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Occurrence of *Bacillus cereus* in raw milk and some dairy products in Egypt

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Abstract

A total of 160 samples (20 each of raw cow's milk and seven dairy products; pasteurized milk, small and large scales-manufactured ice cream, infant formulas, full-fat milk powder, rice pudding and yoghurt) were investigated for the presence of *B. cereus*. Biochemical tests followed by PCR were carried out to confirm the suspected *B. cereus* isolates. Overall 44 samples yielded *Bacillus*-like growth of which 34 were positive for *B. cereus*. The highest occurrence % of *B. cereus* was detected in raw cow's milk (60 %), and rice pudding (55 %). Non-*B. cereus* isolates were identified based on 16S rRNA GS and 7 isolates of *B. licheniformis* and 3 isolates of *B. subtilis* were obtained. No psychrotolerant *B. cereus* strains were detected in all samples. Evidence evolved from our results confirms the need for authorities and producers to adopt *B. cereus* as a reference in microbiological hazard control, particularly for dairy products.

Key words: *Bacillus cereus*, raw milk, dairy products, Food-poisoning, Egypt

Introduction

Milk and different processed dairy products constitute an important nutritional category of human food. However, they also can serve as a good medium for the growth of many undesirable m.os of spoilage or even pathogenic characters. The genus *Bacillus* is ubiquitous in the nature and contained about 305 species (DSMZ web site). This ubiquitous nature may be attributed in some circumstances to that certain toxic *Bacillus* strains are likely to inhabit water-damaged indoor environments and foods[21)]. In this sense, the genus *Bacillus* constituted the majority of aerobic spore-formers flora in raw milk[11)]. Unfortunately, traditional heat treatment methods may be not efficient to completely eliminate all viable bacteria present in foods especially spore-formers, which survive the heat treatment and promote spoilage of the end product. Thus, such thermotolerant spore-formers constitute a considerable risk towards keeping quality of certain thermally-processed dairy products[9,39)].

Among aerobic spore-forming bacilli, *Bacillus cereus* remains of a great concern due to several issues. This bacterium is capable of surviving several heat treatment processes, and in addition, some strains are psychro-tolerant, thus it can limit the keeping quality of both pasteurized and refrigerated dairy products. Furthermore, *B. cereus* represents several crucial risks for both human beings; since its classification as a common food contaminant implicated in both food spoilage and food poisoning (FP)[2,9,39)] as well as for dairy ruminants; where it is well reported as a mastitis-causative pathogen, although less frequent[3,23)].

B. cereus belongs to the Hazard group 2 organisms as defined in the European legislation

(European Commission Council Directive 93/88/EEC). FP caused by such bacterium occurs in two types of illness: the emetic and diarrheal syndromes[18,32].It has been reported that *B. cereus* is responsible for nearly about 5% of food-borne outbreaks in the England, Netherlands, France, and USA[29]. However, the real incidence of FP case attributed to *B. cereus* may be underestimated since the health authorities have not declared *B. cereus* FP as a reportable disease in any country and also due to the fact that symptoms of *B. cereus* FP may be misdiagnosed due to the similarity of symptoms with other types of FP, such as *Staphylococcus aureus* intoxication and *Clostridium perfringens*[18]. Consumption of milk and dairy products has been reported to be strongly connected to *B. cereus* FP outbreaks. As a result of its crucial risk towards food consumers health, the aim of the current study is to investigate the occurrence of *B. cereus* in raw cow`s milk and some dairy products marketed in Egypt.

Materials and methods

Collection of samples: A total of 160 samples of dairy category (20 of each of raw cow's milk, pasteurized milk, small-scale manufactured (SSM) ice cream, large-scale manufactured (LSM) ice cream, infant formulas, full-fat milk powder, rice pudding and yoghurt) were collected randomly from different households and supermarkets in Sharkia, Egypt during the period from June 2015 to August 2015. Full-fat milk powder, infant formulas and LCM ice cream have been transported to laboratory in their original containers. All samples were aseptically collected and transported to our lab. in a 4°C vehicle-mounted refrigerator, except ice cream samples which were transported on ice to lab. within 10 min, stored at -20 °C and kept at 4 °C to melt immediately before analysis.

