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*Research Article*

# Genomic Diversity and Virulence Genes Characterization of *Bacillus cereus sensu lato* Isolated from Processing Equipment of an Algerian Dairy Plant

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*Bacillus* cereus is a ubiquitous spore-forming bacterium causing food spoilage. In this study, seventeen *B. cereus* were isolated from dairy processing equipment in Algeria and characterized. The pathogenic potential genes encoding hemolysin, non-haemolytic enterotoxin, cytotoxin K and emetic toxin were investigated by PCR. Phylogeny and genetic markers for antimicrobial resistance and virulence were analyzed using whole-genome sequencing data using the Illumina MiSeq® technology. The identity of the isolated strains was confirmed as *B. cereus sensu lato* and the sequencing of the *pan*C gene clustered 64.7% of them in phylogenetic group III and 35.3% in group IV. The results of PFGE showed that 8 (47.1%), 4 (23.5%), 3 (17.6%) and 2 (11.8%) were differentiated into four groups A, B, C and D, respectively. Each of the two *pan*C phylogenetic groups contains two different types of isolates corresponding to PFGE group (A and C) and (B and D) for phylogenetic groups III and IV, respectively. None of the 14 isolates carried the emetic toxin (*ces*) gene, whereas other toxin genes were variably detected. Genotyping assigned all isolates to three different sequence types (ST 2226, ST1018 and ST1431) while one clonal complex (CC-142) was identified in three isolates. Hierarchical clustering with a threshold of 500 core genome single nucleotide polymorphism (cgSNPs) differences grouped the strains into three clusters while two strains were less than 20 SNPs different. Three *B. cereus* (25%) contained three enterotoxic HBL complex-encoding genes *hbl*A/C/D. Enterotoxic NHE complex-encoding genes (*nhe*A/B/C) and the *cty*K1 gene were detected in all isolates. The overall results highlight the risk due to toxins produced by the *B. cereus* group in the food safety and dairy industries in Algeria. This study emphasized the potential of whole-genome sequencing for genotyping and predicting the virulence-associated genes and antimicrobial resistance. The present study will help to better assess the health and spoilage risk associated with *B. cereus* in dairy processing plants and to incorporate adequate preventive measures.

## 1. Introduction

The *Bacillus* (*B.*) *cereus* group is composed of several species that are able to put the public health at risk due to the production of toxins. These bacteria also cause economic losses to the food and dairy industries [1]. Consuming contaminated food mainly with pathogens and microbial toxins may lead to severe diseases; hence, food safety is seen as a major concern in any society. For this reason, cleaning and disinfection are important practices for the elimination of both food residues and the reduction of microbial contamination in the food industries [2]. The biofilm-forming bacteria settling in dairy industry materials might influence both the safety and quality of milk and its products [3]. In fact, such bacteria is found on the surfaces of milk transport tools such as pipes and containers and is described as a major cause of contamination of the dairy products. This leads to the corroding of metal pipes, reducing heat transfer, and increasing fluid frictional resistance [4, 5]. Unfortunately, there is no definite method that can radically resolve the biofilm issue in the dairy industry [6].

Bacteria from the *Bacillus cereus* group have been frequently isolated from various food environments including food contact surfaces in the dairy industry, which constitutes a hygienic as well as a technological problem [7, 8]. *Bacillus cereus* is able to form biofilms on many types of substrata, including stainless steel surfaces. The major microbiological challenge for food production lines is the presence of *B. cereus* biofilms and their substantial physiological versatility. The capacity of *Bacillus cereus* to endure in the different parts of the dairy manufacturing plants allows this microorganism to be a potential source of the product's contamination and recontamination. Moreover, the low-temperature and the short period of sterilization are not effective in completing the elimination of *B. cereus* in the milk. This could result in food spoilage which subsequently leads to rejection of the products by the customers and create a financial deficit for the dairy industry [9–11]. Furthermore, the task of removing these bacteria is made challenging because of biofilm and heat-resistant spore formation [10, 11].

The *B. cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the *Bacillus* genus that consists of eight closely related species, i.e., *B. cereus sensustricto*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, *Bacillus toyonensis* and *Bacillus cytotoxicus* [12, 13]. These microorganisms are clustered into seven phylogenetic subgroups according to the *pan*C gene sequence [14–16]. They also include phenotypic features of the strains in each group such as the ranges of temperature growth [15]. *Bacillus cereus* is able to secrete an array of enzymes and virulence factors, such as toxins and enterotoxins. Moreover, the *B. cereus* poisoning effect on food shows two different syndromes: the diarrheal and/or the emetic syndromes [17]. The latter is triggered by a small cyclic heat-stable peptide (cereulide) which leads to vomiting 0.5–6 after ingestion [18]. The diarrheal type is due to enterotoxins, a group of heat-labile proteins. These enterotoxins include the tripartite hemolysin BL (Hbl) constituted by B, $L_2$ and $L_1$ components encoded by *hbl*C, *hbl*D, and *hbl*A genes, respectively [19], the nonhaemolytic enterotoxin (Nhe) encoded by *nhe*A, *nhe*B and *nhe*C genes [20] and the single protein, cytotoxin K encoded by the *cytK* [21, 22]. Products from other genes, such as *ent*FM, *ent*S and *plc*A are equally included in the pathogenesis of *B. cereus* [23–26]. Another significant virulence factor of *B. cereus* is its capacity to adhere to various surfaces [27]. The ability of spores to adhere to stainless steel is linked to various factors such as their hydrophobicity potential, the number of appendages on their surface, the exosporium length and the zeta potential [28]. Various studies have evaluated the prevalence of pathogenic *B. cereus* in milk and dairy products [29–31]. However, data on the presence of such bacteria on the processing equipment, especially on their persistence after the cleaning step, as well as the mechanisms responsible for their ability to withstand the cleaning process are almost inexistent to the best of our knowledge. Therefore, the aim of the current study was to identify potential *B. cereus* isolates from dairy equipment in a processing plant in Algeria and assess their virulence potential.

