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ORIGINAL ARTICLE

Characterization of a *Bacillus cereus* strain associated with a large feed-related outbreak of severe infection in pigs

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Abstract

Aims: *Bacillus cereus* is often responsible for foodborne diseases and both local and systemic infections in humans. Cases of infection in other mammals are rather rare. In this study, we report a *B. cereus* feed-related outbreak that caused the death of 6234 pigs in Italy.

Methods and Results: Massive doses of a Gram-positive, spore-forming bacterium were recovered from the animal feed, faeces of survived pigs and intestinal content of dead ones. The *B. cereus* MM1 strain was identified by MALDI-TOF MS and typified by RAPD-PCR. The isolate was tested for the production of PC-PLC, proteases, hemolysins and biofilm, for motility, as well as for the presence of genes encoding tissue-degrading enzymes and toxins. Antimicrobial resistance and pathogenicity in *Galleria mellonella* larvae were also investigated. Our results show that the isolated *B. cereus* strain is swimming-proficient, produces PC-PLC, proteases, hemolysins, biofilm and carries many virulence genes. The strain shows high pathogenicity in *G. mellonella* larvae.

Conclusions: The isolated *B. cereus* strain demonstrates an aggressive profile of pathogenicity and virulence, being able to produce a wide range of determinants potentially hazardous to pigs' health.

Significance and Impact of Study: This study highlights the proficiency of *B. cereus* to behave as a devastating pathogen in swine if ingested at high doses and underlines that more stringent quality controls are needed for livestock feeds and supplements.

KEYWORDS

Bacillus cereus, outbreak, pathogenicity, strain characterization, swine

INTRODUCTION

The *Bacillus cereus sensu lato* group comprises several closely related species of Gram-positive, rod-shaped, ubiquitously distributed and spore-forming bacteria (Carroll et al., 2020). Amongst them, only *Bacillus anthracis* and *B. cereus sensu stricto* (usually referred to as *B. cereus*) are considered pathogens for mammals.

In humans, *B. cereus* is primarily responsible for two types of food poisoning diseases: The emetic and the

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diarrheal syndromes. The pathogenicity of *B. cereus* is mainly attributed to the secretion of tissue-destructive toxins (i.e. non-hemolytic enterotoxin, NHE; hemolysin BL, HBL; enterotoxin FM; enterotoxin S; enterotoxin T; cytotoxin K) and enzymes (i.e. phosphatidylinositol- and phosphatidylcholine-specific phospholipase C, PI-PLC and PC-PLC; sphingomyelinase, SMase) (Ehling-Schulz et al., 2019; Ramarao & Sanchis, 2013). Cereulide, which is a small peptide responsible for the emetic syndrome, is another important determinant for *B. cereus* pathogenicity (Ehling-Schulz et al., 2019). In addition, flagellum-dependent motility modes (i.e. swimming and swarming) and biofilm production contribute to *B. cereus* pathogenic potential since they are involved in the colonization process of host surfaces (Senesi & Ghelardi, 2010).

Besides being responsible for gastrointestinal infections, *B. cereus* is able to cause severe local and systemic infections in humans (Ehling-Schulz et al., 2019; Jessberger et al., 2020). Furthermore, it has been indicated as causing agent of mammary gland infections in cows and goats (Savini, 2016). Intermediate *B. cereus* strains carrying *B. anthracis* determinants and able to reproduce anthrax-like illness, named *B. cereus* biovar *anthracis* (Brézillon et al., 2015; Klee et al., 2010), were documented in the livestock and wildlife in West and Central Africa (Klee et al., 2006; Leendertz et al., 2006). Nevertheless, only a few reports highlighting the virulence of *B. cereus* in animals are available in the literature.

Herein, we report the first severe feed-related outbreak in pigs due to *B. cereus*. In January and February 2015, a breeding farm located in northern Italy reported an abnormal death of animals, which affected 6234 weaned pigs weighing between 30 and 60 kg. Symptoms, including abdominal pain, tremors, postural instability and breathing difficulties, emerged abruptly and quickly led animals to death. Autopsies showed a critical scenario: severe intestinal congestion with the thickened and oedematous wall, disruption of the intestinal mucosal barrier with scattered bleeding, catarrhal enteritis, heavy neutrophil and lymphocyte infiltration in the intestinal mucosa, liver and kidney, systemic lymphadenopathy, muscle fibre degeneration, interstitial pneumonia with pulmonary oedema, pericardial effusion, meningeal and splenic hyperemia, glomerulonephritis and kidney and liver failure. Investigations carried out by competent authorities resulted in the isolation of *B. cereus* from the faeces of survived animals and the intestinal contents of dead pigs. No other bacteria, viruses, fungi or parasites were detected as presumptive pathogens. Symptoms, histological and anatomopathological lesions and epidemiological distribution suggested that a feed-borne disease occurred. Symptoms and mortality ceased as feed was removed. Perseverance in administering the suspect feed to animals resulted in the same symptomatology and mortality rate.

