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Traceability of potential enterotoxigenic *Bacillus cereus* in bee-pollen samples from Argentina throughout the production process

Ana C. López[a,b], Leticia A. Fernández[c], Adriana M. Alippi[a,d,*]

[a] Unidad de Bacteriología, Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, cc 31, calle 60 y 119, 1900 La Plata, Argentina

[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-CCT La Plata), Argentina

[c] Laboratorio de Estudios Apícolas (LabEA-CIC), Departamento de Agronomía, and Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina

[d] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICBA), Argentina

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ABSTRACT

Bee-pollen is a functional food sold for human and animal consumption but also is a favorable microhabitat for many spore-forming bacteria. Among them, *Bacillus cereus* can produce several toxins and other virulence factors, causing an emetic or diarrheal syndrome after ingestion. The study involved 36 bee-pollen samples obtained from different sampling points throughout the production process (collecting, freezing, drying, and cleaning) in Argentina. Fifty isolates of *B. cereus* yielded 24 different fingerprint patterns with BOX and ERIC primers. Only three fingerprint patterns were maintained throughout the production process. In contrast, others were lost or incorporated during the different steps, suggesting that cross-contamination occurred as shown by differences in fingerprint patterns after freezing, drying, and cleaning steps compared to the initial collection step. Genes encoding for cereulide (*ces*), cytotoxin K (*cyt*K), sphingomyelinase (*sph*), the components of hemolysin BL (*hbl*A, *hbl*B, *hbl*C, *hbl*D) and non-hemolytic complex (*nhe*AB) were studied. All the isolates displayed one or more enterotoxin genes. The most frequent virulence genes detected belong to the HBL complex, being the most abundant *hbl*A (98%), followed by *hbl*D (64%), *hblB* (54%), and *hbl*C (32%), respectively. Ten strains (20%), present at all sampling points, carried all the subunits of the HBL complex. The non-hemolytic enterotoxic complex (*nheAB*) was found in 48 strains (96%), while seven strains (14%) present at all sampling points showed the amplification product for sphingomyelinase (*sph*). One cereulide-producer was isolated at the cleaning step; this strain contained all the components for the hemolytic enterotoxin complex HBL, the NHE complex, and cytotoxin K related to the foodborne diarrhoeal syndrome. In total, 11 different virulence patterns were observed, and also a correlation between rep-fingerprint and virulence patterns. The results suggest that bee-pollen can be contaminated at any point in the production process with potential enterotoxic *B. cereus* strains, emphasizing the importance of hygienic processing.

1. Introduction

Bee-pollen is the result of the agglutination of pollen grains collected from flowers and mixed with nectar and salivary secretions by honeybees (Bertoncelj et al., 2018; Denisow and Denisow-Pietrzyk, 2016). Honeybees (*Apis mellifera* L.) collect pollen from different flowers during collecting trips and pack pollen grains into pollen pellets on their hind legs with the help of several combs and hairs. The pollen transferred to the hive in the form of pollen loads is called “bee-pollen” (Kieliszek et al., 2018), which is stored inside the hive separately from the nectar cells (Almeida-Muradian et al., 2005). Bee-pollen is sold for human and animal consumption as a food supplement in many countries (Soares de Arruda et al., 2017), being a source of energy and protein, containing carbohydrates, lipids, vitamins, minerals, crude fiber, flavonoids, carotenoids, and enzymes (Margăoăn et al., 2010).

Due to structure and nutritive composition, pollen provides a unique microhabitat for bacterial communities, where dominant phyla are *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (Ambika Manirajan et al., 2016). Primary sources of bacterial contamination of pollen include digestive tracts of honeybees, dust, air, earth, and nectar (Ambika

* Corresponding author at: Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICBA), and Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina.

E-mail address: alippi@biol.unlp.edu.ar (A.M. Alippi).

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Table 1
Primers used for the detection of virulence genes and rep-PCR fingerprinting.

