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Prevalence and virulence factor genes of *Bacillus cereus* isolated from milk and some dairy products

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ABSTRACT

Two hundred and fifty samples (Marketable milk, Ras cheese, Domiati cheese and ice cream), were randomly obtained from various dairy shops, supermarkets and ice cream shops in Assiut City, Egypt, during the period of November 2022 to March 2023. These samples were examined for the detection and counting of *Bacillus cereus*. The positive *B. cereus* isolates were analyzed by PCR to detect 16s rRNA gene for *B. cereus*, cytotoxin K (*cytK*) and emetic gene (*ces*). Out of the 250 examined samples, 14 (5.6%) were contaminated by *B. cereus*. The analyzed ice cream had the highest prevalence (11.53%), followed by Ras cheese samples. The prevalence of *ces* gene recorded in the examined isolates was 57.14%, while *cytK* gene was found in all isolates. Moreover, proteolytic and lipolytic activity investigations revealed that nearly all strains released protease enzyme (92.85%), most of which (64.28%) could produce lipase enzyme.

Introduction

Milk and dairy products serve as excellent nutritious foods; however, they also provide an ideal growth media for various microorganisms that could potentially impact product safety and quality for human consumption. Microorganisms can find their way to milk and milk products through inefficient processing, post-heat treatment, or from the initial flora of raw milk (Rajagopal *et al*., 2005).

B. cereus is a significant concern regarding food safety hazards due to its ability to secret various exotoxins and enterotoxins. The food poisoning diarrheal form (infection) is mainly occurred by cytotoxin K (*cytK*), non-haemolytic enterotoxin (*nhe*) and haemolysin BL (*hbl*), which are released in the small intestine during the growth of bacteria itself or its' spores following ingestion of infective dose (10^4–10^9 cells per gram of food) as reported by Granum *et al.* (1993) and Clavel *et al.* (2004). Foods containing the diarrheal toxins are probably not harmful because these toxins are susceptible to the acidity of the stomach, heat, and proteases (Dietrich *et al*., 2021).

Unlike diarrheal toxins, the cereulide is a proteolysis-resistant, thermostable and acid-resistant dodecadepsipeptide (Jeßberger *et al*., 2015). It is secreted by *B. cereus* in contaminated foods and rapidly causes emetic syndrome (intoxication) in a time frame of 30 min to 6 h after ingestion (Granum *et al*., 1993; Clavel *et al*., 2004).

In addition, significant damage and liquefactive necrosis of infected tissues have been ascribed to protease, phospholipase, hemolysins and lecithinase (Ramarao and Sanchis, 2013). When the specified numerical limits are surpassed, these enzymes can cause milk and dairy products to spoil due to (lipolytic and proteolytic activity by *Bacillus* spp.) and the production of acid (i.e., lactic, butyric and acetic acid) (Lopez-Brea *et al.* 2018).

Considering the significant public health implications as well as economic losses, the current work was designed to get the prevalence of *B. cereus*, some of its virulence genes in marketable milk and some dairy products (Ras cheese, Domiati cheese and Ice cream) sold in Assiut City, Egypt and to detect the lipolytic and proteolytic activities of isolated *B. cereus* strains.

Materials and methods

Samples and study area

A total of 250 random samples of marketable milk, Ras cheese, Domiati cheese and ice cream were obtained from different localities of Assiut City, Egypt, during the period of November 2022 to March 2023. Each sample was acquired in the exact form in which consumers purchased it and immediately transferred to the laboratory to minimize any potential delays.

Samples preparation

According to APHA (2004), 11 ml or g from every sample was taken and thoroughly mixed (depending on the type of sample) before analysis.

