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Feasibility of using RFLP of PCR-amplified 16S rRNA gene(s) for rapid differentiation of isolates of aerobic spore-forming bacteria from honey

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ABSTRACT

This study aimed to assess the feasibility of using RFLP of PCR-amplified 16S rRNA gene (s) by using universal primers 27f/1492r and a combination of three restriction enzymes, *Alu*I, *Cfo*I, and *Taq*I, for a low-cost, rapid screen for a primarily differentiation of isolates of the complex of aerobic spore-forming bacteria commonly found in honey samples. The described method produced unique and distinguishable patterns to differentiate among 80 isolates belonging to 26 different species of *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Rummeliibacillus*, and *Paenibacillus* reported in honey and other apiarian sources.

1. Introduction

“Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature” www.fao.org/input/download/standards/310/cxs_012e.pdf. Honey is a supersaturated sugar solution containing small amounts of organic acids, minerals, vitamins, enzymes, proteins, and amino acids (Machado De-Melo et al., 2018). Honey quality is influenced by microorganisms, mainly yeasts, and spore-forming bacteria; nevertheless, commercially sold honey has minimal microbial contamination due to its natural antibacterial properties, including acidity, high osmotic pressure, hydrogen peroxide, and viscosity (Molan, 1992a, 1992b; Mundo et al., 2004; Snowdon and Cliver, 1996). Despite the various inhibitory factors, some microorganisms can survive in honey, particularly spore-forming bacteria, being the primary sources of contamination digestive tracts of larvae and adult bees, brood combs, environmental dust, air, soil, pollen, nectar and flower surfaces (Gilliam, 1979, 1997; Gilliam and Prest, 1978; Gilliam and Valentine, 1976).

The community of aerobic spore-forming bacteria reported in honey comprises *Bacillus amyloliquefaciens*, *Bacillus badius, Bacillus cereus sensu lato, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus flexus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus simplex, Bacillus subtilis, Brevibacillus borstelensis, Brevibacillus brevis, Brevibacillus laterosporus, Lysinibacillus fusiformis, Lysinibacillus sphaericus, Paenibacillus alvei, Paenibacillus apiarius, Paenibacillus larvae, Paenibacillus polymyxa* and *Rummeliibacillus stabekisii* (Alippi, 1995; Alippi et al., 2004; Alippi and Abrahamovich, 2019; Bartel et al., 2018; Evans and Armstrong, 2006; Gilliam, 1979, 1997; Gilliam and Morton, 1978; Gilliam and Valentine, 1976; Iurlina and Fritz, 2005, Piccini et al., 2004; Sinacori et al., 2014; Snowdon and Cliver, 1996; Wen et al., 2017). Within this community, some groups are comprised of close phylogenetic relatives, *i.e.* the *Bacillus cereus sensu lato* consisting of *Bacillus cereus sensu stricto, Bacillus anthracis*, *Bacillus mycoides; Bacillus cytotoxicus*; *Bacillus pseudomycoides*; *Bacillus thuringiensis*, and *Bacillus toyonensis* (Guinebretière et al., 2013; Liu et al., 2018; Vilas-Boas et al., 2007) and *Bacillus subilis* group consisting of *Bacillus subtilis*, *Bacillus amyloliquefaciens; Bacillus athrophaeus, Bacillus licheniformis; Bacillus mojavensis, Bacillus paralicheniformis*, *Bacillus pumilus*, *Bacillus safensis, Bacillus siamensis, Bacillus tequilensis, Bacillus vallismortis, Bacillus velezensis,* and *Bacillus xiamenensis* (Dunlap et al., 2016; Jeyaram et al., 2011; Lai et al., 2014;). Also, *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus* are closely related to each other and with other *Lysinibacillus* species (Ahmed et al., 2007).

Aerobic spore-forming bacteria from honey have been identified using different methodologies, including isolation in selective, differential or chromogenic culture media, microscopy, biochemical tests, and the sequence of the 16S rRNA gene(s) (Alippi, 1995; Alippi et al.,

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Table 1
List of bacterial strains and culture condition used in this study with GenBank accession numbers and references.

Strain	Accession Number	Culture conditions[a]	Culture collection[b]	Reference
Bacillus amyloliquefaciens				
m39	MG004187.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m287b	MG004189.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m163b	MG004188.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m164b	MG004193.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
mv35	MG004186.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
xx	KP177517.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m291b	MG004190.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Bacillus badius				
CCT 0196	N/A[c]	TSA/32 °C	CCT	N/A
Bacillus cereus				
ATCC 11778	AF290546	TSA/32 °C	ATCC	N/A
m6c	KP005456.1	TSA/32 °C	UB-CIDEFI	Minnaard and Alippi, 2016
mv33	KU230015.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
m387	KP005455.1	TSA/32 °C	UB-CIDEFI	Minnaard and Alippi, 2016
m434	KU230027.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
LPcer1	KX431225.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
MexB	KU230012.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
MexC	KU230013.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
Bacillus circulans				
ATCC 4515	N/A	MYPGP/37 °C	ATCC	N/A
Bacillus claussi				
Fr231	KU232014.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
m448b	KX685159.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
BclENT	N/A	MYPGP/37 °C	UB-CIDEFI	N/A
Bacillus coagulans				
ATCC 35670	N/A	TSA/30 °C	ATCC	N/A
Bacillus firmus				
ATCC 8247	N/A	TSA/32 °C	ATCC	N/A
Bacillus licheniformis				
NRRLB-1001	N/A	TSA/30 °C	NRRL	N/A
mv55	KU232018.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
mv68	MF187633.1	TSA/30 °C	UB-CIDEFI	Alippi and Abramovich, 2019
Bacillus megaterium				
NRRL B-939	N/A	TSA/32 °C	NRRL	N/A
m327	MF187637.1	TSA/32 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m435	KU232028.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
Bacillus mycoides				
ATCC 10206	N/A	TSA/32 °C	ATCC	N/A
m336	MF187638.1	TSA/32 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m425	N/A	TSA/32 °C	UB-CIDEFI	N/A
Bacillus pumilus				
ATCC 7061[T]	AY876289.1	TSA/30 °C	ATCC	N/A
mv41aA	MG366818.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
mv49b	KU232016.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
mv74	MF972935.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
mv81	KU232019.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m116	KU232020.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m288	MF187635.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m332	MF187646.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m339	MG366884.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m350	KU232023.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m357	MF187634.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m358	MG345110.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m360	MF187636.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m363	KU232024.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m414	KU232026.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
Bacillus subtilis				
NRRL B-543	N/A	TSA/30 °C	NRRL	N/A
m13	MF187645.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
cm45	MF187639.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m191	MF187644.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m329	KU232021.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m334	KU232022.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m347	KP175515.1	TSA/30 °C	UB-CIDEFI	Bartel et al., 2018
m392	MF187640.1	TSA/30 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Bacillus thuringiensis				
ATCC 10792[T]	D16281.1	TSA/32 °C	ATCC	N/A
mv50b	KU232017.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
m395	KU232025.1	TSA/32 °C	UB-CIDEFI	Bartel et al., 2018
Brevibacillus borstelensis				
RC	MF187641.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
m348	KP177514.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Brevibacillus brevis				

(*continued on next page*)

Table 1 (*continued*)