	Primers designation	Sequence (5′-3′)	Product size (bp)	Annealing temperature (°C)	Target gene	Reference
HBL Complex	Bf	ACGAACAATGGAGATACGGC	622	64	*hbl*A	Granum et al., 1996
	Br	TTGGTAGACCCAAAATAGCACC				
	B′f	ATAACTATTAATGGAAATACA	232	62	*hbl*B	Granum et al., 1996
	B′r	CTCCTTGTAAATCTGTAATCCCT				
	L-1f	ATATTCACCTTAATCAAGAGCTGTCACG	810	60	*hbl*D	Ryan et al., 1997
	L-1r	CCAGTAAATCTGTATAATTTGCGCCC				
	L-2f	TATCAATACTCTCGCAACACCAATCG	977	58	*hbl*C	Ryan et al., 1997
	L-2r	GTTTCTCTAAATCATCTAAATATGCTCGC				
Sphingomyelinase	Ph1	CGTGCCGATTTAATTGGGGC	558	58	*sph*	Hsieh et al., 1999
	Ph2	CAATGTTTTAAACATGGATGCG				
NHE	NheF	GTAAATGCTGCVGATAGYCAAAC	480	54	*nheAB*	Guinebretière et al., 2010
	NheR	GGCATVATRTTYCCTGCTGC				
Cereulide	CESF1	GGTGACACATTATCATATAAGGTG	700	53	*ces*	Ehling-Schulz et al., 2005
	CESR2	GTAAGCGAACCTGTCTGTAACAACA				
Cytotoxin K	CK F2	ACAGATATCGGICAAAATGC	421	49	*cyt*K	Ehling-Schulz et al., 2006
	CK R5	CAAGTIACTTGACCIGTTGC				
ERIC	ERIC1R	ATGTAAGCTCCTGGGGATTCAC	ND	53	N/A	Versalovic et al., 1994
	ERIC2R	AAGTAAGTGACTGGGGTGAGCG				
BOX	BOX1AR	CTACGGCAAGGCGACGCTGACG	ND	53	N/A	Versalovic et al., 1994

Manirajan et al., 2016; Gilliam, 1979; Gilliam et al., 1990; Kačániová et al., 2009), while post-harvest sources of bacteria are likely to be the same as those for other food products, and may include humans, equipment, containers, dust, insects, animals, and water (De-Melo et al., 2015, Estevinho et al., 2012).

Also, bee-pollen is a favorable environment for spore-forming bacteria, among this group, *Bacillus cereus sensu stricto* (*B. cereus s.s*) is a ubiquitous bacterium found in soil, plants, and other niches such as enteric tracts of insects, honey and pollen (Ambika Manirajan et al., 2016; Gilliam, 1979; Gilliam et al., 1990; Heydenreich et al., 2012; Kačániová et al., 2009; López and Alippi, 2007; Moreno Andrade et al., 2019; Snowdon and Cliver, 1996). *B. cereus s.s.* belongs to the *Bacillus cereus* group consisting of *Bacillus cereus*, *Bacillus anthracis*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, and *Bacillus toyonensis* (Guinebretière et al., 2013; Liu et al., 2018; Vilas-Boas et al., 2007).

B. cereus is widely recognized as the etiological agent of gastrointestinal diseases (emetic and diarrheic syndromes) as well as non-intestinal pathologies (Bottone, 2010; Ghelardi et al., 2002; Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008). Emesis is caused by cereulide (Ehling-Schulz et al., 2005, 2006, 2015), whereas diarrhea involves diverse extracellular factors (Stenfors Arnesen et al., 2008).

The emetic syndrome is caused by the thermostable peptide cereulide, encoded by non-ribosomal peptide synthetase genes (*ces*) (Ehling-Schulz et al., 2005). Intoxication is usually caused by the ingestion of toxin pre-formed in food by *ces*-positive *B. cereus* strains (Ehling-Schulz et al., 2015).

The most recurrent toxins reported to produce diarrheic syndrome are hemolysin BL (Hbl), the non-hemolytic enterotoxin (Nhe), and cytotoxin K. Cytotoxin K, encoded by *cyt*K, causes a diarrheal syndrome with necrotic, hemolytic, and cytotoxic effects on the intestinal epithelium (Fagerlund et al., 2004). It has been found in *B. cereus* strains that cause severe necrotic enteritis (Lund et al., 2000). Hbl, encoded by the operon *hbl*DAC, is a tripartite protein complex. All the parts are required to maximize their biological effects, *i.e.*, hemolytic, cytotoxic, dermonecrotic, and vascular permeability activities of Hbl (Beecher and Wong, 1994; Carter et al., 2018; Ryan et al., 1997). Nhe is composed of three genes (*nheA*, *nheB*, and *nheC*) that constitute the *nhe*ABC operon, and it is responsible for the diarrheal food-poisoning syndrome when all the three components are present (Lindbäck et al., 2004). Nevertheless, other authors considered that it is not clear which combination and/or polymorphisms of enterotoxin genes are associated with *B. cereus* strains responsible for diarrhea illness (Ceuppens et al., 2011).

Finally, sphingomyelinase (SMase) has been reported as a virulence factor for septicemia (Hsieh et al., 1999; Oda et al., 2012); while other authors suggested that SMase acts in connection with Hbl (Beecher and Wong, 2000) and that Nhe and SMase complement each other significantly to cause *B. cereus* full virulence (Doll et al., 2013).

The study of correlations between toxigenic profiles and other markers, *e.g.*, rep-PCR fingerprinting, antibiotic sensitivity, RAPD patterns, and multilocus sequence typing, allows gaining further insight into the epidemiology of enterotoxic *B. cereus* strains (Ghelardi et al., 2002; Yu et al., 2020). Previous work evaluated which was the effect of the different stages of bee pollen production that lead to changes in the microbiota throughout the production process in the Southwest of Buenos Aires province, Argentina (Fernández et al., 2020). Within this context, the present work aimed to study the traceability of potential enterotoxic *Bacillus cereus* strains based on colony counts, rep-fingerprinting, and toxigenic profiles at four sampling points of the production process of pollen (collecting, freezing, drying, and cleaning).