Counting and isolation of B. cereus

Counting of *B. cereus* (Tallent *et al*., 2012)

By using the Most Probable Number (MPN) technique, 3 ml of in-

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oculum from previously made dilutions of the sample were divided into three tubes of the MPN series in trypticase soy-polymyxin broth. The tubes were left to incubate for 48 h at 30°C, and the turbidity broth was indicative of *B. cereus*. A loopful from the turbid broth was spread on the surface of MYP plates and incubated for 18–24 h at 30°C using turbid, positive tubes. To further identify *B. cereus*, the lecithinase-positive colonies were streaked on the slants and cultured there for 24 h at 30°C. The count of *B. cereus* cells/ml or g of sample is dependent upon the count of positive tubes utilized for each dilution.

Isolation of *B. cereus* (Vidal *et al*., 2016)

The enrichment procedure included 90 ml of tryptone soy broth (TSB) containing polymyxin β, that inoculated with 10 ml or g of each sample was incubated at 30°C for 24-30 h. A loopful of the incubated broth was spread over mannitol egg yolk polymyxin (MYP) agar to verify the purity. Pink colonies that were lecithinase-positive were seen when the Petri dishes were cultured for 18 to 24 h at 30°C.

Microbiological identification of B. cereus

The growth characters on the solid medium and the results of biochemical testing. Gram staining was used to carry out the microscopical identification (Cruickshank *et al*., 1975), Rhizoid growth (Tallent *et al*., 2012), the catalase test (Macfaddin, 1976), egg yolk lecithinase (Collins and Lyne, 1984), and sugar fermentation (Aruwa and Olatope, 2015).

Molecular identification of B. cereus

Following manufacturer instructions, the Patho Gene-spin TM DNA/RNA Extraction kit (ISO 9001/14001) was used to extract DNA from the suspected bacterial colonies (5 strains), which were cultivated on nutrient broth and incubated overnight at 37°C. At -20°C, the extracted DNA was kept.

16S rRNA, *cytK*, and *ces* genes' DNAs were amplified as (12.5 µl of 2X PCR master mix (Emerald Amp GT PCR mastermix (2x premix)), 5 µl of DNA template, 1 µl (20 pmol) of each primer (Table 1), and 5.5 µl of PCR grade water were added up to 25 µl in a PCR tube). The thermal cycler (T3 Thermal Cycler, Biometra, Germany) was used to carry out the amplification according to the condition described on Table 2. Gel electrophoresis PCR products were electrophoresed in a 1% agarose gel (GX 040.90, Gen AGarose, L.E., Standard DNA/RNA agarose, Inno-Train Diagnostic, D-61476, Kronberg/Taunus) using ethidium bromide as a 1 µl/ml electrophoresis buffer at 100 volts for 60 min. utilizing the (SCiE-PLAS, HU 10, 5636, UK) 100 bp DNA ladder. The results were carried out by a High-performance ultraviolet Transilluminator, (UV, INC, UK). The images of the PCR products were analyzed by gel documentation system software (Alpha Innotech, Biometra, Germany).

Detection of lipolysis and proteolysis activities of the isolated B. cereus

To assess the lipase activity, Tween 80 (Fluka 93781) was added to Trypticase Soy Agar (BBL 211043) to serve as a substrate in this test. Following inoculation, the inoculated plates were left to incubate for 72 h at 25°C. A turbid halo zone around a colony has been observed (Janda and Bottone, 1981; Edberg *et al*., 1996; Pavlov *et al*., 2004). To evaluate proteinase activity, dialyzed Brain Heart Infusion broth (CM0375, Oxoid) was mixed with 1.5% agar and supplemented with skim milk powder (L31, Oxoid, UK). After inoculation, the plates were allowed to stand at 25°C for 72 h. Positive findings were demonstrated by a clear zone that formed around the colonies (Edberg *et al*., 1996).

Results and Discussion

According to the data in Table 3, which was obtained after working on 250 samples of marketable milk and some milk products to detect the

Table 1. Oligonucleotide primers sequences.

Target gene	Sequence (5'-3')	Amplified product	Reference
16S rRNA	CTT (C/T) TT GGC CTT CTT CTA A GAG ATT TAA ATG AGC TGT AA	284 bp	Altayar and Sutherland (2006)
cytK	ACA GAT ATC GGI CAA AAT GC CAA GTI ACT TGA CCI GTT GC	421 bp	Ehling-Schulz *et al*. (2006)
ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271 bp	

Table 2. Cycling conditions of the different primers during cPCR.

