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Original Article

Prevalence and Identification of *Bacillus cereus* in Some Dried Dairy Products Focuses on its Toxigenic Genes

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Abstract: *Bacillus cereus* is a spore-forming, toxin-producing bacterium. It has significant food safety risks, especially in dried dairy products, including powdered and infant milk. This may pose a risk to the consumers. The current study aimed to show the prevalence and toxigenic potential of *B. cereus* in 134 milk samples. They were collected randomly from food stores and various sale points in the local markets from Sulaymaniyah and Halabja provinces between November 2024 and May 2025. Bacterial isolation was performed using Mannitol egg Yolk Polymyxin agar. The isolates were then confirmed by biochemical assays, VITEK 2, and Polymerase Chain Reaction amplification of *gyrB* and 16S rRNA genes. Phylogenetic analysis revealed close genetic clustering of the isolates with reference strains of *B. cereus*, *B. thuringiensis*, and *B. tropicus*. Antimicrobial susceptibility testing using the disk diffusion method presented complete resistance to β-lactam antibiotics. However, all isolates remained susceptible to ciprofloxacin, tetracycline, erythromycin, and vancomycin. Of the tested samples, *B. cereus* was detected in 48% of different types of powdered milk and 11.7% of infant milk samples. Virulence gene analysis displayed high prevalence rates of enterotoxins: *nheA* (100%), *nheB* (80.76%), *cytK* (86.53%), *hblA* (75%), and *hblC* (88.46%). While the emetic toxin gene *ces* was not detected in any milk samples. In conclusion, the presenting of multi-virulent and β-lactam-resistant *B. cereus* in dried milk reinforce the need for improved hygiene during dairy processing. Future studies can employ whole-genome sequencing approaches to better understand the genetic diversity, and virulence mechanisms in *B. cereus* from dairy environments.

1. Introduction

The foodborne pathogenic bacterium *Bacillus cereus* is a rod-shaped, Gram-positive, aerobic or optionally anaerobic bacterium belonging to the Bacillaceae family [1]. It is more commonly present in natural environments and is often available in foods, particularly dairy samples, as well as cooked dishes [2]. The presence of *B. cereus* in food is typically associated with food spoilage [3]. Other *Bacillus* species, including *B. anthracis, B. weihenstephanensis, B. mycoides, and B. thuringiensis,* are closely related. Pasteurization and other traditional heating methods cannot kill *Bacillus* spores. Certain *bacilli* have been reported to withstand cooking [4]. Owing to their widespread resistance to several antimicrobials, *B. cereus* strains with multiple antibiotic resistances are regarded as risk factors for treating infections

[5]. *B. cereus* is thought to be the most common infectious *Bacillus* species discovered in raw milk during dairy processing and storage. These microbes are also robust and widespread spore-formers. Many of these are psychrotolerant [6]. Biofilms can survive in production lines and storage facilities because they act as barriers that protect bacteria from sterilization procedures and cleaning agents [7]. This persistence increases the likelihood of contamination in processed foods, especially those that undergo multiple handling stages, such as dairy products and ready-to-eat items (sandwiches, salads, and sushi) [8]. The capacity of bacteria to create spores is of special concern in powdered milk and infant formula. This is because spores can remain in low-moisture products and proliferate later, posing a serious risk to food safety [8].

Diarrheal and emetic characteristics are the two main forms of food poisoning related to the well-known foodborne bacterium *B. cereus* [2]. The generation of enterotoxins such as hemolysin BL (Hbl), cytotoxin K (CytK), and non-hemolytic enterotoxin (Nhe) causes diarrheal syndrome. After consuming polluted food, these poisons are created in the intestine.[10]. However, emetic sickness results from the consumption of cereulide. It is a cyclic peptide toxin that affects serotonin receptors and vagus nerve. It can withstand cooking and food processing owing to its high thermal stability [9]. Although most cases are mild and resolve on their own, vulnerable individuals experience serious consequences. Under these conditions, liver failure, which underscores the vital significance of keeping the eye out, is predominant [10].

Milk powders have a long shelf life without significantly sacrificing the quality, convenience, or nutritional content. Therefore, they are crucial to global food supply networks [11]. As *B. cereus* spores are more widespread and resilient than vegetative cells, they are a major source of concern for the global dairy industry. They can withstand a variety of environmental stresses including heat, desiccation, freezing, and thawing. Although the origin of the contamination has not been determined, spores in milk can appear when cows are housed indoors. The following potential sources of contamination were considered: soil, dung, bedding, feed, air, and the milking apparatus. Feces and bedding materials can infect teats during the housing phase [12]. Milking materials such as tanks, tubes, pasteurizers, and packaging machines may be important sources of contamination, because spores can stick to and grow [13]. The ability of *B. cereus* to generate spores makes it highly resistant to stressors frequently encountered during dairy processing, and is primarily responsible for its survival in dry dairy products. For example, *B. cereus* spores can withstand temperatures greater than 100°C. Therefore, pasteurization does not completely destroy these cells, even when it successfully lowers vegetative cells [14].

Routine detection and identification of *B. cereus* in food involves the use of selective solid media, such as mannitol egg yolk polymyxin (MYP) agar. It typically forms pink colonies surrounded by an opaque lecithinase halo (egg yolk precipitate) owing to its lecithinase activity [15]. Additional routine identification of *B. cereus* isolates includes catalase and oxidase tests, Voges-Proskauer reaction, starch hydrolysis, evaluation of motility, hemolysis, lecithinase activity, evaluation of lactose fermentation, spore staining, and automated phenotypic identification with the VITEK 2 (BCL) card may also be employed [16]. Polymerase chain reaction (PCR) enables the detection of enterotoxins and emetic toxin genes in *B. cereus*. It improves sensitivity over conventional culture-based methods [17]. Phylogenetic analysis has also been used to resolve genetic relationships within the *B. cereus* group and clarify the links among its members [18].