Considering this overall information, in this study, we analysed the suspect animal feed to isolate the microorganism presumptively responsible for the feed-related outbreak. The isolated microorganism was identified, typified and characterized for its virulence, pathogenicity and microbial susceptibility.

MATERIALS AND METHODS

Bacterial isolation and quantification

In this study, a granular dry feed enriched with 2%–4% of a powder protein supplement declared to contain protein extract from *Corynebacterium glutamicum* and the same feed in the absence of the supplement were analysed. Feed samples (50 g each) were picked up from different points of the feed storage area on three separate days. For each sample, 2 g of feed were dissolved in 20 ml of PBS (1 mol L^{-1} KH_2PO_4, 1 mol L^{-1} K_2HPO_4, 5 mol L^{-1} NaCl, pH 7.2) and divided into two aliquots. To reveal the presence of bacterial spores, an aliquot was thermally treated at 80°C for 15 min before plating. Thermally treated and untreated aliquots were serially diluted in PBS and 100 µl seeded on trypticase soy agar containing 5% horse blood (TSH). Plates were incubated at 37°C for 24 h in aerobic as well as anaerobic conditions and the number of CFU ml^{-1} was then determined.

Molecular identification by MALDI-TOF MS

Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) identification was performed as previously reported (Tirloni et al., 2020). Briefly, well-isolated colonies were directly spotted on the MALDI plate, overlaid with 1 µl of 70% ethanol, 1 µl of formic acid, and 1 µl of acetonitrile, and air-dried. 1 µl of saturated α-cyano-4-hydroxycinnamic acid matrix was subsequently added to each spot and air-dried. Mass spectra were automatically acquired at a laser frequency of 60 Hz with an acquisition range from 1.960 to 20.000 Da and imported into the MALDI Biotyper software (version 3.1, Bruker). A score ≥ 2.00 indicated identification at the species level, a score ranging from 1.99 to 1.70 indicated identification at the *genus* level, whereas any score <1.70 meant no significant similarity of the obtained spectrum with any database entry.

Microscopy for parasporal crystals

Well-isolated colonies previously grown on TSH agar were then streaked on sporulation medium agar (Senesi

et al., 1991) and incubated for 16 h at 30°C. Spores were then microscopically examined for the presence of parasporal crystals in methanol-fixed preparations stained with 0.5% basic fuchsin (Ghelardi et al., 2007). *B. thuringiensis* Cry 407 was used as a positive control.

Extraction of the genomic DNA

Bacterial isolates from contaminated feed underwent a genomic DNA extraction process through the phenol-chloroform method (Celandroni et al., 2000). DNA concentration was calculated by measuring the optical density at 260 nm (OD_{260}) and DNA purity was estimated by determining OD_{260}/OD_{280} and OD_{260}/OD_{230} ratio with the spectrophotometer BioPhotometer D30 (Eppendorf).

Random amplification of polymorphic DNA-PCR (RAPD-PCR)

Genomic DNAs extracted from *B. cereus* isolates were subjected to RAPD-PCR for genetic typing to confirm whether the same strain of *B. cereus* was detected in the animal feed sampled on different days. RAPD-PCR fingerprinting was performed with random primers PRO-UP, RPO2, HLWL85 and HLWL74 (Ghelardi et al., 2002; Senesi et al., 2001) (Table 1). PCR products were visualized after a 1% agarose gel electrophoresis as strain-specific band profiles.

PCR for genes encoding *B. anthracis*-specific virulence factors

The presence of the genes encoding the three components of anthrax toxin (protective antigen, *pag*; oedema factor, *cya*; lethal factor, *lef*) and enzymes involved in capsule biosynthesis (capsule biosynthesis protein A, *capA2*; capsule biosynthesis protein B, *capB*) was assessed by multiplex PCR amplifications of genomic DNA. PCR protocol and primer pairs were selected accordingly to Riojas and colleagues (Riojas et al., 2015) (Table 1). PCR products were visualized after a 1% agarose gel electrophoresis. Due to the impossibility of using a genomic DNA from *B. anthracis* as a positive control, successfully performed amplification was confirmed by targeting *sph* gene in the same extracted genomic DNAs.

PCR for gene encoding *B. cereus* cereulide

The potential to produce cereulide was tested by detecting the presence of the cereulide synthetase gene (*ces*) in the extracted genomic DNA. Primers and PCR protocol used were reported in previous work by Kim and colleagues (2010) (Table 1). PCR products were visualized after a 1% agarose gel electrophoresis. Genomic DNA extracted from the cereulide-producing strain *B. cereus* DSM 4312 was used as a positive control.

PCR for other toxin-encoding genes

For the detection of *B. cereus* toxin-encoding genes (SMase, *sph*; enterotoxin T, *bcet*; enterotoxin FM, *entFM*; enterotoxin S, *entS*; PI-PLC, *piplc*; cytotoxin K, *cytK*; NHE, *nheA*, *nheB* and *nheC*; L_2 component of HBL, *hblC*), PCR reactions were performed on bacterial genomic DNAs as previously reported (Ghelardi et al., 2002) (Table 1). PCR products were visualized after a 1% agarose gel electrophoresis. Genomic DNA extracted from *B. cereus* ATCC 14579 was used as a positive control for each amplification.