Strain	Accession Number	Culture conditions[a]	Culture collection[b]	Reference
ATCC 8246	N/A	MYPGP/37 °C	ATCC	N/A
Brevibacillus laterosporus				
LAT169	KX102627.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
LAT170	KX431223.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
LAT171	KX431224.1	MYPGP/37 °C	UB-CIDEFI	Bartel et al., 2018
Lysinibacillus fusiformis				
mv119	MG004185.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Lysinibacillus sphaericus				
ATCC 245	N/A	MYPGP/37 °C	ATCC	N/A
m533	MG001492.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
LMDZA	MG004191.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Paenibacillus alvei				
NRRL B-383	N/A	MYPGP/37 °C	NRRL	N/A
mv82	MF187643.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m291a	MF187632.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
m420	MF187642.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019
Paenibacillus apiarius				
ATCC 29575	N/A	MYPGP/37 °C	ATCC	N/A
Paenibacillus larvae subsp. larvae				
ATCC 9545[T]	NR_118956.1	MYPGP/37 °C	ATCC	Ash et al., 1991
PL36	N/A	MYPGP/37 °C	UB-CIDEFI	N/A
Paenibacillus larvae subsp. *pulvifaciens*				
ATCC 13537[T]	KT363749.1	MYPGP/37 °C	ATCC	Dingman, 2015, unpublished
SAG 290	N/A	MYPGP/37 °C	UB-CIDEFI	N/A
SAG 10367	KT363748.1	MYPGP/37 °C	UB-CIDEFI	Dingman, 2015, unpublished
Paenibacillus polymyxa				
NRRL B-510	N/A	MYPGP/37 °C	NRRL	N/A
Rummeliibacillus stabekisii				
mv111	MF972934.1	MYPGP/37 °C	UB-CIDEFI	Alippi and Abrahamovich, 2019

[a] TSA: Tryptic soy agar, MYPGP: Müller-Hinton – Yeast –Peptone – Glucose – Pyruvate agar.
[b] ATCC: American Type Culture Collection, Rockville, USA; CCT: Coleção de Culturas Tropical, Fundaçao André Tosello, Brazil; NRRL: Northern Utilization Research and Development Division, Peoria, Illinois, USA; UB-CIDEFI: Unidad de Bacteriología, Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales,Universidad Nacional de La Plata, La Plata, Argentina.
[c] N/A: Not applicable.

2004; Alippi and Abrahamovich, 2019; Sinacori et al., 2014; Wen et al., 2017). Classical microbiological techniques, including microscopy and biochemical tests, are laborious and time-consuming. Only comparisons of complete 16S rRNA gene sequences do allow differentiation between closely related species, in particular within *B. cereus* and *B. subtilis* groups or *Lysinibacillus* species; while partial or low-quality sequences of the 16S rRNA gene(s) can produce erroneous identification (Logan et al., 2009).

Strains of closely related spore-forming bacteria have been identified using various novel or sophisticated methods, including the analysis of the 16S−23S rRNA intergenic transcribed spacers (ITS), *i.e.*, ITS-PCR fingerprinting or ITS-RFLP (Daffonchio et al., 1998; Haque and Russel, 2005; Shaver et al., 2002); Raman spectroscopy (Hutsebaut et al., 2006), Matrix-Assisted Laser Desorption/Ionization- time-of-flight- Mass (MALDI-TOF-MS) (Fernández-No et al., 2013; Pomastowski et al., 2019; Shu and Yang, 2017), and machine learning assisted Fourier Transform Infrared (FTIR) spectroscopy (Bağcıoğlu et al., 2019). However, most of these techniques require a high level of expertise and expensive equipment.

On the other hand, Restriction fragment length polymorphisms analysis (RFLP) of PCR amplified 16S rRNA gene(s) had been employed to examine the diversity of several spore-forming species isolated from different sources (Alippi et al., 2002; Ash et al., 1991; Jeyaram et al., 2011; López and Alippi 2007, 2008; Manzano et al., 2003; Vaerewijck et al., 2001; Vardhan et al., 2011; Wu et al., 2006). Nevertheless, these studies have focused on the differentiation of a limited number of species or specific groups isolated from diverse ecological niches.

In the light of the above considerations, the objective of this study was to assess the feasibility of using RFLP of PCR-amplified 16S rRNA gene (s) by using universal primers 27f/1492r for a low-cost, rapid screen for a primarily differentiation of the complex of aerobic spore-forming bacteria from honey.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

A total of 80 strains of aerobic mesophilic spore-forming bacteria listed in Table 1 were examined in this work. The collection includes 61 isolates from honey or other apiarian sources belonging to the Collection of UB-CIDEFI (Unidad de Bacteriología del Centro de Investigaciones de Fitopatología) and 19 strains from International Culture Collections (Table 1). All isolates were routinely grown either on tryptic soy agar (TSA) (Britania®, Argentina) or on Müller-Hinton – Yeast –Peptone – Glucose – Pyruvate agar (MYPGP) (Dingman and Stahly, 1983) at the appropriate temperature according to the species tested (Table 1).

2.2. DNA preparation, PCR amplification, and RFLP analysis of 16S rRNA genes

Bacterial cells for DNA extraction were grown at the appropriate temperature and medium under aerobic conditions for 24–48 h according to the species used (Table 1). For DNA preparation, a rapid procedure using whole cells from plates was used as previously described (Alippi and Aguilar, 1998). Universal primers 27f (5´AGAGTT TGATCMTGGCTCAG 3′) and 1492r (5´ TACGGYTACCTTGTTACGACTT 3′) described by Yu et al. (2013) were employed. PCRs were carried out in a final volume of 25 μl according to a previously described protocol (Yu et al., 2013). After amplification of the PCR product of approximately 1492 bp, subsamples of 2 μl were incubated with endonucleases *Rsa*I, *Hae*III, *Alu*I, *Hinf*I, *Taq*I, and *Cfo*I, according to the manufacturer's specifications (Promega®, CABA, Buenos Aires, Argentina). RFLP analysis was performed by electrophoresis in a 1.6% agarose gel at 70 V for 2 h. All the isolates listed in Table 1 were analyzed.

Table 2
Accession numbers of selected 16S rRNA sequences and whole-genomes from Type strains (T) used for *in silico* analysis and comparisons.