B. cereus spores can germinate in reconstituted powders. This can threaten the product quality and safety [13, 19]. Clinically, *B. cereus* causes emetic intoxication due to cereulide and diarrheal toxicoinfections. The latter is mainly linked to tripartite enterotoxins Hbl, Nhe, and CytK [14, 20, 21]. Surveys on milk powder and other dairy matrices have frequently recovered *B. cereus*. A broad distribution of enterotoxin genes (*nhe*, *hbl*, *cytK*) was observed. In contrast, the emetic gene (*ces*) is less common in dairy contexts [5, 22-24]. European risk assessments similarly urge *B. cereus* in powdered infant formula to remain at the level of the bacterium as low as reasonably achievable [25]. Based on this and the lack of up-to-date regional data, we studied retail milk powders marketed in the Halabja and Sulaymaniyah provinces to determine the prevalence of *B. cereus*. In addition, the distribution of key toxigenic markers, such as *nhe*, *hbl*, *cytK*, and *ces*, and antimicrobial susceptibility profiles were recovered. Together,

these aims fill a recognized local evidence gap and interpret our findings in light of global concerns about dairy-powder safety.

2. Materials and Methods

2.1. Sample Collection

This study had a cross-sectional design. In this study, 20 powdered trademark milk and 10 infant trademark milk samples were tested. A total of 134 samples were collected from these milks, including 100 powdered milk and 34 infant formula samples, which were randomly collected from various retail outlets across the Sulaymaniyah and Halabja provinces between December 2024 and May 2025.

2.2. Sample Preparation, Isolation and Identification

Ten grams of milk were added to 90 mL of buffered peptone water, and the mixture was homogenized in a blender for 1-2 mins. The mixed sample (10 mL) was placed in a water bath and incubated at 80–85°C for 10 mins to destroy vegetative cells and fungi in the sample. The samples were subsequently cooled in cold water. They were placed in a shaking incubator at 37°C and 150 rpm for 4 hrs to activate the *B. cereus* spores. Afterward, 0.1 ml of the sample was spread onto MYP agar and incubated at 33–35°C for 24–48 hrs. The number of bacterial cells was then counted. *B. cereus* colonies were identified as pink colonies. They were surrounded by egg yolk-like precipitates or a cloudy halo due to lecithinase production. In addition, there was a precipitation zone in the MYP. *B. cereus* isolates were identified using Gram staining. Biochemical tests and amplification and sequencing of specific genes [19, 20].

2.3. Biochemical Tests for Identification of B. cereus

Two methods were used for studying the biochemical tests; one of them is manual, and the other is VITEK2 (BCL), (Densi Chek, version 9.02.3). The first category includes catalase, oxidase, Voges-Proskauer, starch hydrolysis, motility, hemolysis, lecithinase, lactose fermentation, and spore staining. VITEK2 (BCL) was used for the second method. After isolating the bacterial colonies on the MYP medium and identifying the bacterial colonies, pure bacterial cultures were grown on nutrient agar. The cells were then incubated at 34°C for 18-24 hrs. A single colony was transferred to 3 mL of sterile saline and subjected to a McFarland turbidity range of 0.5 for utilization by VITEK 2 (BCL). The VITEK vacuum chamber of BCL cards was wadded automatically, incubated at 35°C in a VITEK 2 (BCL) compact instrument, and read automatically after 13.87 hrs [26].

2.4. PCR Assay for the Detection of Pathogenic B. cereus

2.4.1. DNA Extraction

Suspected isolates were refreshed for 18–24 hrs at 34°C in a nutritional broth. DNA was extracted from 5 ml of incubated broth of each isolate using a Genomic DNA Extraction kit (Fla Pure Bacteria Genomic DNA Extraction Kit; Cat. No. DE703-50) according to the manufacturer's protocol. DNA samples were placed at 2-6°C until further use.

Oligonucleotide primers targeting *gyrB* and 16S rRNA for *B. cereus* confirmation and *hblA*, *hblC*, *nheA*, *nheB*, *cytK*, and *ces* for virulence profiling were used as previously described (Table 1).

Table 1: Oligonucleotide primer sequences to find *B. cereus* and its virulence genes.

Reference	Target	Nucleotide sequence	Product size (bp)
[5]	*gyrB*	F-5′ ATTGGTGACACCGATCAAACA -3′	365
		R-5′ TCATACGTATGGATGTTATTC -3′	
[27]	16S rRNA	F-5′ TCGAAATTGAAAGGCGGC′3	288
		R-5′ GGTGCCAGCTTATTCAAC′3	
[28]	*hblA*	F-5′ GTGCAGATGTTGATGCCGAT′3	320
		R-5′ ATGCCACTGCGTGGACATAT′3	
[29]	*hblC*	F-5′ AATGGTCATCGGAACTCTAT′3	731
		R-5′ CTCGCTGTTCTGCTGTTAAT′3	
	nheA	F-5′ TACGCTAAGGAGGGGCA′3	499
		R-5′ GTTTTTATTGCTTCATCGGCT′3	
	nheB	F-5′ CTATCAGCACTTATGGCAG′3	770
		R-5′ ACTCCTAGCGGTGTTCC ′3	
	cytK	F-5′ CGACGTCACAAGTTGTAACA ′3	565
		R-5′ CGTGTGTAAATACCCCAGTT ′3	
[28]	*ces*	F-5′ GGTGACACATTATCATATAAGGTG′3	1271
		R-5′ GTAAGCGAACCTGTCTGTAACAACA′3	