Detection of secreted PC-PLC, proteases and hemolysins

Microbial cells were grown to the late exponential growth phase in Brain Heart Infusion supplemented with glucose 1% (BHIG) for 6 h at 37°C. Culture supernatants were collected by centrifugation at 10,000*g* and concentrated using Microcon®-10 centrifugal filter units (Merck KGaA). The production of PC-PLC in culture supernatants was evaluated by agar-diffusion assays on TBS agar plates containing 0.15% l-α-phosphatidylcholine (Sigma-Aldrich), as previously described (Ghelardi et al., 2002). PC-PLC activity was extrapolated by comparison with a standard curve obtained from serial dilutions of pure PC-PLC (Sigma-Aldrich) in the same assay. Protease and hemolysin production was checked by directly seeding bacterial cells on 1.5% skim milk (Oxoid) and on TSH, followed by incubation at 37°C for 18 h (Celandroni et al., 2016). The presence of a degradation halo and a clear halo of hemolysis around colonies was indicative of the presence of proteolytic and hemolytic activities, respectively. *B. thuringiensis* Cry 407 and *B. thuringiensis* RM were used as positive controls.

Swimming and swarming motility

Both swimming and swarming assays were performed as previously described (Celandroni et al., 2016) to determine the rate of motility of the isolated bacterial strain. Swimming motility was evaluated by spotting 0.5 μl of

TABLE 1 Primer pairs used in this study

Gene	Primer name	Sequence (5′-3′)	References
—	PRO-UP	gctgctggcggtggt	Ghelardi et al. (2002)
—	RPO2	gcgatcccca	Ghelardi et al. (2002)
—	HLWL85	acaactgctc	Ghelardi et al., 2002
—	HLWL74	acgtatctgc	Ghelardi et al. (2002)
pag	*pag* F *pag* R	ggcatttaatcttgctgtatcagcg tggcagcttatccgattgtacatgt	Riojas et al., 2015
cya	*cya* F *cya* R	tgcccccgacatgtttgagt attcaatccctttgtagccacacc	Riojas et al. (2015)
lef	*lef* F *lef* R	ctatcaacactggagcgattctttatctg ggtacttccaatggattgatgtaataaagc	Riojas et al. (2015)
capA2	*capA2* F *capA2* R	caggagctattgcaacgaaagaacaaccag acatcaaaagattgaagtacatgcggatgg	Riojas et al. (2015)
capB	*capB* F *capB* R	tccggatccaggagcaatga tccctagcaaactgctcagtacgat	Riojas et al. (2015)
ces	CER F CER R	atcataaaggtgcgaacaaga aagatcaaccgaatgcaactg	Kim et al. (2010)
sph	Ph1 Ph2	cgtgccgatttaattggggc caatgttttaaacatggatgcg	Ghelardi et al., 2002
bceT	ETF ETR	ttacattaccaggacgtgctt tgtttgtgattgtaattcagg	Ghelardi et al. (2002)
entFM	ENTA ENTB	atgaaaaaagtaatttgcagg ttagtatgcttttgtgtaacc	Ghelardi et al. (2002)
sph	TY123 TY125	ggtttagcagcagcttctgtagctggcg gtttcgttagatacagcagaaccacc	Ghelardi et al. (2002)
piplc	PC105 PC106	cgctatcaatggaccatgg ggactattccatgctgtacc	Ghelardi et al. (2002)
cytK	F2 R7	aacagatatcggtcaaaatgc cgtgcatctgtttcatgagg	Ghelardi et al. (2002)
nheA	*nheA* 344S *nheA* 843A	tacgctaaggaggggca gtttttattgcttcatcggct	Hansen and Hendriksen (2001)
nheB	*nheB* 1500S *nheB* 2269A	ctatcagcacttatggcag actcctagcggtgttcc	Hansen and Hendriksen (2001)
nheC	*nheC* 2820S *nheC* 3401A	cggtagtgattgctggg cagcattcgtacttgccaa	Hansen & Hendriksen, 2001
hblC	L2A F L2B R	aatggtcatcggaactctat ctcgctgttctgctgttaat	Hansen and Hendriksen (2001)

an overnight culture (2×10^8 cells ml^{-1}) onto the centre of TrM plates (1% Tryptone, 0.5% NaCl, 0.25% agar). Halo diameters were measured after 8 h of incubation at 37°C in a humidified chamber. Swimming motility of the microbial suspension was also observed in phase-contrast microscopy. Swarming migration was tested by spotting 50 µl of a culture (2×10^4 cells ml^{-1}) onto TrA plates (1% Tryptone, 0.5% NaCl, 0.7% agar) and incubating at 37°C in a humidified chamber for 24 h. After the incubation, growth halo diameters were measured. *B. cereus* ATCC 14579 was used as a positive control in both motility assays.