2. Materials and methods

2.1. Sampling

The bee-pollen production process designed by Cooperativa de Trabajo Apícola Pampero Limitada (Bahía Blanca, Argentina) involved four stages separated in time and space. This process comprises collection by using pollen traps, freezing (−10 °C), dehydration (drying at 40 °C, 48 h), cleaning, and packaging. The study involved 36 bee-pollen samples from the production line of three beekeepers from South West of Buenos Aires Province, Argentina. Samples were analyzed by testing three samples from each beekeeper at each sampling point (collecting, freezing, dehydration, and cleaning). All samples were aseptically collected in sterile 100 ml vials and stored refrigerated (4 °C) until processing within 30 days.

2.2. Isolation and identification of *Bacillus cereus s.l.*

For the isolation of *Bacillus cereus s.l.* (*B. cereus* group) a technique adapted and modified from López and Alippi (2007) was used. Briefly, 5 g of each bee-pollen sample was mixed with 5 ml of 0.01 M sodium phosphate buffer saline (pH 7.2) and submitted to agitation for 40 min. Each sample was filtrated to a new centrifuge tube and centrifuged at 6000 ×*g* for 30 min at 4 °C. Most of the supernatant was discarded to leave 3 ml of fluid that was vortex-mixed with the sediment and heated in a water bath at 80 °C for 10 min to kill bacterial vegetative cells and yeasts, and at the same time, activate *Bacillus cereus* spore germination.

Table 2
Source of *Bacillus cereus* strains isolated from bee-pollen at each sampling point and results of rep-PCR fingerprint patterns and characterization of virulence genes.

Strain	Beekeeper	Sampling point	Fingerprint pattern		Toxin pattern	HBL complex[a]				*cytK*[a]	*ces*[a]	*sph*[a]	*nheAB*[a]
			BOX	ERIC		*hbl*A	*hbl*B	*hblC*	*hblD*				
BCP17	FL	Freezing	E	E	I	+	+	+	+	−	−	−	+
BCP18	EA	Freezing	I	I	I	+	+	+	+	−	−	−	+
BCP20	EA	Drying	I	I	I	+	+	+	+	−	−	−	+
BCP21	EA	Drying	I	I	I	+	+	+	+	−	−	−	+
BCP22	EA	Collecting	A	A	II	+	+	−	+	−	−	−	+
BCP23	EA	Collecting	B	B	III	+	−	−	+	−	−	−	+
BCP24	DI	Drying	L	L	I	+	+	+	+	−	−	−	+
BCP25	FL	Drying	N	N	IV	−	−	−	+	−	−	−	−
BCP26	EA	Collecting	C	C	V	+	−	−	+	−	−	+	+
BCP27	EA	Drying	O	O	VI	+	+	−	+	−	−	+	−
BCP28	EA	Drying	A	A	II	+	+	−	+	−	−	−	+
BCP29	FL	Freezing	J	J	II	+	+	−	+	−	−	−	+
BCP30	FL	Freezing	D	D	II	+	+	−	+	−	−	−	+
BCP31	FL	Collecting	D	D	II	+	+	−	+	−	−	−	+
BCP32	EA	Drying	P	P	VII	+	+	−	+	−	−	+	+
BCP33	DI	Cleaning	G	G	VIII	+	+	−	−	−	−	−	+
BCP34	EA	Freezing	A	A	II	+	+	−	+	−	−	−	+
BCP35	DI	Collecting	G	G	VIII	+	+	−	−	−	−	−	+
BCP36	DI	Drying	M	M	IX	+	−	−	−	−	−	−	+
BCP37	DI	Drying	M	M	IX	+	−	−	−	−	−	−	+
BCP38	DI	Collecting	F	F	IX	+	−	−	−	−	−	−	+
BCP39	EA	Freezing	A	A	II	+	+	−	+	−	−	−	+
BCP40	FL	Cleaning	X	X	X	+	+	+	+	+	+	−	+
BCP41	FL	Collecting	E	E	I	+	+	+	+	−	−	−	+
BCP42	FL	Drying	Q	Q	I	+	+	+	+	−	−	−	+
BCP43	LF	Drying	R	R	III	+	−	−	+	−	−	−	+
BCP44	EA	Cleaning	A	A	II	+	+	−	+	−	−	−	+
BCP45	EA	Cleaning	A	A	II	+	+	−	+	−	−	−	+
BCP46	DI	Cleaning	V	V	IX	+	−	−	−	−	−	−	+
BCP47	DI	Cleaning	W	W	IX	+	−	−	−	−	−	−	+
BCP48	FL	Cleaning	K	K	XI	+	−	+	−	−	−	−	+
BCP49	EA	Cleaning	C	C	V	+	−	−	+	−	−	+	+
BCP50	EA	Cleaning	C	C	V	+	−	−	+	−	−	+	+
BCP51	EA	Drying	I	I	I	+	+	+	+	−	−	−	+
BCP52	EA	Drying	I	I	I	+	+	+	+	−	−	−	+
BCP53	EA	Freezing	C	C	V	+	−	−	+	−	−	+	+
BCP54	DI	Collecting	H	H	II	+	+	−	+	−	−	−	+
BCP55	DI	Freezing	G	G	VIII	+	+	−	−	−	−	−	+
BCP56	FL	Freezing	K	K	XI	+	−	+	−	−	−	−	+
BCP57	FL	Freezing	K	K	XI	+	−	+	−	−	−	−	+
BCP58	FL	Freezing	K	K	XI	+	−	+	−	−	−	−	+
BCP59	FL	Freezing	K	K	XI	+	−	+	−	−	−	−	+
BCP60	EA	Cleaning	A	A	II	+	+	−	+	−	−	−	+
BCP61	FL	Cleaning	S	S	IX	+	−	−	−	−	−	−	+
BCP62	FL	Cleaning	T	T	IX	+	−	−	−	−	−	−	+
BCP63	FL	Cleaning	U	U	IX	+	−	−	−	−	−	−	+
BCP64	FL	Drying	R	R	III	+	−	−	+	−	−	−	+
BCP82	EA	Drying	C	C	V	+	−	−	+	−	−	+	+
BCP83	DI	Drying	G	G	VIII	+	+	−	−	−	−	−	+
BCP84	FL	Drying	K	K	XI	+	−	+	−	−	−	−	+