Strains	Accession Number 16S rRNA gene	Accession Number whole-genome
Bacillus amyloliquefaciens ATCC 23350^T	NR_118950.1	FN597644
Bacillus anthracis ATCC14578^T	AE016879	GCA_000007845.1
Bacillus atrophaeus JCM 9070^T	AB021181	GCA_001584335.1
Bacillus badius ATCC 14574^T	X77790.1	JXLP01000009
Bacillus cereus ATCC 14579^T	AF290546.1	AE016877
Bacillus circulans ATCC 4513^T	AY724690.1	AY724690
Bacillus clausii DSM 8716^T	X76440.1	CP019985
Bacillus cytotoxicus NVH 391^T	CP000764	GCA_000017425.1
Bacillus coagulans ATCC 7050^T	AB271752.1	CP009709
Bacillus firmus NBRC 15306^T	NR_112635.1	BCUY01000205
Bacillus flexus NBRC 15715^T	NR_024691.1	BCVD01000224
Bacillus licheniformis ATCC 14580^T	NR_074923.1	AE017333
Bacillus mojavensis RO-H-1^T	JH600280	GCA_000507105.1
Bacillus megaterium ATCC 14581^T	NR_112636.1	JJMH01000057
Bacillus mycoides ATCC 6462^T	NR_115993.1	ACMU01000002
Bacillus paralicheniformis KJ-16^T	KY694465	GCA_001042485.2
Bacillus pumilus ATCC 7061^T	NR_043242.1	ABRX01000007
Bacillus pseudomycoides DSM 12442^T	ACMX01000133	GCA_000161455.1
Bacillus safensis FO-36b^T	ASJD01000027	GCA_003097715.1
Bacilus siamensis KCTC 3613^T	AJVF01000043	GCA_000262045.1
Bacillus simplex NBRC 15720^T	NR_042136.1	BCVO01000086
Bacillus subtilis IAM 12118^T	NR–112116.2	ABQL01000001
Bacillus tequilensis KCTC 13622^T	AYTO01000043	GCA_000507145.1
Bacillus thuringiensis ATCC 10792^T	D16281.1	ACNF01000156
Bacillus toyonensis BCT-7112^T	CP006863	GCA_000496285.1
Bacillus vallismortis DV1-F-3^T	JH600273	N/A
Bacillus velezensis CR-502^T	AY603658	GCA_001461825.1
Bacilus xiamenensis HYC-10^T	AMSH01000114	GCA_000300535.1
Brevibacillus borstelensis NRRL NRS-818^T	AB112721	GCA_003710865.1
Brevibacillus brevis NBRC 15304^T	AB271756.1	GCA_003385915.1
Brevibacillus laterosporus DSM 25^T	NR_112212.1	CP017705
Lysinibacillus fusiformis ATCC 7055^T	NR_112569.1	GCA_003049525.1
Lysinibacillus sphaericus NBRC 15095^T	NR_112627.1	GCA_002982115.1
Paenibacillus alvei DSM 29^T	AJ320491	GCA_000293805.1
Paenibacillus apiarius NRRL NRS-1438^T	NR_118834.1	GCA_002161865.1
Paenibacillus larvae subsp. *larvae* ATCC 9545^T	NR_118956.1	GCA_002003265.1
Paenibacillus larvae subsp.*pulvifaciens* ATCC 13537^T	KT363749.1	GCA_002007765.1
Paenibacillus polymyxa ATCC 842^T	AJ320493.1	AFOX01000032
Rummeliibacillus stabekisii KSC-SF6g^T	DQ870754.1	N/A[a]

[a] N/A: Not applicable.

2.3. *In silico* analysis of 16S rRNA gene(s) sequences

Twenty-six different species belonging to 5 different genera of spore-forming bacteria reported in honey were analyzed. *In silico* RFLP analysis was performed using endonucleases *Rsa*I, *Hae*III, *Alu*I, *Hin*fI, *Taq*I, and *Cfo*I, respectively. A total of 94 theoretical restriction fragment patterns were obtained by using the software http://nc2.neb.com/NEBcutter2/.

We tested thirty-nine 16S rRNA sequences from type cultures retrieved from NCBI GenBank (Tables 2 and 3) and fifty-nine 16S rRNA sequences from strains obtained from honey samples previously reported (Alippi and Abrahamovich, 2019; Bartel et al., 2018; Minnaard and Alippi, 2016) (Tables 1 and 3).

3. Result and discussion

To determine whether species-specific amplicons from *Bacillus, Brevibacillus, Lysinibacillus, Rummeliibacillus,* and *Paenibacillus* strains from honey could be detected, restriction digests were carried out with *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Rsa*I, and *Taq*I and RFLP patterns obtained *in silico* matched those obtained experimentally when 16S rRNA gene(s) sequences were near full lenght (> 1400 nucleotides) (Tables 3 and 4). The sequencing of the 16S rRNA gene provides high-quality results in bacterial identification, depending on the quality of the sequence. As 16S rRNA gene(s) sequences in public databases are sometimes of low-quality; an almost complete sequence (> 1400 nt, < 0.5% ambiguity) from reference strains should be used for comparisons and phylogenetic analysis, particularly in the case of aerobic-spore-forming bacteria (Logan et al., 2009).

Our results corroborated this criterion since when comparing RFLP patterns obtained both *in silico* and experimentally, some differences were observed when sequence lengths were < 1400 nt (Tables 3 and 4). For instance, *B. amyloliquefaciens* strains xx and mv35 showed differences in the signature bands obtained *in silico* for restriction enzymes *Alu*I and *Taq*I (Table 3), while no differences were detectable in a gel (Table 4). A similar situation was observed for *B. cereus* strain LPcer1 and *B. megaterium* strain m435 for *Alu*I, and *B. pumilus* strains m350 and m116 for *Cfo*I (Tables 3 and 4). These discrepancies *in silico* were only found when the sequences analyzed were lower than 1350 bp. Nevertheless, strains belonging to the same species showed a unique RFLP pattern for each restriction enzyme when tested experimentally (Table 4). Similar results have been reported by Jeyaram et al. (2011) when they examined strains belonging to the *B. subtilis* group for restriction enzymes *Rsa*I and *Cfo*I.

In the present study, a simple RFLP by PCR amplification of the 16S rRNA gene using universal primers 27f/1492r followed by restriction digestion using *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Rsa*I, and*Taq*I, distinctly differentiated closely related species. Within the whole collection analyzed here, a total of 88 restriction patterns were detected for all the restriction endonucleases tested (Table 5 and Fig. 1). For instance, the *Alu*I restriction pattern of *B. licheniformis* was found to be unique (Table 4) and allowed us to distinguish it from other closely related bacteria reported in honey and from other species within the *B. subtilis* group (Table S1). Similar results were obtained for *B. badius*, *B. cereus sensu stricto, B. circulans, B. clausii, B. coagulans, B. firmus, B. megaterium, B. mycoides, B. pumilus, B. thuringiensis,* and *Br. borstelensis,* respectively that showed species-specific *Alu*I restriction patterns (Table 5). When using *Cfo*I, eight species-specific restriction patterns unique for *B. cereus sensu stricto, B. megaterium, B. mycoides, B. thuringiensis, Br. borstelensis, L. fusiformis, L. sphaericus, and R. stabekisii,* respectively were observed (Table 5). In the case of *Hae*III, nine unique patterns were obtained for *B. badius, B. circulans, B. clausii, B. coagulans, B. firmus, Br. laterosporus, P. alvei, P. apiarius*, and *P. polymyxa* (Table 5). When using *Hin*fI, 10 unique patterns were detected for *B. badius, B. cereus sensu stricto, B. circulans, B. clausii, B. coagulans, B. firmus, P. alvei, P. larvae* subsp. *larvae, P. larvae* subsp. *pulvifaciens,* and *R. stabekisii.* When using *Rsa*I, nine unique patterns were obtained for *B. amyloliquefaciens, B. coagulans, B. licheniformis, P. alvei, P. apiarius, P. larvae* subsp. *larvae, P. larvae* subsp. *pulvifaciens*, *P. polymyxa,* and *R. stabekisii.* Finally, when testing *Taq*I, nine unique patterns were visualized for *B. circulans, B. coagulans, B. firmus, Br. laterosporus, P. alvei, P. apiarius, P. larvae subsp. larvae, P. larvae* subsp. *pulvifaciens* and *P. polymyxa.*