Biofilm formation

To test biofilm formation, bacteria were grown to the early stationary phase in Luria Bertani (LB) broth at 37°C and diluted in fresh LB to reach an optical density at 600 nm of 0.01 (Celandroni et al., 2016). Two millilitres of the suspension were transferred to wells of polystyrene 24-well plates (Falcon/Becton Dickinson) and incubated in static conditions at 37°C. After 48 h of incubation, non-adherent planktonic bacteria were removed, wells washed three times with PBS, and air-dried. Microbial biofilms were stained with 2 ml of 0.1% crystal violet for 10 min, washed

three times with distilled water and air-dried. Crystal violet was solubilized with 2 ml of 70% ethanol. Aliquots of ethanol-crystal violet solution (200 µl) were transferred to a 96-wells plate and OD_{570} was measured by using a microplate reader (Biorad model 550, Biorad). *B. cereus* ATCC 10987, a strong biofilm producer and sterile LB were used as positive and negative controls in the assays, respectively.

In vivo infection in *Galleria mellonella*

G. mellonella larvae were used as animal models to test the *in vivo* pathogenicity of the isolated species as previously described (Mazzantini et al., 2016). Briefly, bacteria were grown to the late exponential growth phase in BHIG at 37°C and harvested by centrifugation at 5000*g* for 10 min. Microbial suspensions were prepared by suspending pellets in PBS and bacteria were counted using a haemocytometer. Five infectious doses (5, 10, 10^2, 10^3 and 10^4 CFUs $larva^{-1}$) were used to intra-hemocoelically inject groups of 10 larvae. A control group of larvae was injected with PBS only. Infected larvae were kept at 37°C and mortality was recorded after 24 h in terms of a 50% lethal dose (LD_{50}).

Susceptibility testing

Minimal inhibitory concentrations (MICs) to penicillin (PEN), ciprofloxacin (CIP), tetracycline (TET) and vancomycin (VCM) were determined by E-test (bioMérieux) on Mueller-Hinton agar (Oxoid) incubated at 37°C for 24 h, as previously described (Celandroni et al., 2016). CLSI interpretative criteria for PEN (Susceptible, $S \leq 0.12$; Resistant, $R \geq 0.25$), TET ($S \leq 4$; Intermediate, I 8; $R \geq 16$) and VCM ($S \leq 4$) against 'fastidious Gram-positive bacilli' were used (Clinical and Laboratory Standards Institute, 2010). As CIP regards, the interpretative criterion described for *Bacillus* strains by Luna and colleagues ($S \leq 1$; $R \geq 4$) was adopted (Luna et al., 2007).

Statistical analysis

Quantitative data are expressed as the mean ± standard deviation. Three biological replicates with two technical replicates each were performed. All statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc.). Statistical significance was set at a *p*-value of <0.05. The Student's *t* test for unpaired data was applied to ensure a statistically significant difference in biofilm production assays.

RESULTS

Isolation, identification and typing of the infectious agent

To isolate the microorganism potentially responsible for the feed-related outbreak, aliquots of animal feed were seeded on TSH. In parallel, aliquots of the same samples were thermally treated and seeded on TSH to detect bacterial spores. As a result, pure cultures of a Gram-positive, rod-shaped, and spore-forming microorganism were obtained only in feed samples containing the protein supplement, thus indicating that the enriched feed constituted the actual source of swine infection. Quantification of the isolated bacteria revealed massive amounts of total bacterial forms ($2.3 \pm 0.4 \times 10^8$ CFU) and spores ($3.5 \pm 0.6 \times 10^7$ CFU) per gram of animal feed.

Several well-isolated colonies were subjected to MALDI-TOF MS. All the isolates were identified as *B. cereus* with scores ranging from 2.07 to 2.37 for six repetitions of each sample. To verify whether the isolated strains were identical in all three samples of animal feed, these isolates were typified by RAPD-PCR. For each isolate, the amplicons obtained with each of the four primers were identical, thus indicating the identity of the isolates (Figure 1).

To further exclude that the isolated strain belonged to *B. thuringiensis* species, the absence of parasporal crystals typical of *B. thuringiensis* allowed us to confirm that microbial isolates identified by MALDI-TOF MS were actually *B. cereus* sensu stricto.

These overall results indicated that only one *B. cereus* strain was responsible for the food-related outbreak. The strain was named *B. cereus* MM1 and further characterized.

B. cereus or *B. cereus* biovar anthracis?

Since the discrimination between *B. cereus* and *B. anthracis* is sometimes difficult due to the existence of intermediate strains (i.e. *B. cereus* biovar *anthracis*), PCR amplifications targeting specific *B. anthracis* virulence genes were performed for taxonomic resolution (Riojas et al., 2015; Zasada, 2020). Genes encoding neither the components of anthrax toxin (i.e. *cya*, *lef*, *pag*) nor enzymes involved in capsule biosynthesis (i.e. *capA2*, *capB*) were detected in the isolate (Figure 2a), thus indicating that *B. cereus* MM1 did not carry *B. anthracis* determinants.