2.4.2. Application of PCR

The PCR was performed using 2×GS Taq Master Mix (Code No. ST211), and the primer sequences are listed in table 1. The PCR master mix component was used for the PCR detection of specific and virulence genes. prepared as follows: 12.5 µL 2×GS Taq PCR Master Mix, 1 µL of forward primer (10 pmoL), 1 µL of reverse primer (10 pmoL), and 2 µL of template DNA. The final volume was adjusted to 25 µL by adding 8.5 µL of nuclease-free water. The amplification profile was standardized. Denaturation was performed at 94℃ for 30 s, annealing at 61℃ for 30 s, and extension at 72℃ for 30 s, followed by 30–35 cycles. The Final extension was performed at 72 ℃ for 5 mins and held at 4℃. PCR amplifications were performed by gel electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light. The PCR was performed using 2×GS Taq Master Mix (Code No. ST211), with the components and the primer sequences listed in table 2.

Table 2: PCR master mix component used for PCR reaction for detection of specific and virulence genes.

PCR mixture	Volume
2×GS Taq PCR Master Mix (2x premix)	12.5 µL
Forward primer (10 pmoL)	1.0 µL
Reverse primer (10 pmoL)	1.0 µL
Template DNA	2.0 µL
Nuclease free water	8.5µl
Total volume mixture	25 µL

2.5. Antimicrobial Susceptibility Test

The disk diffusion method was used on Mueller-Hinton agar. After growth, *B. cereus* samples were adjusted to 0.5 McFarland standard and distributed onto the agar surface to conduct an antibiotic susceptibility test. Antibiotic disks including amoxicillin, ciprofloxacin, tetracycline, and vancomycin were placed on the plates. The cells were incubated for 18–24 hrs at 35°C. Inhibition zones were measured in millimeters [30].

2.6. Sequence Characterization and Phylogenetic Analysis

Amplified DNA fragments were sequenced using Sanger sequencing (Macrogen Co., Korea). The obtained sequences were aligned using Clustal Omega for multiple sequence comparisons and further

analyzed using the National Center for Biotechnology Information (NCBI) BLAST tool to identify identities with known nucleotide sequences (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using the MEGA-X software to show evolutionary relationships.

2.7. Statistical Analysis

Bacterial enumeration results are presented as the average count of colony-forming units per gram (CFU/g). The prevalence of *B. cereus* and its related virulence genes was expressed as the percentage of positive samples in all tests. The inhibition zone diameters (mm) with standard deviations are given as mean antimicrobial susceptibility profiles. The study was descriptive and the findings are presented in tables and figures for comparison.

3. Results

This study confirmed the species-level identification of *B. cereus* in powdered and infant milk samples from Sulaymaniyah and Halabja provinces. It detects virulence genes, assesses antimicrobial resistance, and analyzes phylogenetic relationships using the *16S rRNA* and *cyrB* sequences.

3.1. Identification and Prevalence of the B. cereus in Milk samples by Cultural and Microscopic Techniques

Initially, colonies grown on MYP agar formed pink colonies with a surrounding cloudy halo due to lecithinase activity (Figure 1-A). Gram staining showed that the bacterial strains were gram-positive and rod-shaped (Figure 1-B). Additionally, spore staining with malachite green confirmed the presence of green endospores, which are the hallmarks of *B. cereus* (Figure 1-C). The mean number of bacteria was 2.98×10^3 CFU/g in powdered milk and 2.2×10^3 CFU/g in infant milk.

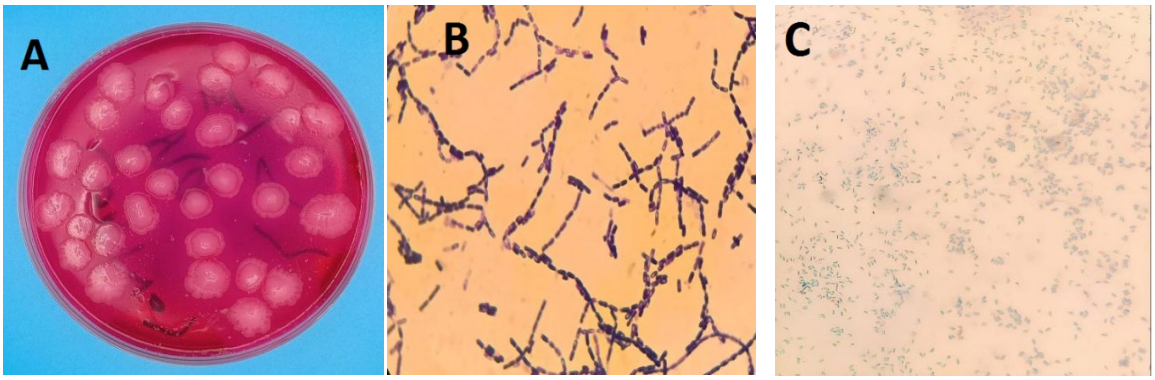

Figure 1: Demonstrate the identification of *B. cereus* by cultural and microscopic techniques. On MYP selective agar (A), colonies appear as pink with an opaque halo. Gram staining (B) presents Gram-positive, rod-shaped cells (100X), while malachite green spore staining (C) demonstrates the presence of green-stained endospores, characteristic of *B. cereus*.