## 2. Materials and Methods

*2.1. Origin of B. cereus Strains.* Seventeen *Bacillus* strains were isolated from 160 samples collected by aseptically swabbing an area of 1 $cm^2$ of the inner surfaces of milk tanks that had contained pasteurized, unpasteurized local raw cow and recombined milk, as well as from packaging lines of dairy plant processing located in Tlemcen (north-west of Algeria) in 2010–2012. Swab premoistened with sterile distilled water containing 3% (v/v) Tween 80 was carefully used to ensure maximum recovery of bacteria from the equipment surface by overlapping horizontal and vertical strokes. Then, the swabs were placed in test tubes containing 10 ml of maximum recovery diluent (tryptone salt broth). The diluent tubes containing the swabs were vortexed for at least one minute to release the attached bacteria. Samples were then heat treated at 80°C for 10 minutes before being incubated at 30°C for 24 to 48 hours.

Recombined milk is obtained by merging water with skim milk powder and carefully adding milk fat in such a way that the target fat content is reached. These strains were collected through different samplings that were performed after the cleaning and sanitizing procedures.

These strains belong to the collection of the Laboratory of Food and Environmental Microbiology (University of Tlemcen-Algeria) and were previously characterized using biochemical tests and physicochemical tests for the surface properties of their spores (hydrophobicity and zeta potential) [32].

*2.2. Molecular Characterization and Identification*

*2.2.1. Molecular Identification of Isolated Strains.* The isolates were grown at 37°C overnight in nutrient broth (NB) (Biokar Diagnostics, Beauvais, France) and genomic DNA was extracted using the method described by Sambrook [33]. The

amplification of the 16S rRNA partial gene was performed using the forward primer 27F (5′-GAG TTT GAT CMT GGC TCA G-3′) and the reverse primer 1492R (5′-GNT ACC TTG TTA CGA CTT-3′) [34]. Amplicons were purified using the High Pure PCR Product Kit (Roche Diagnostics, Mannheim, Germany) and sequenced (GATC Biotech, Heidelberg, Germany). Bacterial identification was performed by analyzing the obtained sequences with the BLAST algorithm (https://www.ncbi.nlm.nih.gov/BLAST/) [35].

*2.2.2. Determination of the Affiliation of the Presumptive B. cereus Isolates.* The genotypic group of the presumptive *B. cereus* was determined as defined previously by Guinebretière et al. [15]. Amplification and partial sequencing of the *pan*C gene were performed using Forward (5′-GAG GCG AGA GAA TAC GGA ATA CG-3′) and reverse (5′–GCC CAT TTG ACT CGG ATC CAC T-3′) primers [15]. The following PCR conditions were applied: 94°C for 5 min as an initial denaturation step, followed by 30 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C, and 7 min at 72°C as a final extension [36]. Amplicons were purified and sequenced as mentioned above. The sequences were analyzed using the Sym'Previus online software (https://www.tools.symprevius.org/bcereus/), a tool that allows a rapid classification of *B. cereus* strains in four groups (from I to IV) by comparing the *pan*C gene sequences of new isolates with reference strains published by Guinebretière et al. [15] and stored in the database [15].

*2.2.3. Pulse Field Gel Electrophoresis (PFGE).* To screen potential intra and interspecies diversity, the isolates were further investigated using PFGE [37]. The fingerprints obtained were analyzed, compared and grouped based on the number and position of the fragments observed using the Bionumerics system (Dice's Coefficient of similarity, UPGMA: Unweighted Pair Group Method with Arithmetic Average; Applied Maths, Saint-Martens-Latem, Belgium).

*2.3. Hemolysis Assay.* For the evaluation of hemolytic activity, the isolates were streaked on Columbia agar (Oxoid, Basingstoke, UK) containing 5% horse-blood according to the technique described by Mulligan et al. [38]. The plates were incubated at 30°C for 24 to 48 h and the presence of hemolytic haloes around the colonies was examined visually. Depending on the ability to degrade red blood cells, three categories can be identified: no hemolysis ($\gamma$-hemolysis), incomplete hemolysis ($\alpha$-hemolysis) and complete hemolysis ($\beta$-hemolysis) [39].