In vitro and *in vivo* characterization of *B. cereus* MM1

The virulence potential of *B. cereus* MM1 was initially evaluated by PCR amplification of many genes encoding

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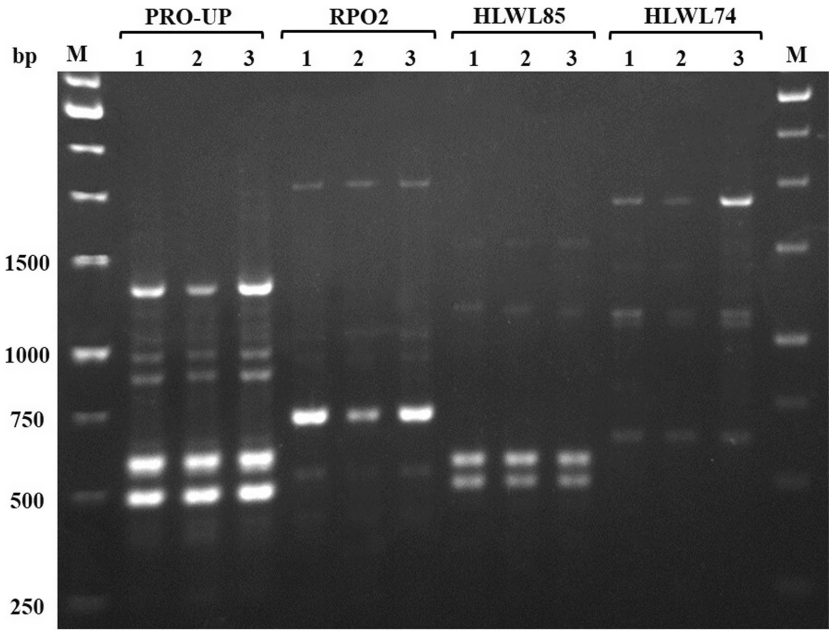

FIGURE 1 RAPD-PCR amplification profiles obtained with the primers PRO-UP, RPO2, HLWL85, and HLWL74. RAPD-PCR reactions were performed on genomic DNA extracted from *B. cereus* isolates obtained from the three samples of animal feed. M: Thermo Scientific GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA). Numbers on the left margin indicate the position of the molecular weight standards (bp).

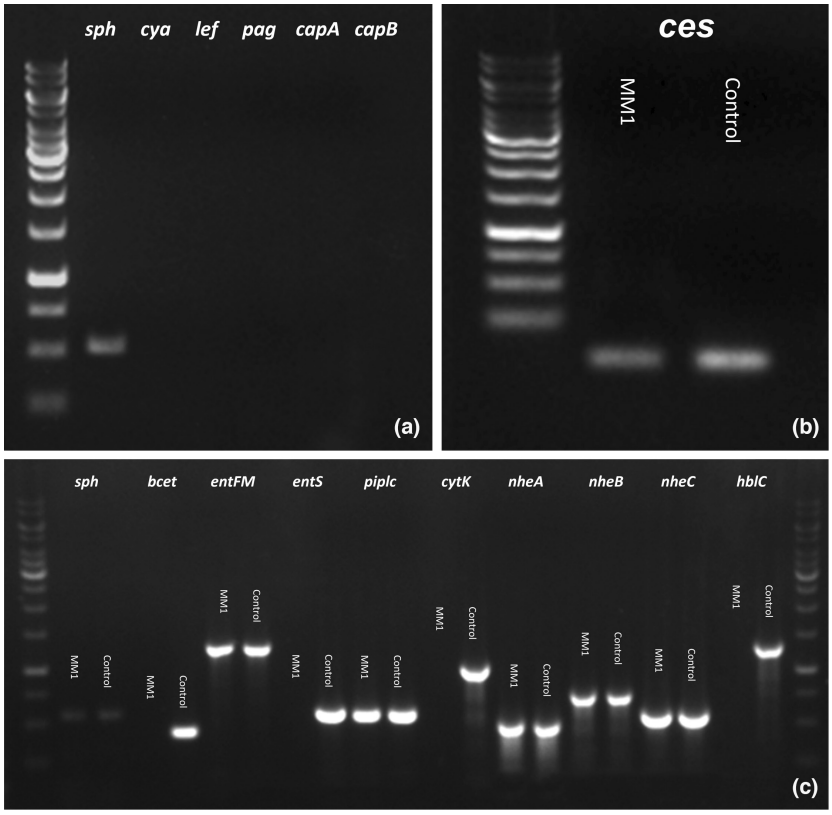

FIGURE 2 (a) PCR targeting genes encoding *Bacillus anthracis* virulence factors (*cya*, *lef*, *pag*, *capA*, *capB*) performed on genomic DNA extracted from *Bacillus cereus* MM1. *sph* gene was used as an amplification control. (b) PCR targeting gene encoding cereulide synthetase (*ces*). Genomic DNA extracted from *B. cereus* DSM 4312 was used as a positive control. (c) PCR targeting genes encoding *B. cereus* virulence factors (*sph*, *bcet*, *entFM*, *entS*, *piplc*, *cytK*, *nheA*, *nheB*, *nheC*, *hblC*) performed on genomic DNA extracted from *B. cereus* MM1. Genomic DNA extracted from *B. cereus* ATCC 14579 was used as a positive control.