Isolation and Enumeration of B. cereus-like organisms: All samples were devitalized at 80ºC for 10 min in a water bath for the aim of killing vegetative cells and only recovering bacterial spores[28]. Primary enrichment was done by adding 1.0 mL of each devitalized sample to 9.0 mL of nutrient broth followed by incubation at 34 °C for 24 hrs. All presumptive positive cultures were further streaked on MYP agar plates (Oxoid, UK) and incubated at 34 °C for 48 hrs[7]. *B. cereus*-like organisms was estimated acc. to standard procedures[29].

Characterization of B. cereus isolates: The suspected colonies on MYP agar plates (pink color surrounded by egg yolk-like precipitate or cloudy halo due to lecithinase production) were picked for further streaking on sheep blood agar plates and incubated at 32-34°C for 24 hrs[34]. Large, grayish to greenish, circular colonies with a β-haemolytic pattern and ground glass appearance were preliminarily proposed as *B. cereus* to be subjected to biochemical characterization acc. to standard protocols[7,8,15,34].

Determination of Psychrotolerant B. cereus strains: *B. cereu*s isolates were inoculated in tryptone soy broth at 32 °C for 24 hrs then streaking on tryptone soy agar plates and examined for growth at 7°C for 10 days[40].

Identificationof B. cereus by PCR: The suspected colonies were also subjected to PCR-based identification, using primers; BcAPR1 (CTT(C/T)TTGGCCTTCTTCTAA) and BcFF2 (GAGATTTAAATGAGCTGTAA)[5].

Identification of non B. cereus isolates by 16S rRNA gene sequencing (GS): All *Bacillus*-like isolates that did not yield PCR products with primers BcAPR1 and BcFF2 were subjected to 16S rRNA GS-based identification as described by[2,4,27] and quantified using the QuantiTTM. A fragment of the 16S rRNA gene was amplified using the universal primer pair: p8FPL (5′-AGTTTGATCCTGGCTCAG-3′) and p806R (5′-GGACTACCAGGGTATCT-AAT-3′) within a "My Cycler" Thermal Cycler (BioRad, Hercules, USA). Direct GS was performed by the "Big Dye Terminator v3.1" Cycle Sequencing Kit and using the same primers used for PCR. The sequencing reactions were analysed in ABI3130 automatic GS sys. (ABs, USA). The entire 16S rRNA gene sequences were analysed using Chromas software and aligned with Clustal-X software. Next, these sequences were identified by sequence homology alignment among published reference sequences using the web tool; NCBI BLAST.

Results and Discussion

A total of 160 samples of raw cow's milk and dairy products were screened for the presence of *B. cereus*, overall 44 samples (27.5%) yielded *Bacillus*-like growth, of which 34 samples (21.25 %) were positive for *B. cereus* based on biochemical and PCR tools as shown in table (1) and Figure (1). The highest occurrence % of *B. cereus* was detected in raw cow's milk (60 %), followed in order by rice pudding (55 %), SSM ice-cream (35 %), pasteurized milk and infant formula (10 % of each). Meanwhile, all LSM manufactured ice-cream, full fat milk powder and yoghurt samples were devoid of *B. cereus*. Remaining 10 non-*B. cereus* isolates (6.25 %) that didn`t yielded PCR products, were identified based on targeting approximately 800-bp fragment of 16S rRNA gene. The obtained sequences were identified within GenBank using the BLAST tool to determine the closest known relative species as shown in Table 2. Non-*B. cereus* isolates were identified as *B. licheniformis* (7 isolates) and *B. subtilis* (3 isolates)

Most *Bacillus* strains are believed to be harmless

Table 1: Occurrence of *B. cereus* in the examined samples

Samples	No.	Positive Bacillus-like growth		Count (CFU/ml)			PCR-Confirmed *B. cereus*	Samples contained *B. cereus (%)*
		No.	%	Min.	Max	Mean		
Raw cow's milk	20	12	60.0	$6.3x10^2$	$2.4x10^3$	$1.83x10^3$	12	60.0
Pasteurized milk	20	3	15.0	2.0x10	$2.0x10^2$	$1.2x10^2$	2	10.0
SSM ice-cream	20	10	50.0	$5.2x10^2$	$1.5x10^3$	$1.27x10^3$	7	35.0
LSM ice-cream	20	0	0.0	0.00	0.00	0.00	0	0.0
Full-fat milk powder	20	3	15.0	$1.0x10^1$	$3.9x10^2$	$2.05x10^2$	0	0.0
Infant formula	20	2	10.0	$4.0x10^1$	$2.1x10^2$	$1.45x10^2$	2	10.0
Rice pudding	20	14	70.0	$3.5x10^2$	$1.6x10^3$	$1.15x10^3$	11	55.0
Yoghurt	20	0	0.0	0.00	0.00	0.00	0	0.0
Total	160	44	27.5	---	---	---	34	21.25