*2.4. Genotypic Determination of Toxin Production Potential.* Genomic DNA was extracted and purified as previously described by Celandroni et al. [40]. The presence of toxin-encoding genes in bacterial genomes was evaluated by PCR as previously described by Thaenthanee et al. [41]. All strains were tested for the presence of *sph* (sphingomyelinase), *plc*A (phosphatidylinositol-specific phospholipase C; PI-PLC), *ent*FM (enterotoxin FM), *ent*S (enterotoxin S), *nhe*A, *nhe*B and *nhe*C (the three components of NHE), *hbl*C (the $L_2$ component of HBL) complexes, *cyt*K (cytotoxin K) and *ces* (emetic toxin) genes. Primers and annealing temperatures used for each PCR are listed in Table 1. Genomic DNA was extracted from *B. cereus* ATCC 14579 and ATCC 10987 and used as positive controls for each amplification.

*2.5. Whole Genome Sequencing.* Genomic DNA was extracted and purified from a 48 h bacterial culture on Mueller-Hinton blood agar plates (Oxoid Deutschland GmbH, Wesel, Germany) using the QIAGEN® Genomic-tip 20/G Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in 200 $\mu$l elution buffer. DNA was quantified spectrophotometrically using a Nanodrop® ND-1000 (Fisher Scientific GmbH, Schwerte, Germany).

Sequencing libraries were created using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA) and 300 pb-long aired-end sequences were generated on an Illumina MiSeq instrument Raw sequencing data from this study were deposited in the European Nucleotide Archive (ENA) under BioProject PRJEB64867. The Linux-based bioinformatics pipeline WGSBAC v. 2.0.0 (https://gitlab.com/FLI_Bioinfo/WGSBAC) was used for data analysis. FastQC v. 0.11.7 [46] was used for quality control, and raw read coverage was calculated. Next, WGSBAC performed assembly using Shovill v. 1.0.4 (https://github.com/tseemann/shovill). The quality of the assembled genomes was controlled by QUAST v. 5.0.2 [47], while Kraken 2 v. 1.1 [48] and the database Kraken2DB were used to determine potential contamination. For the investigation of antimicrobial resistance genes and virulence determinants, WGSBAC used the software ABRicate (v. 0.8.10) (https://github.com/tseemann/abricate) and the databases: ResFinder [49], NCBI [50] and virulence factor database (VFDB) [51]. In addition, AMRFinderPlus (v. 3.6.10) [52] was utilized for the detection of chromosomal point mutations leading to AMR and organism-specific acquired resistance genes.

For genotyping, classical multilocus sequence typing (MLST) based on assembled genomes was performed with the software mlst v. 2.16.1 that incorporates the PubMLST database for *B. cereus* typing database (https://pubmlst.org/bigsdb?db=pubmlst_bcereus_seqdef) which was also used to define clonal complexes. In addition, WGSBAC performed mapping-based genotyping using core-genome single nucleotide polymorphisms (cgSNPs) identified by Snippy v. 4.3.6 (https://github.com/tseemann/snippy) with standard settings. As a reference, the genome of *B. cereus* strain FORC_047 (accession ASM222028v1) was used. For phylogenetic tree construction based on cgSNP analysis, RAxML (Randomized Axelerated Maximum Likelihood) v. 8 [53] was utilized. The phylogenetic tree was rooted to the reference genome and visualized using the interactive Tree of Life (iTOL) v. 4 web tool (https://itol.embl.de/login.cgi) [54]. The strains were partitioned into clusters (subpopulations) based on hierarchical clustering.

Table 1: Genes, primers sequences and PCR conditions used in this study.

| Gene | Primer | Sequence (5′-3′) | Product (bp) | Cycles | Denat. | Anneal. | Elong. | Ref |
|---|---|---|---|---|---|---|---|---|
| *Sph* | Ph1<br>Ph2 | CGT GCC GAT TTA ATT GGG GC<br>CAA TGT TTT AAA CAT GGA TGC G | 558 | 35 | 20 s, 94°C | 20 s, 58°C | 20 s, 72°C | [42] |
| *bceT* | ETF<br>ETR | TTA CAT TAC CAG GAC GTG CTT<br>TGT TTG TGA TTG TAA TTC AGG | 428 | 35 | 20 s, 94°C | 20 s, 56°C | 20 s, 72°C | [43] |
| *entFM* | ENTA<br>ENTB | ATG AAA AAA GTA ATT TGC AGG<br>TTA GTA TGC TTT TGT GTA ACC | 1269 | 35 | 20 s, 94°C | 20 s, 52°C | 20 s, 72°C | [44] |
| *entS* | TY123<br>TY125 | GGT TTA GCA GCA GCT TCT GTA GCT GGC G<br>GTT TCG TTA GAT ACA GCA GAA CCA CC | 581 | 29 | 40 s, 94°C | 60 s, 60.7°C | 60 s, 72°C | [44] |
| *pipIc* | PC105<br>PC106 | CGC TAT CAA TGG ACC ATG G<br>GGA CTA TTC CAT GCT GTA CC | 569 | 30 | 30 s, 94°C | 45 s, 57°C | 90 s, 72°C | [45] |
| *cytK* | F2<br>R7 | AAC AGA TAT CGG TCA AAA TGC<br>CGT GCA TCT GTT TCA T | 623 | 30 | 60 s, 94°C | 60 s, 52°C | 60 s, 72°C | [26] |
| *nheA* | 344S<br>843A | TAC GCT AAG GAG GGG CA<br>GTT TTT ATT GCT TCA TCG GCT | 499 | 30 | 15 s, 94°C | 45 s, 55°C | 120 s, 72°C | [25] |
| *nheB* | 1500S<br>2269A | CTA TCA GCA CTT ATG GCA G<br>ACT CCT AGC GGT GTT CC | 769 | 30 | 15 s, 94°C | 45 s, 55°C | 120 s, 72°C | [25] |
| *nheC* | 2820S<br>3401A | CGG TAG TGA TTG CTG GG<br>CAG CAT TCG TAC TTG CCA A | 581 | 30 | 15 s, 94°C | 45 s, 55°C | 120 s, 72°C | [25] |
| *hblC* | FHC<br>RHC | GAA TGG TCA TCG GAA CTC TA<br>CAT CAG GTC ATA CTC TTG TGT | 672 | 30 | 60 s, 94°C | 60 s, 54°C | 60 s, 72°C | [41] |
| *Ces* | cesF1<br>cesR2 | GGT GAC ACA TTA TCA TAT AAG GTG<br>GTA AGC GAA CCT GTC TGT AAC AAC A | 1.271 | 35 | 30 s, 94°C | 45 s, 58°C | 90 s, 72°C | [23] |