Gene	Primary Denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16S rRNA	95˚C 10 min.	95˚C 15 sec.	50˚C 30 sec.	72˚C 30 sec.	40	72˚C 10 min.
cytK	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
Ces	94˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.3	35	72˚C 12 min.

Table 3. Prevalence of *B. cereus* in marketable milk and some dairy products and the quality of the analyzed samples considering the incidence of *B. cereus* and the Egyptian Standard (2010).

Types of the examined samples	No. of the examined samples	Positive samples		Egyptian Standard			
				Compatible (Negative)		Incompatible (Positive)	
		No.	%	No.	%	No.	%
Marketable milk	88	2	2.27	86	97.73	2	2.27
Ras cheese	52	4	7.69	48	92.31	4	7.69
Domiati cheese	58	2	3.44	56	96.56	2	3.44
Ice cream	52	6	11.5	46	88.5	6	11.5
Total	250	14	5.6	236	94.4	14	5.6

prevalence of *B. cereus* in these samples. It revealed that the analyzed ice cream had the greatest prevalence of *B. cereus* (11.53%) followed by Ras cheese samples. While the samples of Domiati cheese and marketable milk samples were found to be the lowest in the prevalence of *B. cereus*. These results described above are almost similar to the results showed by Abdeen *et al*. (2020) and Bianco *et al*. (2023), they found *B. cereus* in the same proportions from raw milk and Ras cheese samples. However, the occurrence of *B. cereus* in the tested marketable milk, Domiati cheese and ice cream samples is lower than those shown by Mohamed *et al*. (2016); Osama *et al*. (2020) and Elbassiony *et al*. (2021). On the other hand, previous studies conducted by Ibrahim *et al*. (2015) and Torii and Ohkubo (2023) reported lower results for the prevalence of *B. cereus* in raw milk and Domiati cheese, respectively. As shown in Table 4, the majority of *B. cereus* counts in the examined milk and milk product samples fell within the range of $10 \leq 10^2$ cfu/ ml or g.

B. cereus can exist in various environments and is considered a saprophytic bacterium. These bacteria's ability to produce high-temperature resistant spores and their firm adherence to materials such as polymers or stainless steel contribute to their survival and persistence of these bacteria along the lines. The occurrence of bacteria in food is also due to their potential to multiply at low temperatures (Stenfors Arnesen *et al*., 2008). Therefore, it is one factor that significantly influences the shelf-life of milk and milk products (Torii and Ohkubo, 2023).

According to results in Table 5, *cytK* gene was found in all the investigated *B. cereus* isolates. Low percentages were obtained by Hammad *et al*. (2021); Abdeen *et al*. (2020); Saeed *et al*. (2021) and Sánchez-Chica *et al*. (2021) where *cytK* gene was found in 50, 94.87, 85 and 83.33% of the examined *B. cereus* strains, respectively.

Concerning the emetic toxin gene, it is evident that *ces* gene existed in 57.14% of the examined *B. cereus* strains (Fig. 1). A close finding was obtained by Abdeen *et al*. (2020). However, Saeed *et al*. (2021); Bianco *et al*. (2023) and Torii and Ohkubo (2023) could detect *ces* gene in 7.69, 8 and 0.3 % of the examined *B. cereus* strains, respectively. In contrast, Ibrahim *et al*. (2015) and Sánchez-Chica *et al*. (2021) didn't detect any *ces* gene in the examined *B. cereus* isolates.