3.2. Biochemical Tests for Manually Identifying B. cereus

Biochemical assays and VITEK 2 (BCL) analyses were used to confirm the identification of *Bacillus cereus* isolates. The microbes were catalase-positive (bubble production), oxidase-negative (no color change), capable of hydrolyzing starch (clear zones), and motile and lecithinase-positive (opaque zones). In addition, it was positive for the Voges-Proskauer test (color change) but negative for lactose fermentation, indicating its inability to ferment lactose (Table 3). The table summarizes important test data for most essential bacterial characteristics, enzyme activity (catalase, oxidase, and lecithinase), and metabolic functions (starch hydrolysis, lactose fermentation, and Voges-Proskauer. These results confirmed the presence of *B. cereus*. The VITEK 2 (BCL) system further verified the species-level identification. The probability of correct identification ranges from 79% to 96%. The use of this system allowed for rapid, standardized, and highly reproducible results, and strengthened the reliability of the species confirmation process in this study.

Table 3: Biochemical tests were done for the confirmation of *B. cereus*.

Test	Result
Catalase	Positive, Bubbles
Oxidase	Negative, no.color change
Starch hydrolysis	Positive, Clear zone
Lecithinase test	Positive, Opaque zone
Lactose fermentation	Negative, no ferment
Voges-poskauer test	Positive, Color change

3.3. Identification of B. cereus using PCR-based Methods

After examination of the above and molecular identification and characterization of *B. cereus* using PCR-based methods, 48 samples (% 48) were positive for *B. cereus* in 100 powdered milk samples and four samples (%11.7) were positive for *B. cereus* in 32 infant samples.

3.3.1 Identification of Pathogenic B. cereus by PCR Amplification of gyrB and 16S rRNA genes

Biochemically positive strains were confirmed to be *B. cereus* by PCR amplification of crucial *16S rRNA* and *gyrB* genes. Agarose gel electrophoresis of the PCR products showed that the tested isolates formed specific bands at 365 bp for *gyrB* (Figure 2-A) and 288 bp for *16S rRNA* (Figure 2-B). These results are consistent with the expected fragment sizes. The positive control (lane 14) showed similar band sizes, whereas the negative control (lane 15) showed no amplification.

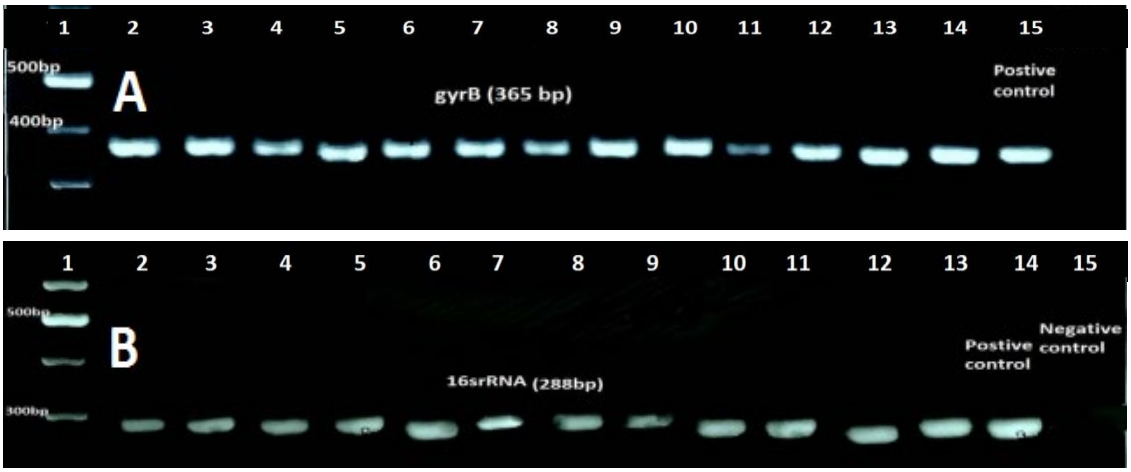

Figure 2: Gel electrophoresis analysis of PCR amplification for the *gyrB* gene (A) and 16S rRNA gene (B). Lane 1 contains a molecular weight DNA ladder (1500–100 bp). Lanes 2–14 of figure 2 (A) show positive isolates of *B. cereus*, each showing a specific amplicon band at 365 bp. It is confirming the presence of the *gyrB* gene. Lane 15 in figure 2 (A) and lane 14 in figure 2 (B) show the positive control (*B. cereus* ATCC 21768) with the same expected band. Lane 15 in figure 2 (B) serves as the negative control. It is presenting no amplification. Lanes 2–13 of figure 2 (B) represent positive isolates of *B. cereus*. They are showing a distinct amplification band at 288 bp. It is indicating the availability of the 16S rRNA gene. Lane 14 is the positive control (*B. cereus* ATCC 21768) with the expected band, and lane 15 is the negative control showing no amplification.

3.3.2 Identification of Pathogenic B. cereus Based on Detecting Virulence-associated Genes

The availability of six virulence-associated genes was examined to determine the pathogenic potential of each bacterial species. The first group was comprised of three enterotoxin genes (*hheA*, *nheB*, and *cytK*), the second group consisted of two hemolysin genes (*hblA* and *hblC*), and the third group was an emetic toxin gene (*ces*). The PCR products for the toxin genes displayed specific band patterns that corresponded to the expected amplicon sizes. As shown in figure 3 (A), lanes 2–6 represent amplification of the *nheA* gene with bands 499 bp. which confirmed the presence of non-hemolytic enterotoxin A. Lanes 7–11 showed bands at 770 bp, confirming the presence of the *nheB* gene. Lanes 12–16 presented 565 bp bands for *cytK*. These data indicated the widespread distribution of diarrheal toxin genes in *B.*

cereus isolates. Figure 3 (B) shows the amplification profiles of the two hemolysin genes, *hblA* (lanes 2–5, 320 bp) and *hblC* (lanes 6–9, 731 bp), with the emetic toxin *ces* gene (lanes 10-13, at 1271 bp). Interestingly, although the majority of the tested *B. cereus* isolates contained *hblA* and *hblC*, none of them tested positive for *ces*. This indicated a low ratio of the emetic toxin gene in *B. cereus* strains from milk sources in the current study.

