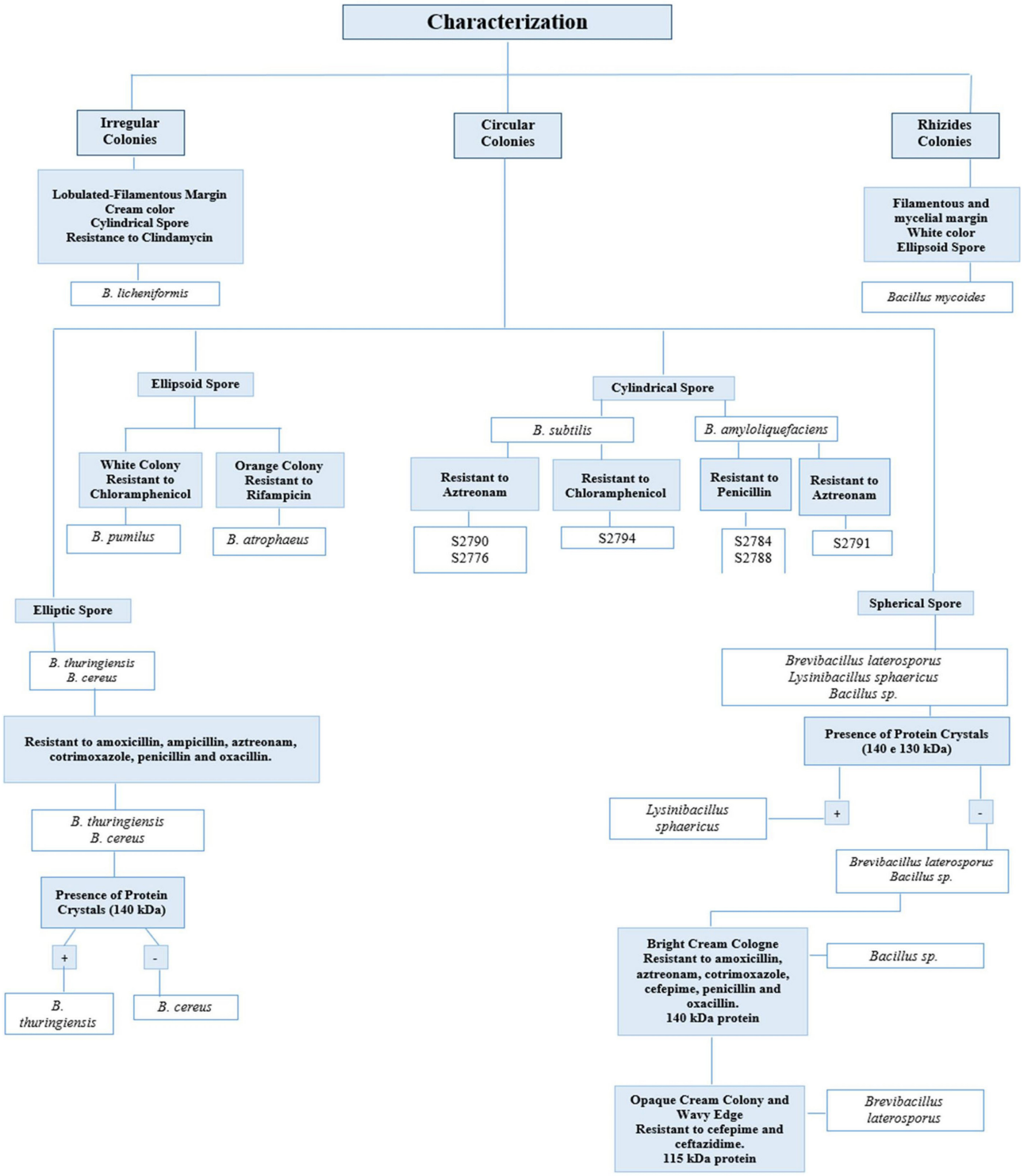

Figure 5: Summary of essential characterizations found in *Bacillus* strains and correlated genus.