toxins and tissue-degrading enzymes that *B. cereus* usually produces. A screening of the genes encoding cereulide (*ces*), SMase (*sph*), enterotoxin T (*bcet*), enterotoxin FM (*entFM*), enterotoxin S (*entS*), PI-PLC (*piplc*), cytotoxin K (*cytK*), the three components of NHE (*nheA*, *nheB*, *nheC*) and the L_2 component of HBL (*hblC*) was performed. These analyses revealed that *B. cereus* MM1 carried the *ces*, *sph*, *entFM*, *piplc*, *nheA*, *nheB* and *nheC* genes in its genome (Figure 2b,c, Table 2). On the contrary, genes *entS*, *bceT*, *cytK* and *hblC* were not detected (Figure 2c, Table 2). Since HBL requires all three components to be active, the last result indicated that *B. cereus* MM1 could not produce HBL.

The analysis of additional virulence factors that can contribute to *B. cereus* virulence (i.e. swimming, swarming, biofilm, PC-PLC, hemolysin and protease secretion) was performed. *B. cereus* MM1 resulted in motile (swimming halo *B. cereus* MM1: 31.0 ± 1.0 mm; *B. cereus* ATCC 14579: 32.9 ± 1.1 mm), but non-swarming proficient (swarming

TABLE 2 Features of *Bacillus cereus* MM1 involved in its pathogenicity and virulence

B. thuringiensis parasporal crystals	–
B. anthracis virulence genes	
pag	–
cya	–
lef	–
capA2	–
capB	–
Toxin-encoding genes	
ces	+
sph	+
bceT	–
entFM	+
entS	–
piplc	+
cytK	–
nheA	+
nheB	+
nheC	+
hblC	–
Additional virulence factors	
Swimming (mm halo)	+ (31.0 ± 1.0)
Swarming (mm halo)	–
Biofilm (OD_{570})	+ (2.24 ± 0.12)
Protease secretion	+
Hemolysins	+
PC-PLC (U ml^{-1})	+ (1.21 ± 0.13)
Pathogenicity in *Galleria mellonella*	
LD_{50} (CFUs $larva^{-1}$)	7.26 ± 0.15
Antibiotic susceptibility	
PEN	Resistant
VCM	Susceptible
TET	Susceptible
CIP	Susceptible

halo *B. cereus* MM1: 4.0 ± 1.0 mm; *B. cereus* ATCC 14579: 18.0 ± 3.0 mm), able to produce biofilm ($p < 0.0001$ compared to *B. cereus* ATCC 10987 and negative control) (Figure 3), hemolysins, proteases, and PC-PLC (*B. cereus* MM1: 1.21 ± 0.13 U ml^{-1}; *B. thuringiensis* Cry 407: 0.2 ± 0.04 U ml^{-1}; *B. thuringiensis* RM: 0.85 ± 0.08 U ml^{-1}) (Table 2). These traits may altogether concur to bacterial virulence of *B. cereus* MM1.

To confirm *in vitro* results, we used *G. mellonella* larvae as model organisms to evaluate *B. cereus* MM1 pathogenicity in vivo. The calculated LD_{50} was 7.26 ± 0.15 CFUs $larva^{-1}$ (Table 2), thus indicating strong pathogenicity of MM1 in this infection model.

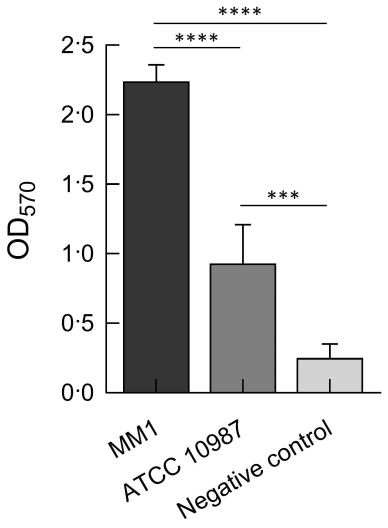

FIGURE 3 Quantification of the biofilm production from *Bacillus cereus* MM1. *B. cereus* ATCC 10987 and sterile LB broth were used as a positive control and negative control, respectively. ****$p < 0.0001$; ***$p < 0.001$.

B. cereus MM1 susceptibility to antibiotics

Finally, besides evaluating bacterial pathogenicity and virulence, *B. cereus* MM1 susceptibility to antibiotics by E-tests was determined, and the resulting MICs were 3 mg l^{-1} for PEN, 1 mg l^{-1} for TET, 0.094 mg L^{-1} for CIP, and 1.5 mg L^{-1} for VCM. Similar to other *B. cereus* strains (Celandroni et al., 2016; Luna et al., 2007), MM1 resulted to be resistant to penicillin and susceptible to vancomycin, tetracycline, and ciprofloxacin (Table 2).