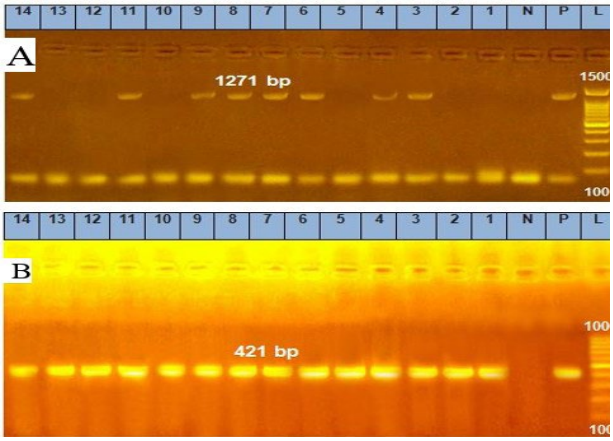

Fig. 1. PCR results for *ces* and *cytK* genes of *B. cereus*. A: *ces* gene. L: molecular marker; lane P: positive control; lane N: negative control; lanes: 1,2,5,10,12 positive isolates and 13: negative isolates. B: *cytK* gene. L: molecular marker; lane P: positive control; lane N: negative control; lanes 1-14: positive isolates.

Considering all the public health significance of *B. cereus* and its toxins, European Food Safety Authority (EFSA, 2016) documented that 11 outbreaks of *B. cereus* were recorded in the EU between 2007 and 2014, which were caused by dairy products. Also, Stenfors Arnesen *et al.* (2008)

Table 4. Frequency distribution of the counted *B. cereus* in the analyzed samples by using MPN technique (MPN/ml or g).

Intervals (CFU)/ ml or g	Marketable milk		Ras cheese		Domiati cheese		Ice cream	
	No.	%	No.	%	No.	%	No.	%
* < 3	-	0	-	0	1	50	-	0
3 -	1	50	2	50	-	-	2	33.33
10 -	1	50	1	25	1	50	3	50
10^2 -	-	0	1	25	-	0	-	0
10^3-	-	0	-	0	-	0	1	16.66
Total	2	100	4	100	2	100	6	100

**B. cereus* could be detected by isolation method.

Table 5. Prevalence of *ces* & *cytK* genes results of the positive *B. cereus* strains in the examined samples.

Milk product	No. of the examined strains	Positive *B. cereus* strains for both *ces* and *cytK* genes		Positive *B. cereus* strains for *ces* gene		Positive *B. cereus* strains for *cytK* gene	
		No.	%	No.	%	No.	%
Marketable milk	2	2	100	2	100	2	100
Ras cheese	4	3	75	3	75	4	100
Domiati cheese	2	1	50	1	50	2	100
Ice cream	6	2	33.33	2	33.33	6	100
Total	14	8	57.14	8	57.14	14	100

Table 6. Lipolysis and proteolysis activities of the isolated *B. cereus* stains.

Milk product	No. of the examined strains	Positive lipolysis activity		Positive proteolysis activity	
		No.	%	No.	%
Marketable milk	2	1	50	2	100
Ras cheese	4	3	75	4	100
Domiati cheese	2	1	50	1	50
Ice cream	6	4	66.66	6	100
Total	14	9	64.28	13	92.85

postulated that the low counts of *B. cereus* at 10^3 cfu/g or ml in food may be enough to cause the infection.

According to data presented in Table 6, it was found that the isolates of *B. cereus* isolated from the examined samples consistently exhibited the potential to release proteases, however only the majority of them (64.28%) showed the capability to produce lipase enzyme. These findings support the previous conclusion made by Tirloni *et al*. (2020) who recorded very high rates of protease and lipase activities of *B. cereus*.

Conclusion

The investigated marketable milk and some milk products sold in Assiut province, Egypt, are contaminated with *B. cereus* bacteria. In addition, the detection of some virulence genes in the isolates may be one of the determinants of the health risk to public health. Moreover, the isolated strains had the ability to deteriorate and spoil milk and milk products because they had lipolysis and proteolysis characterization which eventually led to significant financial loss for food producers. To avoid bacterial contamination, hygienic measures must be performed at different milk production and processing stages.

Conflict of interest

The authors declare that they have no conflict of interest.

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