ACKNOWLEDGMENT

I thank Capes (Coordination for the Improvement of Higher Education Personnel) for the financial support dedicated to this research. There is no conflict of interest in conducting the research and publication of the manuscript.

REFERENCES

ABCBio - Associação Brasileira das Empresas de Controle Biológico (2019) On farm: os riscos de fazer uma produção caseira de biodefensivos e bioestimulantes. Available at: <https://www.abcbio.org.br/blog/on-farm/>. Accessed on: December 30th, 2019.

Abdel-Salam MS, Ameen HH, Soliman GM, Elkelany US & Asar AM (2018) Improving the nematicidal potential of *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* against the root-knot nematode Meloidogyne incognita using protoplast fusion technique. Egyptian Journal of Biological Pest Control, 28:211-216.

Adimpong DB, Sørensen KI, Thorsen L, Stuer-Lauridsen B, Abdelgadir WS, Nielsen DS & Jespersen L (2012) Antimicrobial susceptibility, characterization of bacitracin operon and bacitracin biosynthesis of *Bacillus spp*. strains isolated from primary starters for African traditional bread production. Applied and Environmental Microbiology, 78:7903-7914.

Assis DM, Juliano L & Juliano MA (2011) A espectrometria de massas aplicada na classificação e identificação de microrganismos. Revista da Universidade Vale do Rio Verde, 9:344-355.

Bautista JR & Teves FG (2013) Antibiotic susceptibility testing of isolated *Bacillus thuringiensis* from three soil types around Iligan City, Philippines. African Journal of Microbiology Research, 7:678-682.

Bauer AW (1966) Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology, 45:149-158.

Celandroni F, Salvetti S, Gueye AS, Mazzantini D, Lupetti A, Senesi S & Ghelardi E (2016) Identification and pathogenic potential of clinical *Bacillus* and *Paenibacillus* isolates. PLoS One, 11:e0152831.

Constanski KC, Zorzetti J, Boas GTV, Ricieto APS, Fazion FAP, Boas LV & Neves PMOJ (2015) Seleção e caracterização molecular de isolados de *Bacillus thuringiensis* para o controle de Spodoptera spp. Pesquisa Agropecuária Brasileira, 50:730-733.

CLSI - Clinical & Laboratory Standards Institute (2019) Performance Standards for Antimicrobial Suceptibility Testing. 29º ed. Available: <https://clsi.org/media/2663/m100ed29_sample.pdf>. Accessed on: May 07th, 2020.

De Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH & Whitman W (2009) Bergey's Manual of Systematic Bacteriology. Vol. 3: The Firmicutes. United States, Springer Science. 1450p.

Erlich HA (1989) PCR technology - Principles and Applications for DNA Amplification. New York, Stockton press. 246p.

Galperin MY (2013) Genome diversity of spore-forming *Firmicutes*. Microbiology Spectrum, 1:TBS-0015-2012.

Guimarães DO, Momesso LDS & Pupo MT (2010) Antibióticos: importância terapêutica e perspectivas para a descoberta e desenvolvimento de novos agentes. Química Nova, 33:667-679.

Hire RS, Sharma M, Hadapad AB & Kumar V (2014) An oligomeric complex of BinA/BinB is not formed in-situ in mosquito-larvicidal *Lysinibacillus sphaericus* ISPC-8. Journal of Invertebrate Pathology, 122:44-47.

Lana UDP, Tavares A, Aguiar F, Gomes E & Valicente F (2019) Avaliação da qualidade de biopesticidas à base de Bacillus thuringiensis produzidos em sistema" on farm". Sete Lagoas, Embrapa Milho e Sorgo. 23p. (Boletim de Pesquisa e Desenvolvimento, 191).

Lanna-Filho R, Ferro HM & Pinho RSC (2010) Controle biológico mediado por *Bacillus subtilis*. Revista Trópica: Ciências Agrárias e Biológicas, 4:12-20.