DISCUSSION

The identification of species belonging to *Bacillus* genus may be challenging due to similarities amongst some closely related species that share morphological, biochemical, and genetic features. MALDI-TOF MS has been proven useful as a diagnostic technique for *Bacillus* spp. by overcoming most of the limits associated with the traditional identification of these microorganisms (Al Masoud et al., 2014; Böhme et al., 2011; Celandroni et al., 2016; Farfour et al., 2012; Fernández-No et al., 2013; Hotta et al., 2011; Shu & Yang, 2017; Tirloni et al., 2020). Therefore, in this investigation, MALDI-TOF MS was used to identify the bacterial isolate found in the supplemented animal feed. As result, *B. cereus* was always identified with a very high value of significance. Isolates from animal feed were typified by RAPD-PCR, thus demonstrating that the same strain of *B. cereus*, subsequently named MM1, was associated with the feed-related outbreak.

Considering the histopathological frame similar to the gastrointestinal anthrax infection and given the existence of intermediate *B. cereus* strains carrying *B. anthracis* determinants, the production of virulence factors typical of *B. anthracis* (i.e. capsule, anthrax toxin) was evaluated. Due to the high percentage of identity amongst chromosomal DNA sequences of *B. cereus* and *B. anthracis*, analysing the presence of plasmid-encoded virulence factors is the main distinguishing criterion between the two microbial species (Rasko et al., 2005). None of the *B. anthracis* determinants was detected in *B. cereus* MM1 genome by PCR amplification, thus suggesting that the virulence of the isolated *B. cereus* strain was due to intrinsic *B. cereus* virulence determinants.

To the best of our knowledge, this is the first report attributing to *B. cereus*, a severe epidemic event in pigs. Since the removal of contaminated feed resulted in the total cessation of pigs' symptomatology and *B. cereus* was found in both the intestines of dead animals and faeces of survivors, a highly reasonable cause-effect association between *B. cereus* infection and the onset of the clinical picture was inferred. The isolation of *B. cereus* from blood samples of pigs experimentally infected with the porcine reproductive and respiratory syndrome virus (PRRSV) and with the porcine circovirus type 2 (PCV2) (Niederwerder et al., 2016) has been previously reported, but no other data are currently available as far as the susceptibility of pigs to natural feed-borne *B. cereus* infections concerns. Considering our findings, we can speculate that the present case was due to the ingestion of a high amount of a particularly virulent *B. cereus* strain. Although the *B. cereus* lethal dose for pigs is unknown, it can be reasonably assumed that 2.3×10^8 CFU per gram of food is an exceptionally high number of ingested microorganisms per day, even for pigs.

Tissue invasion and establishment of infection by *B. cereus* are the results of a multifactorial process including microbial motility modes, adhesion factors and production of degrading enzymes and of a variety of toxins (Jessberger et al., 2020). All these traits are shared by most of the strains belonging to the species. However, the toxigenic profile and the pathogenic potential of this germ are strain-specific features.

PCR analysis targeting the cereulide synthetase gene indicated that *B. cereus* MM1 is potentially able to produce cereulide. Acting as a stimulator on the vagus nerve and thus causing nausea and vomiting, cereulide is the causative toxin of the emetic syndrome associated with *B. cereus* food poisoning. Depression, convulsions, tremors and gasping respiration have also been demonstrated to be arisen as a result of acute intoxication (Bauer et al., 2018). Porcine models experimentally intoxicated with purified cereulide showed toxin bioaccumulation in kidneys, liver, muscles and adipose tissue, as well as the ability of cereulide to cross the blood–brain barrier and thus be directly active in the central nervous system (Bauer et al., 2018). However, histopathological examination of swine organs did not reveal any notable morphological and functional aberration after cereulide oral administration (Bauer et al., 2018), and the direct effects of cereulide on animal tissues still remain uncertain. Since the gene encoding cereulide synthetase is localized on the 270 kb plasmid pCER270, it is not detectable in all isolated *B. cereus* strains but only in an under-represented lineage carrying the megaplasmid (Ehling-Schulz et al., 2015). *B. cereus* MM1 appeared to belong to the narrow subgroup of strains producing cereulide. Although quantifying the actual amount of the secreted emetic toxin in the animal feed could have been important, it was not possible since no reliable methods were available at the time analysis was carried out (Ramarao et al., 2020). Nevertheless, the overall clinical picture of infected pigs was hardly attributable to cereulide intoxication alone, whilst it is more plausible that a set of virulence determinants from *B. cereus* MM1, comprising cereulide, was the cause of such a severe clinical picture in pigs.

B. cereus MM1 was impaired in the production of HBL, one of the major virulence factor this species produces (Beecher & Wong, 1995). It has already been demonstrated that cereulide-synthetizing *B. cereus* strains do not commonly carry *hbl* genes (Dietrich et al., 2021), so this result should not be surprising. Similarly, the absence of the cytotoxin K encoding gene is quite common in *B. cereus* (Guinebretière et al., 2002; Wijnands et al., 2006). Finally, *B. cereus* MM1 resulted potentially able to produce other toxins, such as SMase and enterotoxins NHE and FM.