[a] Virulence genes tested: Hemolysin BL (HBL); Cytotoxin K (*cytK*); Cereulide (*ces*); sphingomyelinase (*sph*), Non-hemolytic complex (nheAB).

Samples were vortex-mixed again for 2 min, and 100 µl of the sediment-fluid mixture was poured over the surface of polymyxin-pyruvate-egg-yolk-mannitol-agar (PEMBA) plates (Britania®, Argentina) and spread by using a sterile cotton swab. Plates were incubated at 32 °C and examined daily and up to 5 days for bacterial growth.

Distinct colonies of *B. cereus* group, *i.e.*, turquoise blue crenated colonies (mannitol negative) surrounded by a distinct opaque zone of egg yolk precipitation (lecithinase positive) were counted. Counts were expressed as colony-forming units per g of pollen (CFU.g^{-1}). Colonies growing on PEMBA were identified by their shape, rhizoidal growth, and hemolytic activity as belonging to the *B. cereus* group. Bacterial smears were examined for the presence and location of spores within cells, as well as for the size and shape of vegetative cells (Parry et al., 1983; Priest et al., 1988). Also, the presence of both unstained globules in the cytoplasm and parasporal crystals were examined by using a phase-contrast microscope (1000×, oil immersion) (EFSA BIOHAZ, 2016; López and Alippi, 2007). The number of colonies submitted to phenotypic and genotypic tests corresponds to the square root of the total number of colony-forming units (CFU) per plate. These isolates were tested for catalase, production of lecithinase, Voges-Proskauer reaction, mannitol utilization, anaerobic utilization of glucose, hemolytic activity, and starch and gelatin hydrolysis according to standard protocols (Gordon et al., 1973; Lancette and Harmon, 1980; Pirttijärvi et al., 1996).

2.3. Statistical analysis

Data from colony counts of *B. cereus* between the three beekeepers at each sampling point throughout the production process were analyzed by one-way analysis of variance using Infostat software (Di Rienzo et al., 2013).