Table 2: Identification of other non-*B.cereus* isolates by 16S rRNA GS

No.	Source	Hemolysis	16S rRNA GS-based identification
1	Rice pudding	-	*B. licheniformis*
2	Pasteurized milk	-	*B. licheniformis*
3	SSM ice-cream	-	*B. licheniformis*
4	SSM ice-cream	+	*B. licheniformis*
5	SSM ice-cream	-	*B. licheniformis*
6	Full-fat milk powder	+	*B. licheniformis*
7	Full-fat milk powder	-	*B. licheniformis*
8	Full-fat milk powder	-	*B. subtilis*
9	Rice pudding	-	*B. subtilis*
10	Rice pudding	-	*B. subtilis*

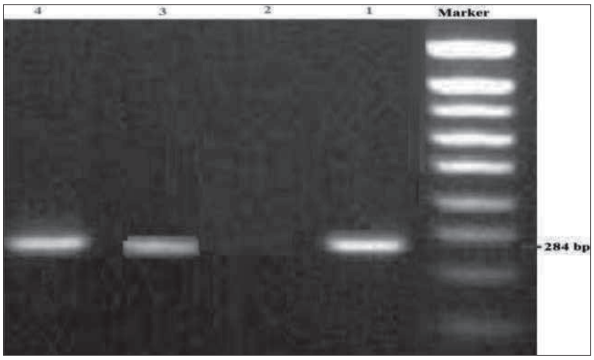

Figure 1: PCR amplification of 284 bp fragments using BcAPR1 and BCFF2 primers

if found in low concentration (< $1.0x10^3$ CFU/g)[21]. However, at suitable temperatures (20-50 °C), *B. cereus* can multiply in 20 min and reach to >$1x10^4$ CFU/g, which is considered as a food spoilage amount in less than 1.5 hrs. Therefore, unsuitable storage conditions after cooking could be a risk factor for both multiplication of such pathogen as well as for production of toxic metabolites[18]. On the other side, some psychrotolerant *B. cereus* strains had been identified in the 1990s[13] with ability to grow quickly at low temperature and with the consequence of greater potential hazard in chilled-foods. Also, these psychrotolerant *B. cereus* strains were found to share the same enterotoxic gene as mesophilic strains, thus their pathogenicity cannot be ignored[40]. In the current study, none of *B. cereus* isolates from examined milk samples showed psychrotolerant feature.

Presence of *B. cereus* in higher percentage of examined raw cow's milk samples may be attributed to environmental contamination since the bacterium is ubiquitous in the nature and/or the fact that it can adapt and inhabit intramammary environment causing mastitis. In this sense, *B. cereus* has been identified as an occasional mastitis pathogen[3,23]. IMIs caused by *B. cereus* can exist in several forms, but subclinical form remains of greater safety concern as the bacterium can still excreted without existence of clinical symptoms, thus the secreted milk is not excluded and passes for human consumption or undergoes further processing. For this reason, the exact incidence of *Bacillus*-incriminated mastitis cases in not known. Compared to our results, a study by[36] reported that *B. cereus* constituted 27.2% of all *Bacillus* isolates from raw milk, while *B. cereus* was present in 56 % and 35.67% of raw milk samples by[22] and [35], respectively.

On the other hand, in pasteurized milk samples, *B. cereus* occurred in 2 (10 %) out of 20 samples. Our results were lower than those documented by[39], where the occurrences of *B. cereus* were 71.4% and 33.3% in spring and in autumn full-fat pasteurized milk samples, respectively and high percentage of the isolates were carriers of enterotoxins genes. Also, in another study[16] found that Psychrotrophic *Bacillus* spp isolates were present in more than 70% of pasteurized milk samples, and of these, over 75% contained *B. cereus*.