From our analysis, *B. cereus* MM1 was found to be a high producer of PC-PLC. Phospholipases are important virulence factors since they can enhance bacterial tissue colonization and infection progression, as well as immune response hindering (Flores-Diaz et al., 2016). Extracellular proteases, which are actively secreted by *B. cereus* MM1, might have an additional role in tissue penetration during animal infection or in processing other host proteins (Marshall et al., 2017). Likewise, due to their cytolytic activity, hemolysins can act on erythrocytes and several other cell types, enhancing tissue destruction and germ dispersion (Ramarao & Sanchis, 2013).

Besides the role of toxins and exoenzymes, additional virulence factors may favour the establishment and maintenance of a bacterial infection. Infections by organisms able to form biofilms are often persistent and difficult to resolve, as microbial cells constituting biofilm are less susceptible to both drugs and the immune response (Clayton & Thien-Fah, 2017). As many other *B. cereus* strains (Majed et al., 2016), *B. cereus* MM1 was found to be a strong biofilm producer. Its ability to form biofilm could in part explain the presence of high amounts of vegetative

cells in the dry feed. It is also reasonable that the production of biofilms can provide the strain with a better resistance during the infectious process in the host (Majed et al., 2016). In addition, motility modes swimming and swarming can facilitate penetration and spread of a microorganism inside host tissues. Swimming motility is traditionally considered as a classification criterion of *B. cereus*, differentially from non-motile *B. anthracis* and *B. mycoides*, and *B. cereus* MM1 was found to be typically motile. Swarming is a strain-specific ability that is not shared by all *B. cereus* strains, instead. Indeed, *B. cereus* MM1 was non-swarming proficient.

Globally, the pathogenic potential of *B. cereus* MM1 was rather peculiar. Therefore, its virulence was assessed in vivo by intra-hemocoelically injecting *G. mellonella* larvae to mimic systemic infections. This model of infection possesses the benefits of being not subjected to regulation by the Ethics Committee and to be easily preserved and undertaken without anaesthesia. Although being an invertebrate, *G. mellonella* has a relatively advanced defensive system (Browne et al., 2013). For this reason, this arthropod is considered useful for getting information about the infection process in mammals (Mazzantini et al., 2016; Ramarao et al., 2012). The obtained LD_{50} of 7.26 CFUs $larva^{-1}$ was lower compared to the LD_{50} of other Gram-positive species (i.e. *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Listeria monocytogenes*; reviewed in Tsai et al., 2016). In particular, the LD_{50} of *B. cereus* MM1 resulted to be 30-fold, 350-fold, and 580-fold lower than the LD_{50} of other *B. cereus* strains (Brillard et al., 2008; Daou et al., 2009; Kavanaugh et al., 2022), thus indicating the very high virulence of *B. cereus* MM1. This result is in line with the findings that *B. cereus* MM1 secretes high levels of PC-PLC, is swimming proficient, and potentially produces SMase, EntFM and PI-PLC. In fact, these virulence factors have been described to have an important impact on larvae mortality (Doll et al., 2013; Frenzel et al., 2015; Mazzantini et al., 2016; Tran et al., 2010).

In order to limit bacterial infections and to increase the feed conversion ratio and weight gain, breeding animals are often subjected to a diet enriched with sub-therapeutic doses of antibiotics, although these procedures are strongly discouraged by the EU (directive 2001/82/EC and regulation 726/2004). Therefore, we evaluated the sensitivity/resistance of the isolated strain to antibiotics. No significantly important resistance was found. Whilst the E-test highlighted that *B. cereus* MM1 is resistant to penicillin, a widespread feature of the species, it was shown to be susceptible to vancomycin, ciprofloxacin, and tetracycline.

Considering that the analysis of the feed alone did not reveal the presence of any pathogen whilst the feed enriched with the protein supplement showed a heavy contamination by *B. cereus*, we can reasonably assume that the source of feed contamination was the supplement itself, even if our data do not provide direct evidence about it. The outbreak severity and the number of involved animals highlight the importance of ensuring high sanitary standards in the veterinary field. Although the information supporting the importance of *B. cereus* in the veterinary field is fragmentary, this epidemic event due to *B. cereus* most likely constitutes an isolated outbreak.

CONCLUSIONS

The case here reported is the first demonstrating the ability of *B. cereus* to cause severe systemic infections in pigs. Although the isolate was not tested in alive pigs to reproduce the disease, this paper provides very strong evidence that the ingestion of substantial amounts of a virulent *B. cereus* strain can lead swine to death as a consequence of multi-organ injury. This finding corroborates the fact that more accurate microbiological quality controls for feeds and supplements are essential requirements for livestock safety and wellness, in order to exclude any possible risk even if the receiving animal is not commonly considered highly susceptible to feed-related diseases.

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CONFLICT OF INTEREST

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