Logan NA & Halket G (2011) Developments in the Taxonomy of Aerobic, Endospore-forming Bacteria. In: Logan NA & De Vos P (Eds.) Endospore-forming Soil Bacteria. Soil Biology. Berlin, Springer. p.01-29.

Lozano LC & Dussán J (2017) Synergistic activity between S-layer protein and spore–crystal preparations from *Lysinibacillus sphaericus* against *Culex quinquefasciatus* larvae. Current microbiology, 74:371-376.

Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT & Cattani J (2007) Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. Journal of Antimicrobial Chemotherapy, 60:555-567.

Madigan M, John M, Kelly B, Daniel B & David S (2016) Microbiologia de Brock. 14ª ed. Porto Alegre, Artmed. 960p.

Melatti V, Batista A, Demo C, Praça L, Brod C & Monnerat RG (2005) Determinação da susceptibilidade de *Spodoptera frugiperda* a diferentes subespécies de *Bacillus thuringiensis*. Brasília, Embrapa Recursos Genéticos e Biotecnologia. 14p. (Boletim de Pesquisa e Desenvolvimento, 88).

Monnerat R, Praça LB, Silva ES da, Montalvão SCL, Martins ES, Soares CMS & Queiroz PR (2018) Produção e controle de qualidade de produtos biológicos à base de *Bacillus thuringiensis* para uso na agricultura. Brasília, Embrapa Recursos Genéticos e Biotecnologia. 32p. (Documentos, 360).

Monnerat RG, Batista AC, Medeiros PTS, Martins E, Melatti V, Praça L, Dumas V, Demo C, Gomes ACM, Falcão R, Brod CS, Silva-Werneck JO & Berry C (2007) Characterization of Brazilian *Bacillus thuringiensis* strains active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*. Biological Control, 41:291-295.

Muniady S, Rathinam X & Subramaniam S (2011) Quick isolation and characterization for the confirmation of a novel *Bacillus thuringiensis* strains from chicken manure samples. African Journal of Microbiology Research, 5:3131-3137.

Praça LB, Batista AC, Martins ES, Siqueira CB, Dias DGS, Gomes ACMM, Falcão R & Monnerat RG (2004) Estirpes de *Bacillus thuringiensis* efetivas contra insetos das ordens Lepidoptera, Coleoptera e Diptera. Pesquisa Agropecuária Brasileira, 39:11-16.

Rabinovitch L & Oliveira EJ (2015) Coletânea de procedimentos técnicos e metodologias empregadas para o estudo de *Bacillus* e gêneros esporulados aeróbios correlatos. Available at:<http://www.fiocruz.br/ioc/media/Coletanea%20de%20Procedimentos%20Tecnicos%20para%20Bacillus.PDF>. Accessed on: March 25th, 2020.

Ramírez LCC, Leal LCS, Galvez ZYA & Burbano VEM (2014) *Bacillus*: género bacteriano que demuestra ser un importante solubilizador de fosfato. Nova, 12:165-177.

Romeiro R da S (2001) Métodos em bacteriologia de plantas. Viçosa, UFV. 279p.

Ruiu L (2013) *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. Insects, 4:476-492.

Silva SMB da, Silva-Werneck JO, Falcão, R, Gomes AC, Fragoso RR, Quezado MT, Oliveira-Neto OB, Sá MFG de, Bravo A & Monnerat RG (2004) Characterization of novel Brazilian *Bacillus thuringiensis* strains active against *Spodoptera frugiperda* and other insect pests. Journal of Applied Entomology, 128:102-107.

Valicente FH, Lana UG de P, Pereira ACP, Martins JLA & Tavares ANG (2020) Riscos à produção de biopesticida à base de *Bacillus thuringiensis*. Sete Lagoas, Embrapa Milho e Sorgo. 20p. (Circular Técnica, 239).