## 3. Results

*3.1. Bacillus cereus Identification and Differentiation.* Using 16S rRNA gene sequencing, the isolates were identified as belonging to the *B. cereus sensu lato* group. The microscopic observation of the isolates did not reveal the presence of parasporal crystals characteristic of *B. thuringiensis*. Sequencing of the *pan*C gene phylogenetic classified the isolates into 64.7% group III and 35.3% group IV (Table 2).

The results of PFGE showed that 8 (47.1%), 4 (23.5%), 3 (17.6%) and 2 (11.8%) were differentiated into four groups A, B, C and D, respectively, based on the number and position of the fragments observed, as shown in Figure 1. It was noticed that each of the two *pan*C phylogenetic groups contain two different types of isolates corresponding to the PFGE group (A and C) and (B and D) for phylogenetic group III and IV, respectively (Table 2). Group A was the dominant group (47.1%) and contains bacteria isolated for all the different samples screened except from the sample of the pipelines of pasteurized recombinated milk. Isolates from groups B, C and D were also detected in various samples (Table 2 and Figure 1).

*3.2. Hemolysis Capacity of the Isolates.* Hemolysin production was evaluated by seeding bacterial cells on blood agar plates. All bacteria investigated exhibited a $\beta$-hemolytic characteristic as a clear zone around the colonies due to the complete degradation of red blood cells that was observed.

*3.3. Putative Pathogenic Potential of B. cereus Strains.* The presence of *sph*, *nhe*A/B/C, *hbl*C, *plc*A, *ent*T, *ent*FM, *ent*S, *cyt*K and *ces* genes in the bacterial genomes was verified by PCR. As shown in Table 3, none of the 17 isolates carried the emetic toxin-encoding gene (*cse*), while the other genes were commonly amplified. *ent*FM and *ent*S were amplified in all isolated strains. Nhe components and SMase-encoding genes were detected in most strains (*sph* 88.2%, *nhe*A 88.2%, *nhe*B 94.1% and *nhe*C 94.1%). For other genes, the *hbl*C, *plc*A, *ent*T and *cyt*K genes were detected in 29.4%, 94.1%, 35.3% and 41.2%, respectively. The obtained results indicate a noticeable interstrain differences in the toxigenic profiles of the isolates. One isolate (*Bacillus cereus sensu lato*100) can be assumed to be the most virulent strain, since it possesses hemolytic activity and 10 out of 11 (90.9%) virulence genes (Table 3). On the other hand, the least virulent potential can be attributed to the isolate *Bacillus cereus sensu lato*18, with four genes out of 11 (36.4%).

*3.4. Genetic Characterization of Bacillus cereus sensu lato Using WGS Analysis.* Sequencing of the 12 *Bacillus cereus sensu lato* isolates, yielded an average of 183.625.4 total reads (range: 889.720–295.942,2) per sample, leading to an average read-coverage of 82-fold (range: 37–137). The assembled genomes consisted on average of 83 contigs (range: 35–204). The GC content averaged at 35.15%, and the genome size of the isolates was 525.439.4 bp on average (range: 518.571.5–541.973.2 bp).

The phylogenetic and genotyping analysis displaying relatedness between the *Bacillus cereus sensu lato* isolates based on cgSNP distances rooted to the reference genome revealed three different groups according to how the isolates were closely related (Figure 2).