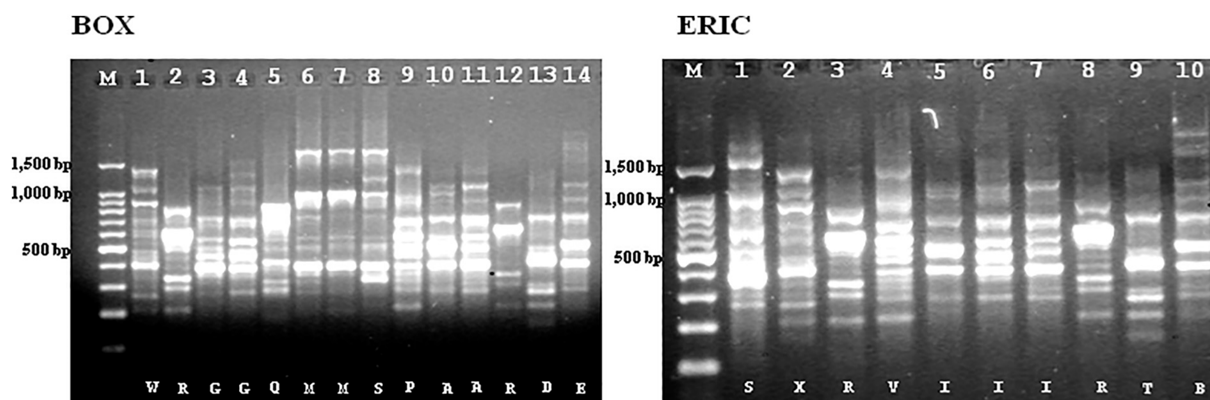

Fig. 1. Fingerprints generated from template DNA of *Bacillus cereus s.s.* isolates using BOX and ERIC primers. BOX: M, Molecular size marker 100-bp DNA Ladder Promega®, Argentina, lanes 1 to 14 *Bacillus cereus* isolates (BCP47, BCP43; BCP33, BCP55, BCP42, BCP36, BCP37, BCP61, BCP32, BCP22, BCP34, BCP64, BCP30, and BCP17). ERIC: M, Molecular size marker 100-bp DNA Ladder Promega®, Argentina, lanes 1 to 10 *Bacillus cereus* isolates (BCP61, BCP40, BCP43, BCP46, BCP18, BCP20, BCP21, BCP64, BCP62, and BCP23).

2.4. DNA preparation

Suspected *Bacillus cereus* isolates were cultured on PEMBA plates for 24 h at 32 °C under aerobic conditions. For the preparation of bacterial DNA template, a rapid procedure was used (Alippi and Aguilar, 1998). Briefly, bacterial colonies were picked up by using a 1-μl plastic disposable loop and suspended in 200 μl double-distilled sterile water. The sample was vortex mixed and centrifuged at 10,000 ×*g* for 4 min, the supernatant was removed, and the pellet was resuspended in 150 μl of an aqueous suspension of 6% resin Chelex 100 (Bio-Rad). The mixture of cells and resin was incubated at 56 °C for 20 min, vortex mixed, incubated at 99 °C for 15 min, and vortex mixed for 1 min. Finally, bacterial debris and resin were removed by centrifugation.

2.5. RFLP analysis of PCR-amplified 16S rDNA

Fifty isolates of the *B. cereus* group from bee-pollen were identified at the species level by RFLP analysis of PCR-amplified 16S rRNA as previously described (López and Alippi, 2019). Briefly, universal primers 27f and 1492r were employed (Yu et al., 2013). PCRs were carried out in a final volume of 25 μl (Yu et al., 2013). After amplification of the approximately 1492 bp PCR product, subsamples of 2 μl were incubated with endonucleases *Alu*I and *Cfo*I, according to the manufacturer's specifications (Promega®). RFLP analysis was performed by electrophoresis in a 1.6% agarose gel at 70 V for 2 h.

2.6. Analysis of the diversity of isolates by rep-PCR

The rep-PCR method with BOX (BOXA1R) and ERIC (ERIC1R and ERIC2) primers was used (Versalovic et al., 1994). PCR amplifications were done according to López and Alippi (2007). For the analysis of amplification products, 5 μl of each PCR reaction was run on a 1.6% (W/V) agarose gel in TBE buffer and visualized by using ethidium bromide and UV light. A digital image of each gel was analyzed using GelcomparII® software (v. 5.1, Applied Maths). Cluster analysis was performed using the DICE similarity coefficient and the UPGMA clustering algorithm with a band tolerance of 5% for a combined gel.

2.7. Detection of virulence genes by PCR

All strains were evaluated for the presence of sequences associated with virulence genes by PCR. Genes encoding for cereulide (*ces*) (Ehling-Schulz et al., 2005), cytotoxin K (*cyt*K) (Ehling-Schulz et al., 2006), sphingomyelinase (*sph*) (Hsieh et al., 1999); the components of hemolysin BL (*hbl*A, *hbl*B, *hbl*C, *hbl*D) (Granum et al., 1996; Ryan et al., 1997) and the non-hemolytic complex (*nhe*AB) (Guinebretière et al., 2010) were studied. Primers used and conditions of amplification are listed in Table 1. DNA amplifications were performed in a thermal cycler (Mastercycler personal; Eppendorf Hamburg, Germany). The amplification products were separated in 1.6% (W/V) agarose gel in 0.5× TBE buffer, stained with ethidium bromide, and visualized with a UV transillluminator (UVP, Upland, California, USA). Gel images were digitalized by using a digital image capture gel documentation system (DigiDoc-It, UVP, v.1.1.25, Upland, California, US).