In the case of closely related groups, *i.e.*, *B. cereus* and *B. subtilis* (Bağcıoğlu et al., 2019; Fan et al., 2017, Guinebretière et al., 2013; Dunlap et al., 2016; Hutsebaut et al., 2006; Haque and Russel, 2005; Jeyaram et al., 2011; Manzano et al., 2003; Shaver et al., 2002; Vilas-Boas et al., 2007), the technique described here allowed us to differentiate closely related species. Such as, species within the *B. cereus* group reported in honey (*B. cereus sensu stricto, B. mycoides*, and *B. thuringiensis*) can be separated by using *AluI* (Table 5 and Fig. 2A) or *Cfo*I (Table 5 and Fig. 2B). *B. cereus sensu stricto, B. thuringiensis,* and *B. mycoides* showed distinct *Alu*I-fragments of about 600 bp (Fig. 1A, lane C and Fig. 2A, lane 1); 593 bp (Fig. 1A, lane L and Fig. 2A, lanes 3 and 4), and 550 bp (Fig. 1A, lane J and Fig. 2A, lane2), respectively. In addition, when using *Cfo*I, *B. cereus sensu stricto* showed a distinct

Table 3
Theoretical prediction of restriction fragments size (in base pairs) generated by *Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Rsa*I, and *Taq*I based on published 16S rRNA sequences from GeneBank.

Species	Signature bands obtained with six restriction enzymes with a 4-bp recognition site					
	*Alu*I	*Cfo*I	*Hae*III	*Hinf*I	*Rsa*I	*Taq*I
Bacillus amyloliquefaciens						
ATCC 23350^T	430-265-207-201-186-173-68.	869-426-235.	599-457-289-105-22.	605-372-316-154-25.	501-466-406-99.	907-359-214-50.
m39-m287b-m163b-m164b-m291b	430-265-207-186-173-134-24.	869-359-191	599-457-260-81-22.	606-372-287-129-25.	501-437-406-75.	907-359-147-6.
mv35	430-207-187-186-173.	869-165-149.	599-457-266-65-22.	605-372-293-114-25.	501-443-406-59.	891-292.
xx	430-265-207-186-173-94	869-319-97	599-457-166-41-22	605-372-193-90-25	501-406-343-35	819-359-107
Bacillus badius						
ATCC 14574^T	429-392-206-186-176-53	352-345-336-223-182-2-2.	564-365-292-91-74-34-2.2	975-319-123-25	439-406-354-146-68-18-11.	909-499-34.
Bacillus cereus						
ATCC 14579^T	599-224-186-174-169-81-58-21	586-394-346-182-2-2.	565-457-291-117-34-22.	977-345-165-25	495-406-355-146-110	771-541-138-62
ATCC 11778	599-224-186-174-156-64-58-21	569-381-346-182-2-2	565-457-301-103-34-22	977-328-172-25	478-406-355-146-97	771-528-138-45
m6c-mv33 434	599-224-186-174-168-60-58-21	585-393-346-182-2-2	565-460-297-112-34-22	977-324-164-25.	444-406-355-149-106.	771-540-138-41
LPcer1	599-224-186-174-112-79-9	530-346-337-182-2-2	565-457-262-59-34-22	977-289-108-25	439-406-355-146-53.	771-484-129-9-6
m387	600-398-186-93-58-21-8	513-347-318-182-2-2	566-457-212-40-34-33-22	978-272-89-25	422-407-355-146-34	772-465-127
MexB	601-224-174-114-29	534-351-182-71-2-2	565-266-255-34-22	824-293-25	443-406-293.	771-223-138-10
MexC	599-224-186-174-111	511-346-336-182-2-2	565-457-243-58-34-22	977-270-105-25	420-406-355-146-52	771-483-425
Bacillus circulans						
ATCC 4513^T	824-265-170-84-56-50-38	392-348-336-216-182-2-2	438-406-357-146-111-18-11	607-351-318-166-25-20	438-406-357-146-111-18-11	770-541-122-31-14
Bacillus clausii						
DSM 8716^T	454-419-218-186-88-86-54	414-346-337-214-182-6-2-2-2	627-264-216-208-85-78-22-5	790-372-318-25	433-406-355-146-130-19-11-5	561-409-380-120-35
Fr231	419-394-219-186-88-85-26	354-346-338-193-182-2-2	567-264-217-184-85-78-22	730-373-289-25	409-406-355-146-70-20-11	561-408-380-120-8
m448b	428-419-218-186-88-70	388-346-337-182-152-2-2	601-264-216-143-85-78-22	764-372-248-25	406-368-355-146-104-19-11	535-380-374-120
Bacillus coagulans						
ATCC 7050^T	616-473-209-174-77	385-348-336-222-182-2-2	404-367-236-155-92-85-76-44	979-366-204	491-406-357-149-146	908-534-35
Bacillus firmus						
NBRC 15306^T	824-425-124-53-51	395-348-336-222-182-2-2	564-459-213-107-44-34-34-22	547-371-318-156-60-25	438-406-357-146-101-18-11	904-312-232-39
Bacillus flexus						
NBRC 15715^T	615-265-209-199-88-86-67	433-348-245-236-182-101-2-2	598-459-226-146-78-22	978-331-195-25	451-406-357-146-140-18-11	582-500-467
Bacillus licheniformis						
ATCC 14580^T	823-265-210-141-73-33	380-346-337-219-182-2-2	599-457-288-102-22	605-372-315-151-25	501-435-406-96-19-11	500-408-359-168-33
mv55-mv68	823-265-163-141-69-33	388-346-337-237-182-2-2	599-457-306-108-22	605-372-333-155-25-4	503-453-406-102-19-11	500-359-176-51
Bacillus megaterium						
ATCC 14581^T	615-265-209-134-86-54	531-350-348-182-2-2	598-459-200-120-78-22	978-318-165-25	438-406-357-146-110-18-11	908-573-48
m327	615-265-209-123-88-86-24-10	528-358-348-182-2-2	598-459-183-80-78-22	978-288-129-25	438-406-357-146-74-18-11	907-507-6
m435	388-265-209-123-48	396-348-181-77-27-2-2	459-319-137-118	866-167	405-357-146-112-11-2	545-488
Bacillus mycoides						
ATCC 6462^T	552-224-186-174-169-58-47-30-21	535-394-346-182-2-2	565-457-267-116-34-22	977-294-165-25	444-406-355-146-110	771-541-138-11
m336	552-224-186-174-134-58-47-25-21	530-359-346-182-2-2	565-457-262-81-34-22	977-289-130-25	439-406-355-146-75	771-506-138-6
Bacillus pumilus						
ATCC 7061^T	428-265-207-186-160-88-85-62	867-385-229	598-457-260-97-22	605-371-287-146-25	501-436-406-91	905-359-173-44
m330-m288-m339-m354-m357-m358	429-265-207-186-134-88-85-24	868-359-191	598-457-260-79-22	605-371-287-128-25	501-407-406-75-19-11	906-359-147-6
mv41aA-m363-m414-m360-mv49b-						
mv74	429-265-207-186-88-85-56-25	868-250-223	598-429-292-22	605-371-319-25-21	468-467-406	906-359-38-38
mv81	429-265-207-186-132-88-85-4	868-357-171.	598-457-240-79-22	605-371-267-128-25.	501-416-406-73.	892-359-145.
m350	429-186-126-88-85-51	747-218	598-287-58-22	371-314-25-25	463-406-96	906-33-26
m116	429-265-186-130	900-250-223	598-459-240-79-22	605-314-25-25	470-406-96	882-359-145
Bacillus simplex						
NBRC 15720^T	615-463-208-88-85-63	423-347-336-230-182-2-2	479-458-221-145-85-78-34-22	917-275-194-60-51-25	446-406-356-146-139-18-11	896-572-45-9
Bacillus subtilis						
IAM 12118^T	430-265-207-186-173-130-51	869-342-218	599-457-269-140-22	605-372-326-189-25	501-446-406-134-19-11	500-407-359-110-33
m392-cm45-m191-m13-m347-329-334	430-265-207-186-173-134-24	869-359-191	599-457-260-81-22	605-372-287-130-25	501-407-406-75-19-11	500-407-359-147-66
Bacillus thuringiensis						
ATCC 10792^T	599-224-186-174-170-58-54-21	559-395-346-182-2-2	556-457-291-117-34-22.	977-318-166-25	468-406-355-146-111	771-542-138-35.