Table 2: Phylogenetic group and isolate origin of *Bacillus cereus sensu lato* isolated in this study.

| Specie | Strain code | Phylogenetic group (*pan*C) | Phylogenetic group PFGE | Isolate origin |
|---|---|---|---|---|
| *Bacillus cereus sensu lato* | 18 | III | A | Pipeline of pasteurized milk |
| *Bacillus cereus sensu lato* | 44 | III | A | Pipeline of pasteurized milk |
| *Bacillus cereus sensu lato* | 80 | III | A | Raw recombined milk storage tank |
| *Bacillus cereus sensu lato* | 82 | III | A | Raw milk storage tank |
| *Bacillus cereus sensu lato* | 103-2 | III | A | Pasteurized milk storage tank |
| *Bacillus cereus sensu lato* | 109 | III | A | Pasteurized recombined milk storage tank |
| *Bacillus cereus sensu lato* | 110 | III | A | Pipeline of raw recombined milk |
| *Bacillus cereus sensu lato* | 126-2 | III | A | Pipeline of raw milk |
| *Bacillus cereus sensu lato* | 14 | III | C | Pasteurized milk storage tank |
| *Bacillus cereus sensu lato* | 89 | III | C | Pasteurized recombined milk storage tank |
| *Bacillus cereus sensu lato* | 126-3 | III | C | Pipeline of raw milk |
| *Bacillus cereus sensu lato* | 100 | IV | B | Raw milk storage tank |
| *Bacillus cereus sensu lato* | 103-1 | IV | B | Pasteurized milk storage tank |
| *Bacillus cereus sensu lato* | 107 | IV | B | Pipeline of pasteurized recombined milk |
| *Bacillus cereus sensu lato* | 120 | IV | B | Pipeline of pasteurized recombined milk |
| *Bacillus cereus sensu lato* | 123 | IV | D | Pipeline of pasteurized recombined milk |
| *Bacillus cereus sensu lato* | 126-1 | IV | D | Pipeline of raw milk |

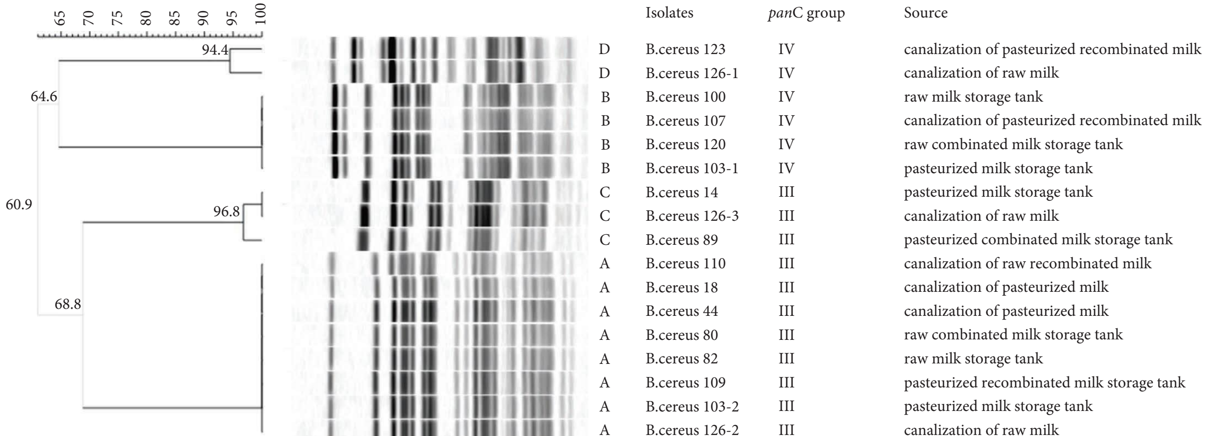


Figure 1: Dendrogram of cluster analysis of PFGE fingerprints of *B. cereus sensu lato* isolated from different processing equipment of an Algerian dairy plant. The dendrogram based on Dice's coefficient of similarity with the unweighted pair method with arithmetic averages clustering algorithm (UPGMA).

The MLST analysis based on WGS revealed three different sequence types (STs) (1431, 2226, and 1018) (Figure 2). Three isolates belonged to clonal complexes (CC-142) (Figure 2). For eight isolates, so far, an unknown clonal complex was identified. Hierarchical clustering with a threshold of 500 cgSNPs, grouped eleven strains into three distinct clusters while two strains were less than 20 SNPs different (Figure 2).

The genomic analysis of *Bacillus cereus sensu lato* isolates revealed different virulence-associated genes related to motility, chemotaxis, adhesion and invasion. All isolated *B. cereus sensu lato* harbored *inh*A, *nhe*A/B/C, *ent*FM, *cyt*K2, *plc*A, *alo*, *sph* and *cer*A while *hbl*A/B/D, *hbl*C, *hlyI*IR, *hly*II and *bps*C were detected in 25% of the isolates. Hemolysin BL-binding components represented by *hbl*A/C/D genes were detected in the strains with the same PFGE profile as sequence type 1431 and clonal complex CC-142. A pore-forming toxin of *Bacillus cereus* that has been linked to a case of necrotic enteritis (cytK) was detected in all isolated *B. cereus*.

WGS analyses identified 4 acquired antimicrobial-resistant-associated genes that code for resistance to $\beta$-lactamase (*bla*1/2), fosfomycin (*Fos*B), macrolide (*mph*L), streptothricin (*sat*A/B) and vancomycin (*van*Z/F) (Table 4). The chromosomally-encoded macrolide phosphotransferases (*mph*L) that inactivate 14- and 15-membered macrolides such as erythromycin, clarithromycin and azithromycin were detected in 9 (75%) isolated *Bacillus cereus sensu lato*. *bla*Z gene-encoded penicillin resistance was not detected in all isolates.