3. Results and discussion

3.1. Prevalence and levels of Bacillus cereus s.l.

All pollen samples analyzed ($n = 36$) contained spores of *B. cereus s.l.* A total of about 3×10^2 CFU of *B. cereus* equivalent to 2×10^3 CFU/g were counted on PEMBA plates. Colony counts revealed no statistically significant differences among means at the different sampling points ($p = 0.5$). However, *B. cereus* incidence (total of CFU/g at each sampling point) increased from collection (7×10^1 CFU/g) to freezing (5×10^2 CFU/g) and drying (9×10^2 CFU/g) and slightly decreased at the final step of cleaning (3×10^2 CFU/g). In spite that *B. cereus* was present at the different sampling points, the spore countings complied with the food safety criteria for *B. cereus* in food ($< 10^5$ CFU/g) (EFSA BIOHAZ, 2016; Lücking et al., 2013).

The differences obtained in colony counts per g of pollen suggest that bee-pollen can be contaminated at any point in the production process, as shown in bacterial counts after freezing, drying, and cleaning steps compared to the initial collection step, where higher counts occurred at freezing and drying.

As reported by De Melo and co-workers (De-Melo et al., 2015), the presence of any microorganism in dehydrated bee-pollen is related to inadequate hygienic practices during manipulation at the steps of collection and processing, and contamination of floral pollen grains on the plant or by bees. In the production process, the time that bee-pollen remains in the collection traps is critical because pollen grains are in contact with air, dust, and other dirt. The presence of viable microorganisms in samples of dehydrated bee-pollen after the collection step could be related to two hypotheses: that the low temperature used in the dehydration process is insufficient to remove microorganisms or that contamination occurs during the processing steps after dehydration (De-Melo et al., 2015). Other authors (Estevinho et al., 2012) affirmed that freezing and drying steps allow the multiplication of microorganisms.

Fifty strains were selected from the isolation plates, corresponding to the square root of the total number of colony-forming units (CFU) per plate (Section 2.2), for further analysis (collecting = 8; freezing = 12;