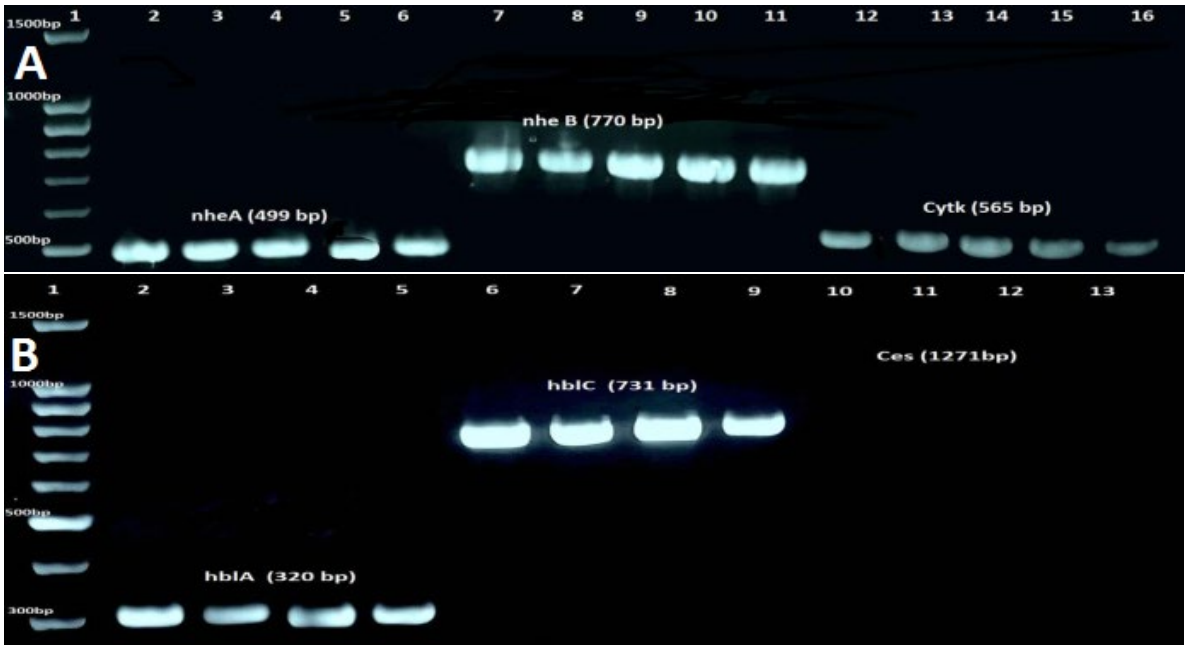

Figure 3: Gel electrophoresis examination of PCR products of virulence genes: *nheA*, *nheB*, and *cytK* (A), and *hblA*, *hblC*, and *ces* (B) of *B. cereus*. Lane 1 presents a DNA molecular weight ladder (1500–100 bp). In figure 3 (A): Lanes 2–6 present positive amplification of the *nheA* gene with bands at 499 bp. Lanes 7–11 show positive isolates for the *nheB* gene (770 bp). Lanes 12–16 present amplification of the *cytK* gene (565 bp), while lanes 2–5 of figure 3 (B) present positive amplification of the *hblA* gene, with bands at 320 bp. Lanes 6–9 show positive results for the *hblC* gene (731 bp). Lanes 10-13 display no amplification. This is displaying negative results for the *ces* gene (1271 bp).

3.4 Distribution of Toxigenic Genes in Powdered and Infant Milks

Table 4 summarizes the distribution of virulence genes in *B. cereus* in milk samples. All isolates (100%) tested positive for *gyrB*, 16S rRNA, and *NheA*. This indicated the conserved nature of these genes among species from different sources of dairy products. The *nheB* gene was found in 81.25% of powdered milk isolates and 75% of infant milk isolates. *cytK* was present in a high proportion (85.4%) of powdered milk isolates and in 100% of infant milk isolates (86.53%). While *hblA* was observed at a slightly lower rate of 77% in powdered milk and 50% in infant milk isolates, *hblC* was detected in 89.58% of powdered milk isolates and 75% of infant milk isolates. However, *ces* was not present in any of the samples. These data highlight the high prevalence of diarrheal toxin genes compared to emetic toxin genes in dried milk.

Table 4: Gene markers and virulence gene distribution among isolated *B. cereus* from dried dairy products**.** Among 100 tested powdered milks, 48 milks contained *B. cereus*, and among 32 tested infant milks, 4 milks contained *B. cereus*.