(*continued on next page*)

Table 3 (*continued*)

Species	Signature bands obtained with six restriction enzymes with a 4-bp recognition site					
	*Alu*I	*Cfo*I	*Hae*III	*Hinf*I	*Rsa*I	*Taq*I
mv50b	600-224-186-174-122-58-26-21	531-347-347-182-2-2	566-457-263-69-34-22.	978-290-118-25	440-406-356-146-63	772-494-137-8
m395	599-224-186-174-162-26	531-346-182-122-2-2	565-301-263-34-22	870-290-25	440-406-339	771-269-138-7
Brevibacillus borstelensis						
NRRL NRS-1438^T	403-229-210-200-98-81-33-4	412-346-337-181-175-31-2-2	598-457-177-134-44-34-22	1001-263-183-39	412-400-355-146-119-19-11-9	499-395-357-202-33
RC-m348	403-212-207-205-189-81-74-33-4	358-346-337-181-151-31-1-1	598-457-173-80-44-34-22	1001-263-129-15	415-398-355-146-74-19-11	499-395-303-202-9
Brevibacillus brevis						
NBRC 15304^T	425-403-212-161-159-46-43-12	385-346-337-208-181-2-2	598-457-199-107-44-34-22	1001-252-156-41-9-2	424-405-355-146-101-19-11	499-395-320-202-35
Brevibacillus laterosporus						
DSM 25^T	452-403-212-181-179-46-33	394-346-337-181-177-31-2-2	457-410-199-188-116-78-22	1001-304-165	454-405-355-146-110	894-339-202-35
lat169-lat170-lat171	403-370-212-161-152-46-33	346-337-330-181-148-31-2-2	457-410-188-170-78-52-52	1001-275-101	425-405-355-146-46	894-275-202-6
Lysinibacillus fusiformis						
ATCC 7055^T	615-246-209-207-174-64	413-348-336-232-182-2-2	598-459-223-135-78-22	978-328-184-25	503-448-406-129-18-11	879-562-45-29
mv119	615-209-207-190-174-27	357-348-336-195-182-2-2	598-459-186-79-78-22	978-291-128-25	503-411-406-73-18-11	908-506-8
Lysinibacillus sphaericus						
NBRC 15095^T	615-216-209-207-174-64.	400-348-336-232-182-2-2	598-459-190-78-72-22	978-295-121-25	503-415-406-66-18-11	908-562-45
m533-LMDZ	615-209-207-189-174-25	356-348-336-193-182-2-2	598-459-184-78-78-22	978-289-127-25	503-409-406-72-18-11	908-505-6
Paenibacillus alvei						
DSM 29^T	461-419-216-186-157-88	421-346-336-239-181-2-2	1124-308-73-22	1167-335-25	484-340-170-146-144-137-91	789-568-120-50
mv82-m420-m291a	419–216-186-116-88	357-346-336-198-181-2-2	1133-267-22	1103-294-25	443-340-235-170-146-73-15	789-504-120-9
Paenibacillus apiarius						
NRRL NRS-1438^T	587-422-186-160-88-46	348-344-336-242-181-34-2-2	596-526-311-34-22	801-338-325-25	596-526-311-34-22	645-528-260-56
Paenibacillus larvae subsp. *larvae*						
ATCC 9545^T	637-364-186-163-73-14	348-336-324-244-181-2-2	286-247-235-231-215-123-78-22	1072-332-25-8	489-405-344-146-38-15	788-473-120-49-7
Paenibacillus larvae subsp.*pulvifaciens*						
NRRL B-14154	637-406-186-135-74-14	366-348-336-217-181-2-2	286-247-240-231-208-123-78-22	919-313-195-25	462-405-344-146-80-15	789-515-120-28
ATCC 13537^T	637-404-186-73-20-14	364-348-336-181-101-2-2	286-247-240-231-208-123-78-22	919-313-195-25	462-405-344-146-80-15	701-513-120
Paenibacillus polymyxa						
ATCC 842^T	419-390-216-186-88-87-72-62	423-346-336-231-181-2-2	689-288-222-222-44-34-22	799-370-327-25	476-405-342-147-136-15	789-570-120-42
Rummeliibacillus stabekisii						
KSC-SF6g^T	615-215-209-207-86-85-32	382-360-348-182-165-8-2-2	598-459-209-70-44-37-34-22	893-339-156-85	434-406-357-146-101-18-11	904-531-14
mv111	615-209-207-192-86-85	380-359-348-182-161-8-2-2	598-459-184-70-44-34-22-11	893-314-130-85	409-406-357-146-75-18-11	904-508-10

Table 4
Gel detectable restriction fragments size (in base pairs) of a PCR-amplified 16S rRNA fragment of 1490 bp digested with *Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Rsa*I, and *Taq*I.