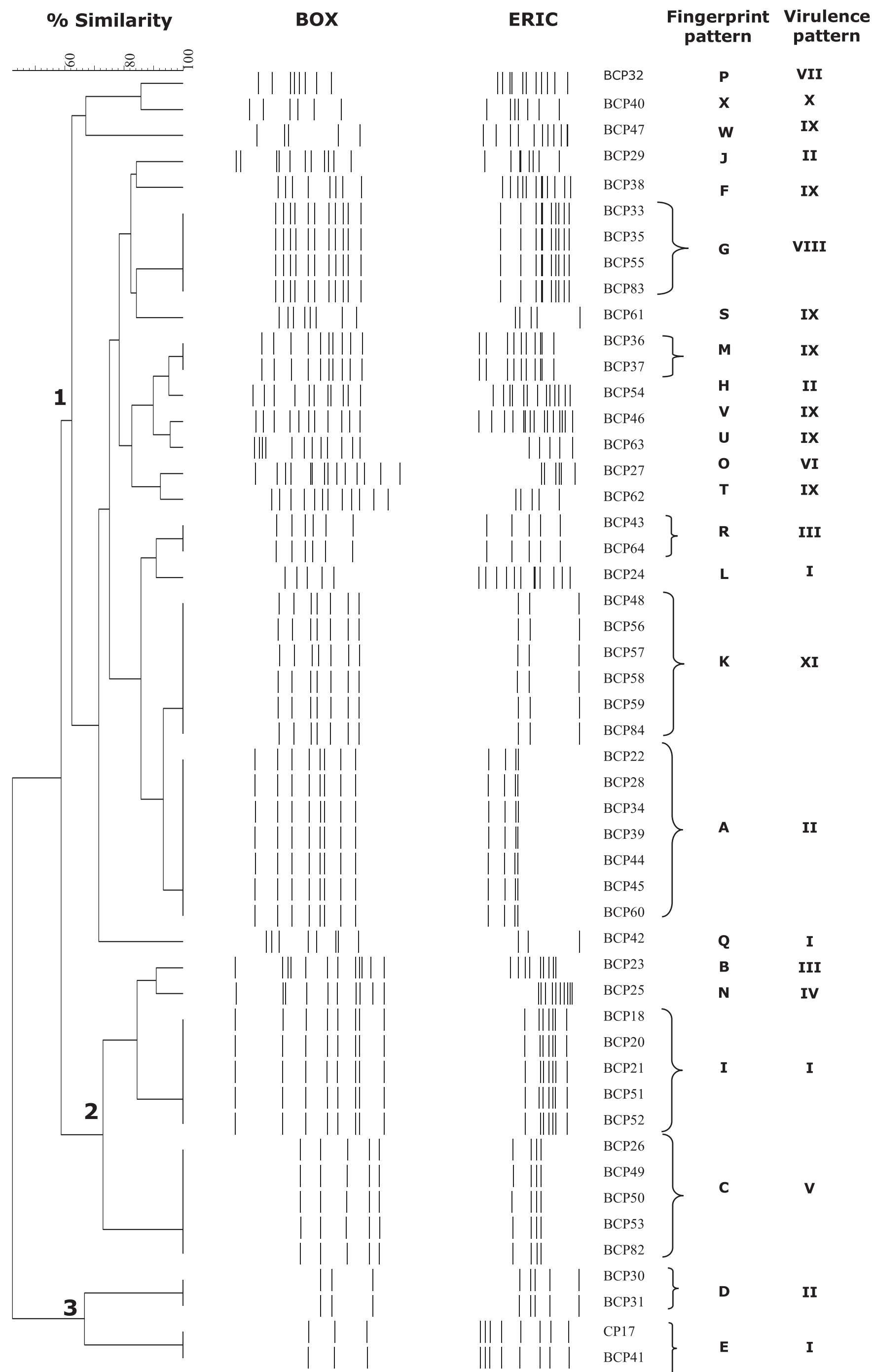

Fig. 2. Dendrogram from computer-assisted analysis of rep-PCR profiles of 50 *B. cereus* s.s. isolates obtained from the different sampling points. Cluster analysis was performed using the DICE similarity coefficient and the UPGMA clustering algorithm with a band tolerance of 5%.

drying = 17 and cleaning = 13) (Table 2).

3.2. Identification of selected isolates

Typical *B. cereus* isolates produced crenated colonies retaining the turquoise blue of the pH indicator because of their inability to ferment mannitol acidifying the medium, and generated an egg-yolk precipitation halo as a result of lecithinase activity. The 50 selected strains were Gram-positive, catalase positive, and showed ellipsoidal spores in a central position, not distending the sporangia. No parasporal crystals were detected, and the cytoplasm was filled with unstained globules. All the isolates were facultatively anaerobic, grew in 7% NaCl, hydrolyzed gelatin and starch, and were positive for Voges Proskauer, reduction of nitrates to nitrites, and hemolytic activity. These characteristics correspond with the phenotypic features typical for *Bacillus cereus* (Gordon et al., 1973; Parry et al., 1983; Priest et al., 1988). Besides, all the strains showed the expected restriction patterns for *Bacillus cereus s.s.* when using a combination of *AluI* and *Cfo*I enzymes (López and Alippi, 2019).

3.3. Analysis of the diversity and traceability of isolates by rep-PCR

The fingerprints generated by BOX- and ERIC-PCR were analyzed by using GelcomparII software, being the number of polymorphic bands

Table 3
Traceability of rep-fingerprint patterns in *Bacillus cereus* strains isolated from bee-pollen throughout the different sampling points tested for all the bee-keepers.

Collecting	Freezing	Drying	Cleaning	
A	A	A	A	
B	–	–	–	
C	C	C	C	
D	D	–	–	
E	E	–	–	
F	–	–	–	
G	G	G	G	
H	–	–	–	
	I	I	–	
	J	–	–	
	K	K	K	
		L	–	
		M	–	
		N	–	
		O	–	
		P	–	
		Q	–	
		R	–	
			S	
			T	
			U	
			V	
			W	
			X	
8	8	12	10	Totals

Table 4
Prevalence of virulence genes in *Bacillus cereus* isolated from bee-pollen throughout the different sampling points tested.

Virulence genes	Number of strains (%) positive for the target gene
Hemolysin BL genes	
hblA	49 (98%)
hblB	27 (54%)
hblD	32 (64%)
hblC	16 (32%)
All *hbl* genes	10 (20%)
Non-hemolytic enterotoxin genes	
nheAB	48 (96%)
Cytotoxin K gene	
cytK	1 (2%)
Cereulide synthetase gene	
Ces	1 (2%)
Sphingomyelinase	
sph	7 (14%)

between 5 and 14, ranging between 200 bp and more than 1500 bp for BOX (Fig. 1), and between 3 and 14 bands, ranging between 150 bp and more than 1500 bp for ERIC (Fig. 1). Among the 50 isolates of *B. cereus s. s*, 24 different rep-fingerprint patterns by using BOX and ERIC were identified (Table 2).

The BOX and ERIC data were combined and used to generate a dendrogram (Fig. 2). Fifteen strains yielded unique fingerprint patterns, while the rest ($n = 35$), were grouped into nine different clusters (Fig. 2). All the isolates clustered together at 46% similarity level, while clusters 1, 2, and 3 were separated at about 60%, 70%, and 65%, respectively. The results of the cluster analysis of fingerprints generated by rep-PCR with BOX and ERIC primers revealed a high genetic diversity among *B. cereus* strains in coincidence with results reported by other authors working with isolates from other foods (Chaves et al., 2011; Lee et al., 2012; López and Alippi, 2007).