## 4. Discussion

The ecological adaptations play a major role in the emerging of *B. cereus*. The temperature tolerance is considered a factor that leads the strains of various groups of *B. cereus* to adjust to new environments [15]. Microorganisms belonging to different groups possess different physiological properties and safety potentials. For example, microorganism of groups

Table 3: Toxin gene profiles of the screened *Bacillus cereus sensu lato* isolated in this study using PCR.

| Strain code | *sph* | *nhe*A | *nhe*B | *nhe*C | *hbl*C | *plc*A | *ent*T | *ent*FM | *ent*S | *cyt*K | *ces* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (14) | − | + | + | + | − | + | + | + | + | − | − |
| (18) | + | − | − | − | − | + | − | + | + | − | − |
| (44) | + | + | + | + | − | − | − | + | + | − | − |
| (80) | + | + | + | + | − | + | − | + | + | + | − |
| (82) | + | − | + | + | + | + | − | + | + | + | − |
| (89) | + | + | + | + | − | + | + | + | + | + | − |
| (100) | + | + | + | + | + | + | + | + | + | + | − |
| (103-1) | + | + | + | + | − | + | − | + | + | + | − |
| (103-2) | + | + | + | + | − | + | − | + | + | + | − |
| (107) | − | + | + | + | + | + | + | + | + | − | − |
| (109) | + | + | + | + | − | + | − | + | + | − | − |
| (110) | + | + | + | + | + | + | − | + | + | − | − |
| (120) | + | + | + | + | + | + | + | + | + | − | − |
| (123) | + | + | + | + | − | + | − | + | + | − | − |
| (126-1) | + | + | + | + | − | + | − | + | + | − | − |
| (126-2) | + | + | + | + | − | + | − | + | + | − | − |
| (126-3) | + | + | + | + | − | + | + | + | + | + | − |
| Total positive (%) | 15/17 (88.2) | 15/17 (88.2) | 16/17 (94.1) | 16/17 (94.1) | 5/17 (29.4) | 16/17 (94.1) | 6/17 (35.3) | 17/17 (100) | 17/17 (100) | 7/17 (41.2) | 0/17 (0) |