Species	Signature bands obtained with six restriction enzymes with a 4-bp recognition site					
	*Alu*I	*Cfo*I	*Hae*III	*Hinf*I	*Rsa*I	*Taq*I
Bacillus amyloliquefaciens						
m39-m287b-m163b-m164b-m291b-mv35-xx	430-265-210-186-170	869-359-191	600-457-260	606-372-287-130	501-437-406	907-359-147
Bacillus badius						
CCT 0196	430-390-205-186-170	352-345-336-223-182	564-365-292	975-319-123	438-406-354-146	910-500
Bacillus cereus						
ATCC 11778-m6c-mv33 -434LPcer1-m387-MexB-MexC	600-224-186-170	585-393-346-182	565-460-297-112	977-324-164	444-406-355-149-106	771-540-138
Bacillus circulans						
ATCC 4515	824-265-170	395-348-336-216-182	438-406-357-146-111	607-351-318-166	438-406-357-146	770-541-122
Bacillus clausii						
Fr231-m448b-BcIENT	428-420-220-190.	354-346-338-193-182	600-264-216-143	764-372-248	409-406-355-146	561-408-380-120
Bacillus coagulans						
ATCC 35670	620-470-200-170	395-348-336-222-182	404-367-236-155	979-366-204	491-406-357-149-146	910-535
Bacillus firmus						
ATCC 8247	824-425-125	395-348-336-222-182	564-459-213-107	547-371-318-156	438-406-357-146	904-312-232
Bacillus licheniformis						
NRRL B 1001-mv55-mv68	823-265-210-170	388-346-337-237	600-457-260	606-372-287-130	503-453-406-102	500-359-176
Bacillus megaterium						
NRRL B-939-m327-m435	615-265-210-170	571-424-348-182	598-457-173	978-288-130	438-406-357-146	910-500
Bacillus mycoides						
ATCC 10206-m336-m425	552-224-186-170	535-394-346-182	565-460-297-112	978-289-130-	439-406-355-146	771-540-138
Bacillus pumilus						
ATCC 7061^T-m330-m288-m339-m354-m357-m358 mv41aA-m363-m414-m360-mv49b-mv74-mv81-m350-m116	430-265-210-186	868-359-191	600-457-260	606-372-287-130	501-407-406	907-359-147
Bacillus subtilis						
NRRL B-543-m392-cm45-m191-m13-m347-329-334	430-265-210-186-170	869-359-191	600-457-260	606-372-287-130	501-407-406	500-359-176
Bacillus thuringiensis						
ATCC 10792^T -mv50b-m395	593-220-186-170	531-347-347-182	565-460-297-112	978-288-130	440-406-356-146	771-540-138
Brevibacillus borstelensis						
RC-m348	400-210-190	358-346-337-181-151	598-457-173	1001-263-129	405-398-355-146	499-395-323-202
Brevibacillus brevis						
NBRC 15304	425-403-210-160	385-346-337-208-181	598-457-173	1001-263-129	425-405-355-146	499-395-323-202
Brevibacillus laterosporus						
LAT169-LAT170-LAT171	425-403-210-160	395-348-336-216-182	457-410-188-170	1001-263-129	425-405-355-146	894-275-202
Lysinibacillus fusiformis						
mv119	615-210-200-170	415-350-336-232-182	598-457-173	978-288-130	503-410-406	910-500
Lysinibacillus sphaericus						
ATCC 245-m533-LMDZA	615-210-200-170	360-350-336-190	598-457-173	978-288-130	503-410-406	910-500
Paenibacillus alvei						
NRRL B-383-mv82-m420-m291a	420-390-220-190	420-350-335-232-182	1133-267	1100-294	443-340-235-170-146	789-504-120

(*continued on next page*)

Table 4 (*continued*)

Species	Signature bands obtained with six restriction enzymes with a 4-bp recognition site					
	*Alu*I	*Cfo*I	*Hae*III	*Hinf*I	*Rsa*I	*Taq*I
Paenibacillus apiarius						
ATCC 29575	590-420-186-160	352-345-336-220-182	596-526-311	800-338-325	487-339-234-170	645-528-260
Paenibacillus larvae subsp. *larvae*						
ATCC 9545^T-PL36	640-400-186	352-345-336-220-182	286-247-235-231-215	1072-332	489-405-344-146	788-473-120
Paenibacillus larvae subsp. *pulvifaciens*						
ATCC 13537^T-SAG290-SAG 10367	640-400-186	352-345-336-220-182	286-247-240-231-215	920-313-195	462-405-344-146	701-513-120
Paenibacillus polymyxa						
NRRL B-510	420-390-220-190	420-350-335-232-180	689-288-222	800-338-325	476-405-342-147-136	789-570-120
Rummeliibacillus stabekisii						
mv111	615-215-210-200	380-360-350-180-160	598-457-173	893-314-130	434-406-357-146-101	910-500

Table 5
Restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rRNA genes among aerobic spore-forming species used in this study.

Species	Pattern obtained with restriction enzyme:					
	*Alu*I	*Cfo*I	*Hae*III	*Hinf*I	*Rsa*I	*Taq*I
Bacillus amyloliquefaciens	A	A	A	A	A	A
Bacillus badius	B	B	B	B	B	B
Bacillus cereus sensu stricto	C	C	C	C	C	C
Bacillus circulans	D	D	D	D	B	D
Bacillus clausii	E	B	E	E	D	E
Bacillus coagulans	F	D	F	F	E	F
Bacillus firmus	G	D	G	G	B	G
Bacillus licheniformis	H	E	A	A	F	H
Bacillus megaterium	I	F	H	H	B	B
Bacillus mycoides	J	G	C	H	B	C
Bacillus pumilus	K	A	A	A	G	A
Bacillus subtilis	A	A	A	A	G	H
Bacillus thuringiensis	L	H	C	H	C	C
Brevibacillus borstelensis	M	I	H	I	D	I
Brevibacillus brevis	N	E	H	I	H	I
Brevibacillus laterosporus	N	D	I	I	H	J
Lysinibacillus fusiformis	O	J	H	H	I	B
Lysinibacillus sphaericus	O	K	H	H	I	B
Paenibacillus alvei	P	L	J	J	J	K
Paenibacillus apiarius	Q	B	K	K	K	L
Paenibacillus larvae subsp. *larvae*	R	B	L	L	L	M
Paenibacillus larvae subsp. *pulvifaciens*	R	B	L	M	M	N
Paenibacillus polymyxa	P	L	M	K	N	O
Rummeliibacillus stabekisii	O	M	H	N	O	B
Total	18	13	13	14	15	15

fragment of about 585 bp (Fig. 1B, lane C, and Fig. 2B, lane 1), while *B. thuringiensis* and *B. mycoides* showed distinct *Cfo*I-fragments of about 531 bp (Fig. 1B, lane H, and Fig. 2B, lane 3) and 535 bp (Fig. 1B, lane G, and Fig. 2B, lane 2), respectively. It is interesting to point out that all the species belonging to the *B. cereus* group present a distinct *Taq*I pattern (C) different to the rest of the *Bacillus* and relatives from apiarian sources (Table 4 and Fig. 1F, pattern C). Members of this group showed a high degree of similarity with only 7–9 dispersed nucleotides differences in the 16S rRNA gene sequence (Ash et al., 1991; Daffonchio et al., 1998; Wu et al., 2006), these differences were enough to allow PCR-RFLP identification in the present study. Also, we tested *in silico* the rest of the species of the group, *i.e.*, *B. anthracis, B. cytotoxicus, B. pseudomycoides,* and *B. toyonensis* and were able to discriminate among them by using *Cfo*I (Table S2). Manzano et al. (2003) found differences among these species by using enzyme digestion of *gyrB* by *Sau3A*I, while Daffonchio et al. (1988) did not find apparent differences in their analysis of the 16S-23S rRNA ITS. In previous studies, we found differences between *B. cereus* and *B. mycoides* with a PCR-RFLP assay using Bacillus-specific primers U1/U2 followed by *Alu*I or *Hae*III digestion (Alippi et al., 2002).