The rep-fingerprint patterns obtained at the different sampling points throughout the production process were compared (Table 3). At collecting, eight patterns (A, B, C, D, E, F, G, and H) were observed, while at freezing, patterns A, C, D, E, and G were maintained; three new patterns (I, J, and K) were incorporated and three patterns (B, F, and H) were lost. Besides, at drying, 12 patterns were observed, where five patterns (A, C, G, I, and K) were maintained; seven new patterns were incorporated (L, M, N, O, P, Q, and R) and three were lost (D, E, and J). Finally, during the cleaning step, ten patterns were visualized, where four patterns were maintained (A, C, G, and K), six new were incorporated (S, T, U, V, W, and X), and the eight patterns which were incorporated at the drying step were lost.

Only three fingerprint patterns (named A, C, and G) were maintained throughout the production process, while others were lost or incorporated during freezing, drying, and/or cleaning (Table 3). These results suggested that cross-contamination occurred as shown by differences in fingerprint patterns after freezing, drying, and cleaning steps compared to the initial collection step.

3.4. Detection of virulence genes by PCR

An overview of all virulence genes detected by PCR is provided in Tables 2 and 4. All the isolates displayed one or more enterotoxin genes. The most frequent virulence genes detected belong to the Hbl complex, being the most abundant *hbl*A (98%), followed by *hbl*D (64%), *hbl*B (54%), and *hbl*C (32%), respectively. Besides, ten strains (20%), present at all sampling points, carried all the subunits of the Hbl complex. The non-hemolytic enterotoxic complex (*nhe*) was found in 48 strains (96%) that were present at all sampling points, while six strains (12%), isolated at the freezing, drying, and cleaning steps showed the amplification product for sphingomyelinase (*sph*) (Table 4). One cereulide-producer (BCP40) was isolated at the cleaning step; this strain also contained all the components for the hemolytic enterotoxin complex Hbl, and cytotoxin K related to the foodborne diarrhoeal syndrome. Interestingly, strain BCP40 showed a unique fingerprint pattern named X (Figs. 1 and 2).

Most strains of *B. cereus* isolated from various food sources contained both *hbl* and *nhe*-encoding genes (Chaves et al., 2011; Lee et al., 2012; López and Alippi, 2010; Yu et al., 2020); similar results were obtained from strains isolated from bee-pollen in this study. The incidence of *B. cereus* strains from pollen carrying cytotoxin K (*cyt*K) and cereulide (*ces*) genes was lower than reported by other authors for other types of foods (Guinebretière et al., 2002; Lee et al., 2012; Yu et al., 2020).

In total, 11 different virulence gene distribution patterns, named I (*hbl*A-*hbl*B-*hbl*C-*hbl*D-*nheAB*); II (*hbl*A-*hbl*B-*hbl*D- *nheAB*); III (*hbl*A-*hbl*D-*nheAB*); IV (*hbl*D); V (*hbl*A-*hbl*D-*sph*-*nheAB*); VI (*hbl*A-*hbl*B-*hbl*D-*sph*);VII (*hbl*A-*hbl*B- *hbl*D-*nheAB*); VIII (*hbl*A-*hbl*B- *nheAB*); IX (*hbl*A-*nheAB*); X (*hbl*A-*hbl*B-*hbl*C-*hbl*D-*cyt*K-*ces*-*nheAB*); and XI (*hbl*A-*hbl*C-*nheAB*) according to the different combinations of genes were observed (Table 2). The most abundant patterns were II, I, and IX, which were present in 22%, 18%, and 16% of strains, respectively (Table 4). Only two virulence patterns (II and VIII) were maintained throughout the production process, while the others were lost or incorporated during freezing, drying, and/or cleaning. It is interesting to point out that the drying step was the one that showed the highest number of virulence patterns (10 out of a total of 11), and the same situation was observed for fingerprint patterns (12 out of a total of 24) (Table 3).

A certain degree of correlation between rep-fingerprinting and virulence gene patterns were found. For instance, all the isolates showing fingerprint pattern C correlated only with virulence pattern V, and the same situation occurs with fingerprint pattern G with virulence pattern VIII, K with virulence pattern XI, fingerprint pattern N with virulence pattern IV, fingerprint pattern O with virulence pattern VI, fingerprint pattern P with virulence pattern VII, and fingerprint pattern X with virulence pattern X, respectively. However, virulence patterns I, II, III, and IX correlated with more than one fingerprint pattern, being the most promiscuous, virulence pattern IV that were present in fingerprint patterns F, M, S, T, U, V, and W (Table 2).

The ubiquity, resistance, and persistence of *B. cereus* spores favor their survival from the environment to food processing facilities. Moreover, due to their strong adhering properties and ability to form biofilms, contamination of food products, including pollen, is almost impossible to avoid (Carlin, 2011).

The results obtained here emphasize the importance of hygienic processing to avoid spore contamination at all steps of the production process. This work represents the first analysis of potential enterotoxic *B. cereus s.s.* strains contamination at all steps during the production process of bee-pollen. It reinforces the idea that appropriate management and practices would improve the microbiological quality of bee-pollen for human consumption.

Declaration of competing interest

The authors declare that no conflict of interest exists.

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