Gene	Powder milk (n=48)	Infant milk (n=4)	Total (n=52)
gyrB	48 (100%)	4 (100%)	52 (100%)
16srRNA	48 (100%)	4 (100%)	52 (100%)
nheA	48 (100%)	4 (100%)	52 (100%)
nheB	39 (81.25%)	3 (75%)	42 (80.76%)
cytk	41 (85.4%)	4 (100%)	45 (86.53%)
hblA	37 (77%)	2 (50%)	39 (75%)
hblC	43 (89.58%)	3 (75%)	46 (88.46%)
ces	0 (0%)	0 (0%)	0 (0%)

3.5 Phylogenetic Analysis Based on 16S rRNA and gyrB genes

Phylogenetic analysis was performed to assess the genetic relatedness between the *B. cereus* isolates and the reference *Bacillus* species, based on 16S rRNA and *gyrB* gene sequences. The resulting maximum-likelihood trees are shown in figure 4. The 16S rRNA phylogenetic tree (Figure 4-A) showed that the *B. cereus* isolate clustered tightly with other *B. cereus* reference strains, including MK910262 and MH588136. It also clustered with closely related species such as *B. thuringiensis*. This provides a strong evolutionary correlation within the *B. cereus* group. Comparably, the *gyrB*-based tree (Figure 4-B) showed close genetic proximity between the test isolate and known *B. cereus* strains P00414611 and AF136388. The tree also includes other species of *B. tropicus* and *B. thuringiensis* branches. However, they exhibit higher divergence values. This reinforces the discriminatory power of *gyrB* for identifying species within the genus *Bacillus*.

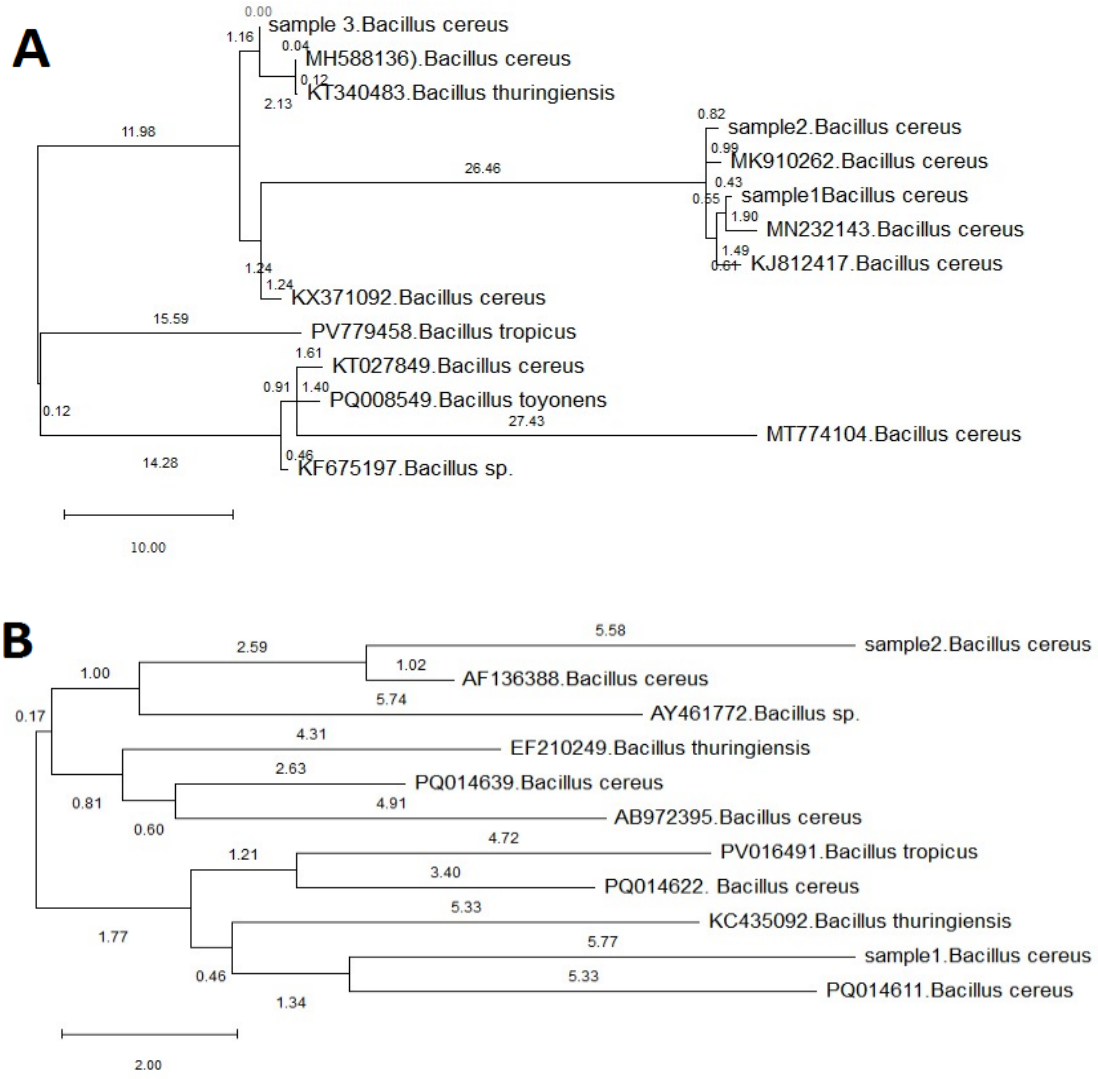

Figure 4: Correlation between *B. cereus* isolate (labeled as sample. *Bacillus cereus*) and other closely related *Bacillus* species is presented by maximum likelihood phylogenetic tree. The tree is according to nucleotide sequence alignment of PCR-amplified 16S rRNA (A) and *gyrB* gene (B). Bootstrap values and evolutionary distances are shown at the nodes and branches, respectively. They are highlighting the genetic identity between the isolate and reference strains, such as *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. tropicus*.