Also, within the closely related *B. subtilis* group, the combination of *Alu*I and *Taq*I or *Rsa*I restriction patterns obtained for *B. subtilis, B. amyloliquefaciens, B. pumilus,* and *B. licheniformis* allowed us to differentiate them within the group and from those generated by the rest of the species tested and reported in honey (Table 5, Fig. 1A patterns A, H, and K). For instance, a fragment of 825 bp after *Alu*I digestion was found in all *B. licheniformis* strains tested, while absent in the rest of the group (Table 4 and Fig. 1A, pattern H). Also, *B. licheniformis* strains lacked a 430 bp fragment that was present in the rest of the group (Table 4 and Fig. 1A, pattern H). On the other hand, *B. pumilus* strains lacked an *Alu*I fragment of 170 bp present in the rest of the group (Table 4 and Fig. 1A, pattern K). When using *Taq*I, the strains of *B. subtilis* and *B. licheniformis* showed a distinct pattern (H) different from the pattern (A) observed in *B. pumilus* and *B. amyloliquefaciens* strains (Table 4 and Fig. 1F). Also, *Rsa*I restriction patterns obtained allowed

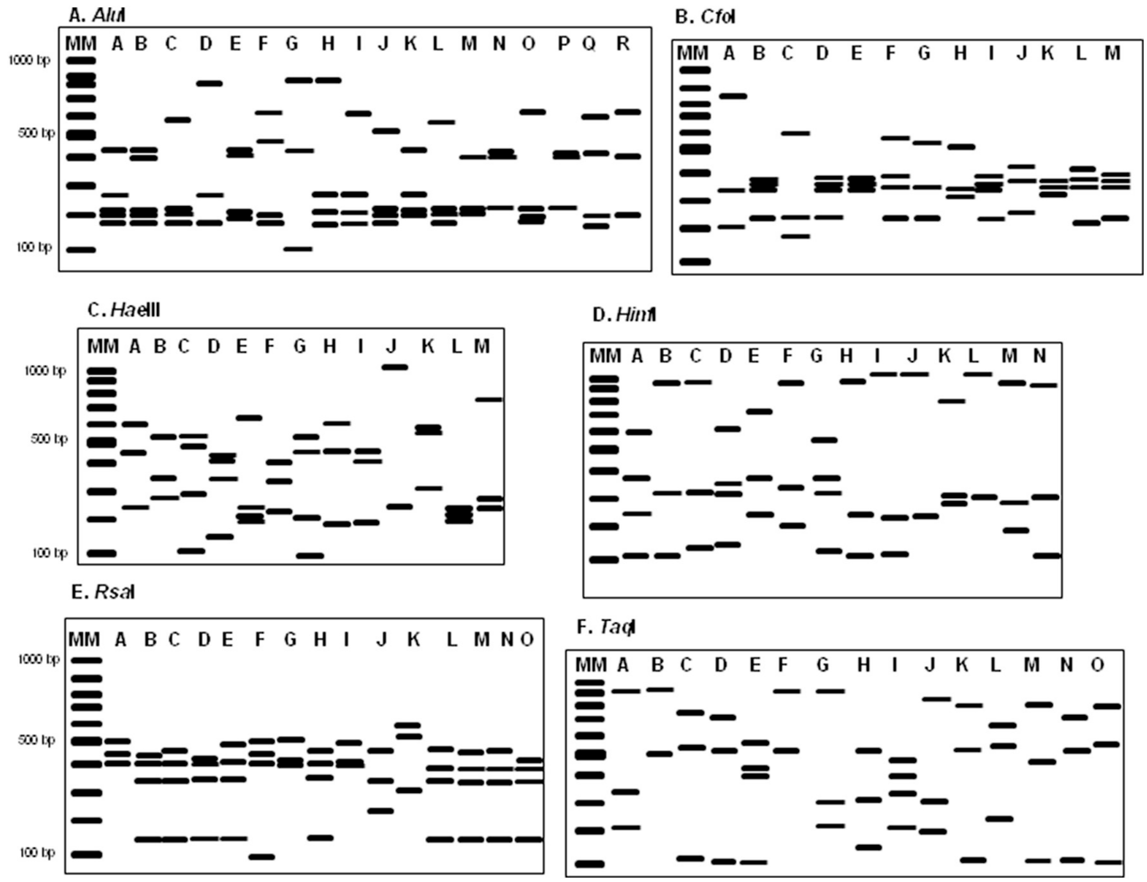

Fig. 1. Restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rRNA genes found among all the aerobic spore-forming isolates analyzed (n = 80) digested with (A) *Alu*I, (B) *Cfo*I, (C) *Hae*III, (D) *Hinf*I, (E) *Rsa*I, and (F) *Taq*I, respectively. (A) *Alu*I: Lane MM: Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina) (size is indicated on the left in bp). Lanes: A, *B. amyloliquefaciens* m39; B, *B. badius* CCT 0196; C, *B. cereus* m6c; D, *B. circulans* ATCC 4515; E, *B. clausii* Fr231; F, *B. coagulans* ATCC 35670; G, *B. firmus* ATCC 8247; H, *B. licheniformis* mv55; I, *B. megaterium* m327; J, *B.mycoides* m425; K, *B. pumilus* m330; L, *B. thuringiensis* mv50b; M, *Br. borstelensis* RC; N, *Br. laterosporus* LAT170; O, *L. sphaericus* m533; P, *P. alvei* m291a; Q, *P. apiarius* ATCC 29575; R, *P. larvae* subsp. *larvae* PL36. (B) *Cfo*I: Lane MM: Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina). Lanes: A, *B. amyloliquefaciens* m39; B, *B. badius* CCT 0196; C, *B. cereus* ATCC11778; D, *B. circulans* ATCC 4515; E, *B. licheniformis* mv68; F, *B.megaterium* m435; G, *B. mycoides* m425, H, *B. thuringiensis* ATCC10792^T; I, *Br. borstelensis* m348; J, *L. fusiformis* mv119; K, *L. sphaericus* LMDZA; L, *P. alvei* mv82; M, *R. stabekisii* mv111. (C) *Hae*III. Lanes: MM: Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina) (size is indicated on the left in bp); A, *B. amyloliquefaciens* m163b; B, *B. badius* CCT 0196; C, *B. cereus* mv33; D, *B. circulans* ATCC 4515; E, *B. clausii* Fr231; F, *B. coagulans* ATCC 35670; G, *B. firmus* ATCC 8247; H, *B. megaterium* m327; I, *Br. laterosporus* LAT170; J, *P. alvei* m420; K, *P. apiarius* ATCC 29575; L, *P. larvae* subsp. *larvae* PL36; M, *P. larvae* subsp. *pulvifaciens* ATCC13537^T. (D) *Hinf*I. Lanes: MM, Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina); A, *B. subtilis* m191; B, *B. badius* CCT 0196; C, *B. cereus* LPcer1; D, *B. circulans* ATCC 4515; E, *B. clausii* Fr231; F, *B. coagulans* ATCC 35670; G, *B. firmus* ATCC 8247; H, *B. thuringiensis* ATCC10792^T; I, *Br. laterosporus* LAT169; J, *P.alvei* m291a; K, *P. apiarius* ATCC 29575; L: *P. larvae* subsp. *larvae* PL36; M, *P. larvae* subsp. *pulvifaciens* ATCC13537; N, *R. stabekisii* mv111. (E) *Rsa*I: MM: Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina) (size is indicated on the left in bp); lane A, *B. amyloliquefaciens* m39; B, *B. badius* CCT0 196; lane C, *B. cereus* m6c; D, *B. clausii* Fr231; E, *B. coagulans* ATCC 35670; F, *B. licheniformis* mv68; G, *B. pumilus* m360; H, *Br. laterosporus* LAT169; I, *L. fusiformis* mv119; J, *P. alvei* mv82; K, *P. apiarius* ATCC 2957; L, *P. larvae* subsp. *larvae* PL36; M, *P. larvae* subsp. *pulvifaciens* ATCC13537^T; N, *P. polymyxa* NRRLB-510; O, *R. stabekisii* mv11. (F) *Taq*I: Lanes: MM, Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina), A, *B. amyloliquefaciens* mv35; B, *B. badius* CCT0196; C, *B. cereus* m387; D, *B. circulans* ATCC 4515; E, *B. clausii* m448b; F, *B. coagulans* ATCC 35670; G, *B. firmus* ATCC 8247; H, *B. subtilis* m347; I, *Br. borstelensis* RC; J, *Br. laterosporus* LAT171; K, *P. alvei* m420; L, *P. apiarius* ATCC 29575; M: *P. larvae* subsp. *larvae* PL36; N, *P. larvae* subsp. *pulvifaciens* SAG 290; O, *P. polymyxa* NRRL B-510.