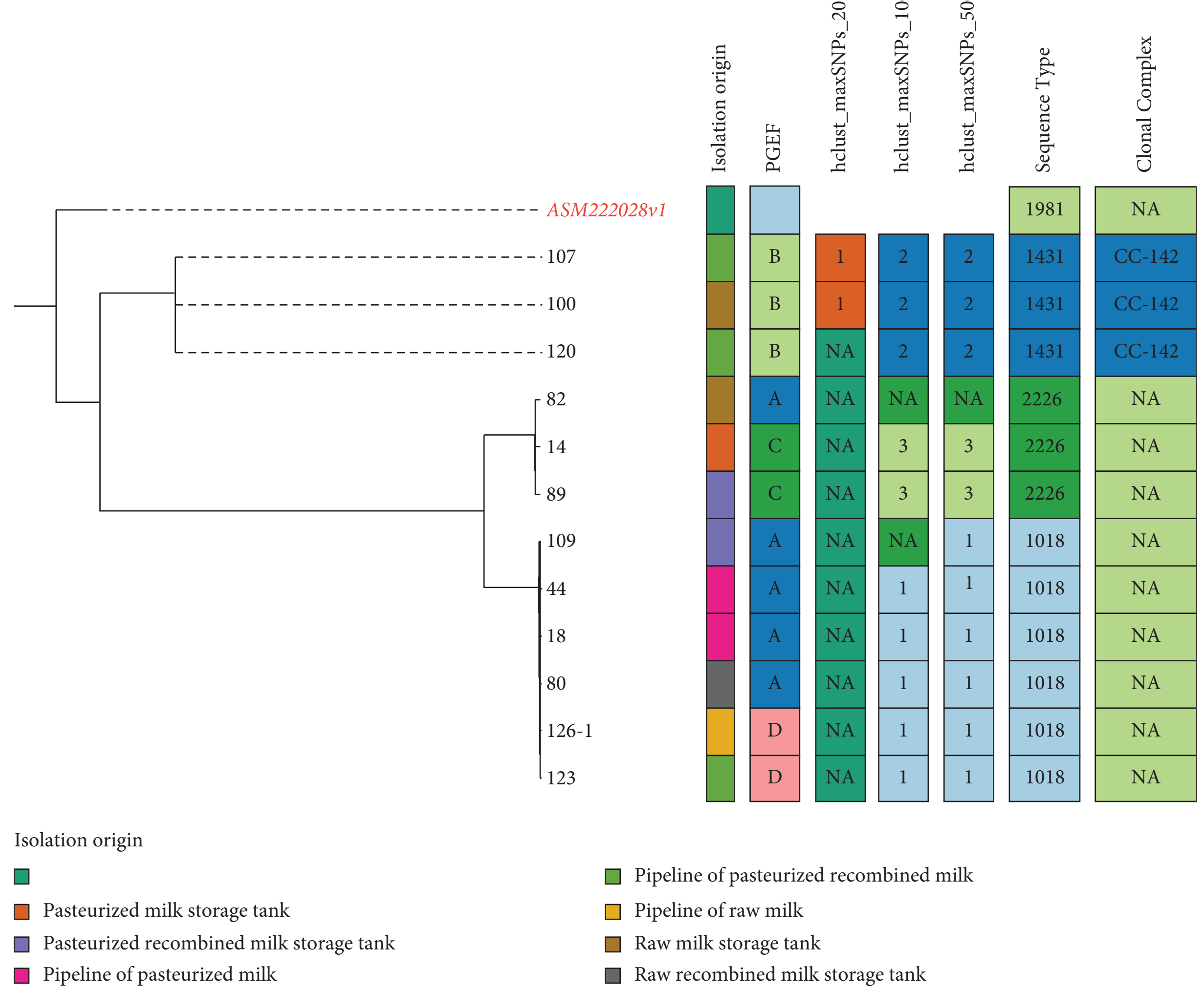


Figure 2: Phylogenetic relation and genotypes of 12 *Bacillus cereus sensu lato* isolated in this study rooted to the reference genome with regard to the sequence types and clonal complex.

Table 4: Genetic characterization of antimicrobial resistance of the tested *Bacillus cereus sensu lato*.

| Strain code | *bla*1/2 ($\beta$-lactamase) | *Fos*B (fosfomycin resistance) | *mph*L (macrolide resistance) | *sat*A/B (streptothricin resistance) | vanZ/F (vancomycin resistance) | *bla*Z (penicillin resistance) |
|---|---|---|---|---|---|---|
| 14 | + | + | + | − | + | − |
| 18 | + | + | + | + | + | − |
| 44 | + | + | + | + | + | − |
| 80 | + | + | + | + | + | − |
| 82 | + | + | + | − | + | − |
| 89 | + | + | + | − | + | − |
| 100 | + | + | − | + | + | − |
| 107 | + | + | − | + | + | − |
| 109 | + | + | + | + | + | − |
| 120 | + | + | − | + | + | − |
| 123 | + | + | + | + | + | − |
| 126-1 | + | + | + | + | + | − |
| Total | 12 (100%) | 12 (100%) | 9 (75%) | 9 (75%) | 12 (100%) | 0 |

II and VI are psychrotrophic, microorganism of groups III, IV and VII are mesophilic and those of group V have an intermediate behavior [15]. Group III seems to be involved in the highest risk of food-borne diseases, while groups VII, IV and II correspond to a moderate risk, and finally group VI represents a very low risk. The strains belonging to group III are mostly cytotoxic, and this group particularly includes the emetic strains [55, 56].

Most isolates screened in the current study belong to Group III, thus indicating that their presence in the milk environments constitutes a serious safety issue. A smaller amount of group IV isolates as compared to Group III was noticed which could be explained by the fact that isolates of group IV are rather sensitive to heat treatments in comparison to those in group III [57, 58]. The strains that are highly toxic and are mostly involved in food-borne disease belong to mesophilic groups III and IV, that is, consistent with the results of the present study [59].

The existence of *B. cereus* in dairy plants can be attributed to different sources such as the cow udders, collecting containers, transportation systems, and milk powder in the case of recombined milk [60]. Their presence in the processing equipment demonstrates that the cleaning and disinfection procedures have not been performed carefully on the production equipment [61] or that the methods and products used are not efficient enough. In a previous study, the spores of the isolates herein investigated were shown to have different lengths of appendages, the surface of the exosporium and spore hydrophobicity which enable these spores to adhere to stainless steel surfaces [32].

In order to trace the sources and comprehend the epidemiology of food-borne pathogens, a number of molecular characterization methods are used such as random amplification of polymorphic DNA (RAPD) [62], multilocus sequence typing (MLST) [63] and amplified fragment length polymorphism (AFLP) [64]. Among these methods, pulse-field gel electrophoresis (PFGE) is an effective way to clearly analyze the genetic variation among strains and the relations between phenotypic and genotypic features [65]. The fingerprints gained by PFGE allow us to observe the presence of similar isolates in different samples, thus suggesting a cross-contamination with similar strains across the production chain.

The findings of this study revealed that pathogens can create a recontamination risk on stainless steel surfaces for a long time because of their ability to survive there. Besides, the outstanding food on these surfaces may well encourage the bacterial survival. Hence, two major aspects are responsible for the risk of food-borne illnesses: the degree of contamination on the surfaces and the possibility of its transmission to food [66].

Furthermore, since milk packaging is commonly performed using very clean, but not strictly aseptic fillers, the danger of postpasteurization recontamination of processed milk is considered [67]. The factors that determine the shelf life of pasteurized milk include the existence and evolution of aerobic psychrotrophic endospore formers where members of *B. cereus* are key spoilers beside other species of the genus *Bacillus.* The resistance of endospores to the pasteurization process reduces the shelf life of the pasteurized milk through the underlying germination and the production of spoilage enzymes [68], such as lipases (SMase, PI-PLC, PC-PLC) and proteases, can lead to food degradation and spoilage, thus economically impacting the food produced [69, 70]. In a previous study, all strains were shown to possess proteolytic and lipolytic activities [32].

Contamination of dairy processing plants by *B. cereus* constitutes a serious health hazard for the consumers, since this microorganism is mainly responsible for food-borne diseases [13]. The pathogenicity of *B. cereus* is primarily due to its ability to secrete a plethora of toxins [71, 72].