3.6 Antimicrobial Susceptibility Profiles

The bacterial strain profiles were examined using disc diffusion. *B. cereus* isolates showed complete resistance to β-lactams, including amoxicillin, ampicillin, and penicillin G (Table 5 and Figure 5). This demonstrates the availability of β-lactamase activity and decreased susceptibility to cell wall synthesis inhibitors. In comparison, none of the bacterial strains was resistant to other antibiotics, including ciprofloxacin (30.1 mm), gentamicin (23.2 mm), chloramphenicol (23.9 mm), erythromycin (28.7 mm), tetracycline (25.2 mm), and vancomycin (17.1 mm). These data indicate that while β-lactam antibiotics may not be effective in treating *B. cereus*, alternative antibiotics, including fluoroquinolones and glycopeptides, may play an effective role.

Table 5: Antibacterial susceptibility profile of *B. cereus* strains from powdered and infant milk food samples**.** Different antibiotics were tested against the bacterium following the disc diffusion method to present resistance or susceptibility based on zone diameters (in mm).

Antimicrobial Test	Zone Diameter (mm) ± standard deviations	Interpretation
Amoxicillin	**4.3 ± 4.76**	Resistant
Ampicillin	0 ± 0	Resistant
Penicillin-G	0 ± 0	Resistant
Ciprofloxacin	30.1 ± 2.9	Susceptible
Gentamicin	23,2 ± 2.64	Susceptible
Chloramphenicol	23.9 ± 1.22	Susceptible
Erythromycin	28.7 ± 2.28	Susceptible
Tetracycline	25.2 ± 1.84	Susceptible
Vancomycin	17.1 ± 1.66	Susceptible

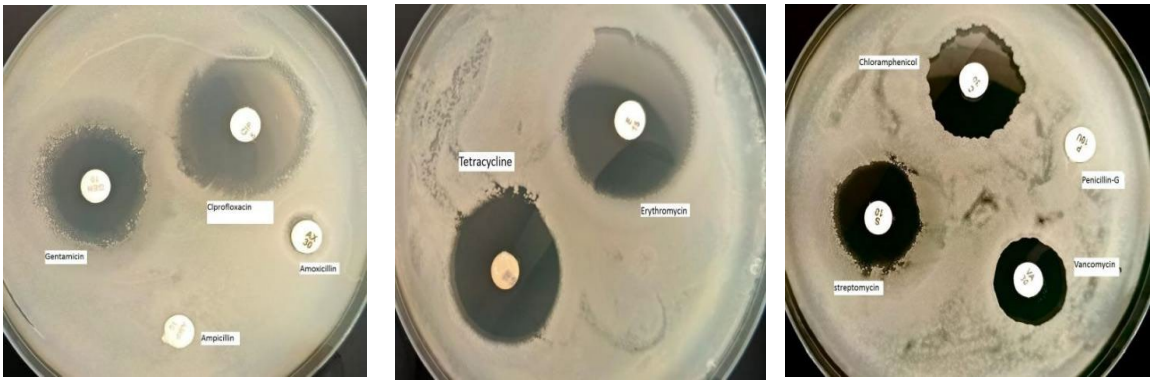

Figure 5: Representative images of the antibacterial activity of different antibiotics against *B. cereus.* The test was performed using the well diffusion method. Clear zones of inhibition around each well indicate the effectiveness of the antibiotic in suppressing bacterial growth.

4. Discussion

This study demonstrated a significant distribution of *B. cereus* in powdered milk-dried dairy products, with a particular focus on its toxigenic gene profile and antibacterial susceptibility patterns. The results underscore not only the availability of the bacterium in powdered and infant milk samples available in the local markets of Sulaymaniyah and Halabja but also raise critical concerns regarding food safety, public health, and the need for stringent hygiene and monitoring protocols in dairy production.

In this study, 48% of powdered milk and 11.7% of infant milk formula samples tested positive for *B. cereus*. These data are in agreement with those of other studies that have shown the widespread contamination of dried dairy products. Kumari and Sarkar [31] reported that 52% of the dairy samples

in India contained *B. cereus*. In contrast, Giffel *et al.* [32] reported contamination rates ranging from 15 to 75% in milk powders. The high proportion of these dairy products may be related to the ability of the bacteria to produce spores that persist after pasteurization and other thermal processing methods [33]. The powdered milk was passed through several processing stages. They are prone to contamination, particularly during post-pasteurization, handling, drying, and packaging [1]. Furthermore, this can also be related to the formation of biofilms on the heat exchangers and evaporators. These biofilms contain spores that survive the cleaning-in-place processes and recontamination subsequent product batches [34]. The low proportion of bacteria in infant milk (11.7%) may be related to improved hygienic standards in its production governed by international guidelines. However, even this low rate is alarming because infants are commonly immunocompromised and highly susceptible to foodborne pathogens [35]. Comparable data were obtained from Hashemi *et al.* [36]. reported an 11% contamination rate in infant milk, confirming that stringent production standards do not eliminate the risk of *B. cereus* contamination completely. Another study reported in Egypt a prevalence of 26.7% in powdered infant milk formula contaminated with *B. cereus* [5].