us to differentiate *B. subtilis* (G) and *B. licheniformis* (F) from *B. amyloliquefaciens* (A) and *B. pumilus* (A) (Table 5 and Fig. 1E). Similar results were observed by Jeyaram et al. (2011) with a PCR-RFLP assay using primers fD1/rD1 followed by a restriction with *Rsa*I or *Cfo*I. However, Wu et al. (2006) found some limitations when trying to distinguish among members of the *B. subtilis* group and also three species in the *B. cereus* cluster by using Amplified Ribosomal DNA Restriction Analysis (ARDRA) of PCR amplicons obtained with B−K1 primers. On the other hand, when tested *in silico,* all the species belonging to the *B. subtilis* group that have not been reported in honey, the combination of *Alu*I, *Cfo*I, *Rsa*I, and *Taq*I allowed us to differentiate them (Table S1).

Within the rest of the *Bacillus* species, *B. badius* showed unique *Alu*I, *Hae*III, and *Hinf*I patterns (Table 4 and Fig. 1A lane B, Fig. 1C, lane B, and Fig. 1D, lane B); *B. circulans* and *B. firmus* showed distinct patterns with *Alu*I, *Hae*III, *Hinf*I, and *Taq*I (Table 4 and Fig. 1A, C, D, and F, lanes D and G, respectively); *B. clausii* showed unique *Alu*I, *Hae*III, *Hinf*I, and *Taq*I patterns (Table 4 and Fig. 1A, C,D, and F, lanes E); *B. coagulans* also showed *Alu*I, *Hae*III, *Hinf*I, *Rsa*I, and *Taq*I patterns (Table 4 and Fig. 1A, C, D, E, and F, lanes F, F, F, E, and F, respectively), and finally, *B. megaterium* strains showed unique *Alu*I and *Cfo*I patterns (Table 4 and Fig. 1A, line I and Fig. 1B, lane F). Similar results were observed by Wu et al. (2006) with isolates of *B. badius, B. clausii,* and *B. coagulans* for *Alu*I and *Taq*I.

When testing *Brevibacillus* species, *Br. borstelensis, Br. brevis,* and *Br. laterosporus* showed unique *Alu*I, *Taq, and Hae*III patterns respectively that allowed us to differentiate them from the rest of aerobic spore formers found in honey (Table 5). Also, the three species can be differentiated among them by using *Cfo*I (Table 5). It was previously

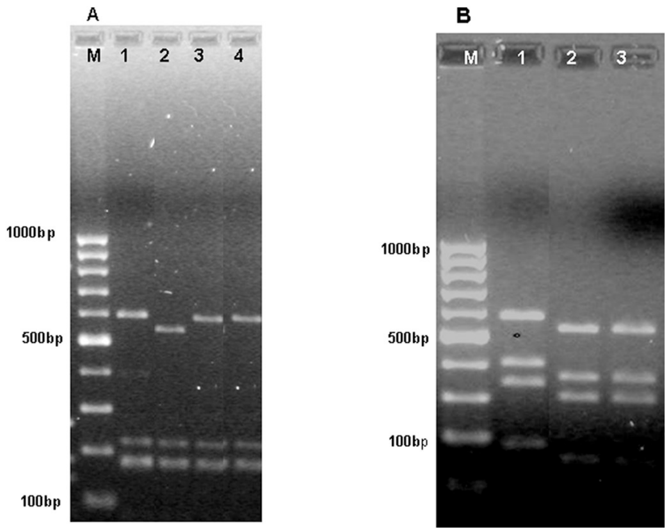

Fig. 2. Distinct differentiation among species of *Bacillus cereus* group reported in honey by PCR-RFLP. (A): Gel electrophoresis of a PCR-amplified 16S rRNA gene fragment of 1492 bp digested with *Alu*I. Lanes: M, Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina) (size is indicated on the left in bp); 1, *B. cereus* m6c; 2, *B. mycoides* m425; 3, *B. thuringiensis* mv50b; *B. thuringiensis* ATCC 10792^{T}. (B) Gel electrophoresis of a PCR-amplified 16S rRNA gene fragment of 1492 bp digested with *Cfo*I. Lanes: M, Molecular size marker 100 bp ladder (InbioHighway®, Tandil, Buenos Aires, Argentina) (size is indicated on the left in bp); 1, *B. cereus* m387; 2, *B. mycoides* m336; *B. thuringiensis* mv50b.

reported that *Br. brevis* and *Br. laterosporus* can be separated by using *Taq*I (Wu et al., 2006). As far as we know, there is no previous information about RFLP patterns for *Br. borstelensis*.

In the case of *Lysinibacillus* reported in honey, we were able to differentiate the species *L. sphaericus* from *L. fusiformis* by using *Cfo*I (Table 5 and Fig. 1B, patterns J, and K, respectively). Concerning *R. stabekisii,* unique restriction patterns with *Cfo*I, *Hinf*I, and *Rsa*I were obtained (Table 5 and Fig. Fig. 1B pattern M, Fig. 1D pattern N, and Fig. 1E pattern O). As far as we know, this paper is the first report for differentiation of *R. stabekisii* by PCR-RFLP.

On the other hand, species of *Paenibacillus* reported in apiarian sources, including honey ($n = 6$), can be separated by using *Taq*I or *Rsa*I. It was previously reported that a combination of seven restriction endonucleases (*Alu*I, *Msp*I, *Hae*III, *Hinf*I, *Cfo*I, *Rsa*I, and *Taq*I) and specific primers U1/U2 could separate 25 different species of *Paenibacillus* between them except in the case of *P. borealis* and *P. macquariensis* (Alippi et al., 2002).

Wu et al. (2006) developed a PCR-ARDRA protocol by using *Bacillus*- specific primers B-K1F/B-K1R for PCR amplification and two restriction enzymes (*Alu*I and *Taq*I) to differentiate 15 reference strains belonging to *Bacillus* ($n = 10$), *Paenibacillus* ($n = 3$) and *Brevibacillus* ($n = 2$) species, but the procedure was restricted by their inability to differentiate closely related species within *B. subtilis* and *B. cereus* groups and some *Paenibacillus* and *Bacillus* species.

In conclusion, we have found that a PCR-RFLP assay using universal primers 27f/1492r and a combination of three restriction enzymes, *Alu*I, *Cfo*I, and *Taq*I, was suitable in distinguishing 26 different species of *Bacillus, Brevibacillus, Lysinibacillus, Rummeliibacillus, and Paenibacillus.* The method is simple and can be used for a prescreening and isolate differentiation for the aerobic spore-forming species, which are commonly found in honey samples. With a similar preliminary survey, this technique could be used on a variety of other food-related samples, taking into account that a potential problem will be the finding of new or undescribed species of bacteria in a sample.

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Declaration of Competing Interest

None.

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