The high occurrence of the *nhe* genes observed is not unusual, however, the occurrence of the *hbl*C gene is quite low compared to that reported (45%–65%) in previous studies on *B. cereus* isolates from other origins [73, 74]. Consequently, based on the various published literature on this matter [18, 73], the amount of strains holding only *cyt*K is assumed to be fairly limited, yet the latter (*cyt*K) might well be toxic [22]. *Bacillus cereus* 14 did not exhibit *hbl*C or *sph* that are linked to hemolysis potential, suggesting that other factors are associated to such a characteristic. In fact, several hemolytic factors are expressed in the *B. cereus* group

of bacteria such as cereolysin [75], sphingomyelinase [76], cereolysin AB [77] and cereolysin-like hemolysin [78].

Although few emetic toxin encoding genes are found in dairy food [79, 80]. In this study, *B. cereus* revealed no inclusion of the emetic toxin encoding gene. In controversy, these genes were detected in *B. cereus* in 10.2%; [81]; 9%, [82]; 1.0–3.8%, [80]; 2%, [83]; and 1.1% [84]. The isolates used in the present work revealed no inclusion of the emetic toxin encoding gene.

In addition, nonhaemolytic enterotoxin (Nhe), pore-forming cytotoxins hemolysin BL (Hbl) and cytotoxin K (CytK) were found to be the reasons behind the diarrheal disease [22]. Hence, in the present work, 10 enterotoxigenic genes with the following proportions (*nhe*A (88.2%), *nhe*B (94.1%), *nhe*C (94.1%), *cyt*K (41.2%), *hbl*C (29.4%), *sph* (88.2%), *ent*FM (100%), *ent*S (100%), *plc*A (94.1%) and *ent*T (35.3%) were detected. Such proportions are similar to those detected in previous studies conducted in France (*nhe* 96%, *hbl* 40% and *cyt*k 42%) [85] and in China (*nhe* 100% and *hbl* 78.3%) [86]. However, the proportions found in a Turkish study were relatively lower (*hb*l 13%, *nhe* 60% and *cyt*k 75%) [87].

The findings of the present research show that the *B. cereus* investigated in this study are likely to represent a significant health risk because these isolates contain the enterotoxin gene and have the ability to adhere to stainless steel surfaces. In addition, the presence of proteolytic and lipolytic enzymes in these isolates increases the chances of cross-contamination and hinders the decontamination of food industry surfaces.

In this study, the detection of genetic factors for AMR was performed using different tools and databases (AMR-Finder, ResFinder and NCBI) in order not to miss any loci, as there is no single method that might be sufficient for the purpose alone (Table 4) and we found that AMRFinder appears to be a highly accurate AMR gene detection system, as previously mentioned [88].

Therefore, it is crucial to include *B. cereus* group of isolates in routine food quality control in laboratories in this region of Algeria. Also, evaluating and upgrading the cleaning and disinfecting procedures of the production materials in the milk processing environment are helpful ways to avoid economic losses and detrimental health consequences for consumers. Finally, eliminating *B. cereus* in the food processing industries requires taking the appropriate hygiene measures. These measures include both the availability of sufficient cleaning equipment and the reassurance of the employees' personal hygiene, and importantly, increasing the points of control with a regular check of the sanitization products and the temperature of the pasteurization process [24].

## 5. Conclusion

While the health risks resulting from the recontamination of pasteurized milk by *B. cereus* adhered to pipelines are well known, little attention has been given to molecular analysis of genotypes and virulence in the Algerian diary industry. In the present work, we highlighted the high virulence factors of *B. cereus* in the pipeline of dairy plant in Algeria. The *pan*C and PFGE analyses clearly showed the genetic diversity of *B. cereus* isolated in this study. To the best of our knowledge, this is the first study concerned with the analysis of virulence factors and definition of genotypes by *Pan*C and PFGE of different *B. cereus* isolates from the pipeline of the dairy industry in Algeria that revealed a possible high risk of *B. cereus* to the dairy industry and public health. From food safety perspectives, the presence and pathogenicity of *B. cereus* in the dairy industry play a key role and should not be disregarded.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Ethical Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the Medical Ethics Research Committee of the Yahia Farès University, Urban Pole, Médéa, Algeria, (protocol code 037/LCEPC/2010 at 08.02.2010).

## Conflicts of Interest

The authors declare that there are no conflicts of interest.

## Authors' Contributions

Nassima Didouh curated the data. Nassima Didouh, Francesco Celandroni, Emilia Ghelardi, Diletta Mazzantini, Florence Postellec, Boumediene Moussa-Boudjemaa, L. Irène I Ouoba, Jörg Linde, and Hosny El-Adawy performed an investigation. Nassima Didouh, Francesco Celandroni, Emilia Ghelardi, Diletta Mazzantini, Florence Postellec, Rachid Achek, Boumediene Moussa-Boudjemaa, and L. Irène I Ouoba proposed a methodology. Emilia Ghelardi and Boumediene Moussa-Boudjemaa supervised the study. Nassima Didouh wrote the original draft. Francesco Celandroni, Emilia Ghelardi, Diletta Mazzantini, Florence Postellec, Boumediene Moussa-Boudjemaa, L. Irène I Ouoba, Herbert Tomaso, Jörg Linde, and Hosny El-Adawy wrote, reviewed, and edited the manuscript.

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