Molecular confirmation using PCR, targeting two important *gyrB* and 16S rRNA genes, validated the initial phenotypic identification of this microbe and resolved members of the *B. cereus* group that can appear biochemically similar during routine testing [5, 37, 38]. All 52 isolates tested positive for *gyrB* and 16S rRNA genes. These genetic markers are well-established for species-level identification. This is because of their high sequence conservation and discriminatory power [28, 29]. In a survey of powdered infant formulas and other ready-to-eat products, Hwang and Park used *gyrB* to confirm *B. cereus* isolates following an initial culture-based screening. This explains why a housekeeping gene marker with a higher phylogenetic resolution than 16S rRNA is valuable in food isolates [37]. Similarly, in dairy powders, Ibrahim *et al.* [5] verified their *B. cereus* panel using *gyrB* amplification as a core confirmation step before downstream characterization of toxigenic profiles and antimicrobial susceptibility. This reinforces the current practice for low-moisture dairy commodities [38]. In this context, the dual-target approach of PCR confirmation using *gyrB* together with 16S rRNA aligns with the above methodologies by combining a conserved bacterial marker with a discriminatory housekeeping gene to confirm phenotype-based identification, and provides a foundation for subsequent toxin gene and antibiotic resistance profiling [5, 37-39].

Virulence-associated genes are associated with a high prevalence of diarrheal symptoms caused by enterotoxins. The non-hemolytic enterotoxin gene *nheA* was found in 100% of the isolates, whereas *nheB* was present in 80.76% of the isolates. The hemolytic enterotoxin genes *hblA* and *hblC* were present in 75% and 88.46% of the strains, respectively. *cytK* was also dominant in 86.53% of the isolates, and the widespread occurrence of *nhe* and *hbl* gene clusters in dairy-derived *B. cereus* isolates has been previously reported [40, 41]. This finding supports their critical role in foodborne diarrhea. Lindback *et al.* [42] showed that *nheA* is the primary contributor to cytotoxicity in intestinal epithelial cells. Furthermore, *cytK* encodes cytotoxin K, which has been implicated in severe diarrhea outbreaks [14]. This gene was also present in a relatively high proportion of the isolates. This indicates the potential for more severe symptoms upon the ingestion of contaminated milk powder. Interestingly, *ces* gene, which encodes the emetic toxin cereulide, was not detected in the isolated bacterium. These data were consistent with those reported in previous studies. Emetic isolates are more often associated with high-carbohydrate food matrices, including rice and pasta, than dairy products [43].

Antibiotic resistance profiling showed that none of the isolates were sensitive to β-lactam antibiotics, including amoxicillin, ampicillin, and penicillin G, but were susceptible to ciprofloxacin, gentamicin, chloramphenicol, erythromycin, tetracycline, and vancomycin. These results are consistent with the findings of Kwarteng *et al.* [44], and Savic *et al.* [45]. reported similar resistance profiles in dairy-derived isolates of *B. cereus*. Resistance to β-lactams is associated with β-lactamase production, which damages antibiotic molecules and renders them ineffective. This property is widespread among environmental *B. cereus* strains and poses a therapeutic challenge. This problem is particularly evident in immunocompromised patients during outbreaks of foodborne diseases. In contrast, susceptibility to fluoroquinolones and glycopeptides such as vancomycin can be used as an alternative treatment for the clinical management of *B. cereus*-related infections [46]. Based on this data, some isolates exhibited

multiple enterotoxin genes and antibiotic resistance. Therefore, their potential impact on public health is substantial. Phylogenetic trees constructed using *gyrB* and 16S rRNA sequences showed that the bacterial strains belonged to the *B. cereus* group. The isolates clustered closely, 98% with *B. cereus* and 93% clustering with *B. thuringiensis*. These data also reinforce the genetic similarity between *B. cereus* strains [10, 47]. *B. thuringiensis* strains are also pathogenic to humans. They share virulence genes with *B. cereus* [28]. The tree also showed the potential role of horizontal gene transfer in the dissemination of virulence and resistance determinants. This aspect requires additional investigations using whole-genome sequencing and comparative genomics to elucidate the evolutionary dynamics of *B. cereus* in different food environments.

The high ratio of enterotoxigenic bacteria in powdered milk confirmed the quick action of the control measures in dairy products. It is essential to target contamination sources at various critical stages at the farm level, during processing, and throughout storage and distribution, because spores of *B. cereus* can resist heat treatment and dry conditions. Contamination risks can be minimized by measures, such as improved equipment sanitation and regular monitoring of microbial loads. In addition, this study presents the role of implementing advanced molecular diagnostics in routine food safety testing. PCR-based identification allows for the rapid and accurate detection of both the organism and its virulence genes. This can significantly enhance the reliability of foodborne risk assessments [48]. Public awareness efforts and industry training programs should be introduced to improve hygiene practices and to understand microbial risks. The results of this study provide crucial insights. However, further research is required to determine the environmental reservoirs and genetic diversity of *B. cereus* in dairy farms. Metagenomic and transcriptomic analyses can reveal the expression dynamics of enterotoxin genes in various environmental conditions.

5. Conclusions

This study showed a high distribution of this bacterium in powdered and infant milk samples from the Sulaymaniyah and Halabja regions. The majority of isolates harbored multiple diarrheal toxin genes. The absence of the emetic gene *ces* potentially indicates a low risk of poisoning in these products. However, the availability of different virulence genes and resistance to β-lactam antibiotics are significant public health concerns. Molecular identification techniques such as PCR for *gyrB* and 16S rRNA genes are effective and reliable for the detection and confirmation of *B. cereus* isolates. These data reinforce the importance of improving sanitation practices and routine microbial screening in dairy industry. Dried dairy products must be free of microbes, because they are crucial for saving infants and immunocompromised individuals. Molecular testing and antimicrobial studies should be prioritized to control the spread of toxigenic and drug-resistant bacterial isolates in the food chain.

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