Rice pudding is very popular dessert worldwide. The *B. cereus* occurrence in examined samples was high (55%) compared to other products. Similar results were reported by[29,35]. Also, in a study[24], *B. cereus* constituted 75 % of overall isolated bacilli from raw rice. It was reported that rice containing dishes have been known to cause *B. cereus* food poisoning of the emetic kind[17]. Furthermore, *B. cereus* was found to increase rapidly in boiled rice to 10^7-10^8 cfu/g and produced emetic toxin at both 30-35 ºC[1]. For that reasons, several *B. cereus* food-borne outbreaks in which rice was implicated have been described.

An extreme variation for the presence of *B. cereus* was detected between SSM and LSM samples of ice cream that harbored the pathogen in high percentage (50 %) and samples of LSM ice cream which was free from such food-borne bacterium, and this variation may be regarded to the application of strict hygienic procedures and implementation of (HACCP) within LSM type of production. This variation was in concurrence with a study by[37] where the occurrence of *B. cereus* was more frequent in open samples (40%) than in packed-industrial ones (26.6%). Occurrence of *B. cereus* in ice cream samples was reported in higher percentages by [29,40], while lower results were reported by [38].

Heat treatment is not completely efficient to eliminate spore-forming bacteria in foods, among *Bacillus spp*. The investigated dairy products that harbored *B. cereus* in our work are all known to be subjected to different types of heat treatment during processing acc. to each product. Survival and/or growth of *B. cereus* in a wide variety of foods could be traced to several reasons. Among, the non-fastidious nature of the organism, the high resistance of both the endospores and the toxins to desiccation and a variety of heat preservation processes like pasteurization (74°C/15-20 sec) and spray evaporation (50-70°C), besides great ability to survive normal cleaning procedures, and to resist several commercial disinfectants[23,33]. Furthermore, *B. cereus* spores are very hydrophobic, such feature allows them to attach the surface of the pipelines of the dairy processing plant, where they can withstand the pasteurization temperature, multiply and resporulate[6]. For its great resistance to heat treatment, *B. cereus* could be a determinant of keeping quality for pasteurized milk and other heat-treated dairy products. It must be mentioned that for some dairy products where additives, vegetative materials or other ingredients are incorporated, the existence of *B. cereus* can be traced not only for dairy components, but may be also attributed to non-dairy components[40], even if the environmental contamination was absent.

B. cereus has been shown to cause two different forms of food-borne illness; diarrheal and emetic[18,32]. The diarrheal form resembles to *Cl. perfringens* FP, develops within 8-16 hrs following ingestion of contaminated foods and characterized by diarrhea, abdominal pain and vomiting in some cases. This form is attributed to production of extracellular heat-labile protein toxins (Haemolysin [BL], Non-haemolytic enterotoxin [Nhe], Cytotoxin K [CytK]). The diarrheal form is not severe and complete recovery occurs within 24-48 hrs, but large no. of *B. cereus* may be isolated from patient faeces 2 days post-ingestion of contaminated food. Heating of food at high temperature may prevent this intoxication by killing the vegetative bacteria and destroying the heat-labile enterotoxins. Whereas, the emetic form, resembles to *S. aureus*-FP, of a shorter incubation period, and characterized by acute attack of nausea and vomiting while, diarrhea is fairly common. The latter form is attributed to the emetic heat-stable non-protein toxin, cereulide produced by growing cells in foods[20]. Therefore it may be found in milk and pass through many food-processing steps even when no live *B. cereus* is found after heating[21,26]. This toxin was also detected in milk even at the farm level[31]. The outcome of emetic FP may be fatal[21].

In addition to cereulide, another toxin of *B. cereus* with a known connection to fatal FP in human is cytotoxin-K[19]. Several other virulence factors have been found in *B. cereus* contributing to the virulence are: phospholipase-C which gives the capability to hydrolyse lecithin, the major component of the mammalian cell membrane[14], cereolysin AB and haemolysin BL which contribute to the haemolytic activity and tyrosinmono-oxygenase responsible for the formation of melanins protecting the cells and spores of *B. cereus* from various environmental factors[10].

B. licheniformis and *B. subtilis* are members of *B. subtilis* group which include in addition *B. pumilus*. Relevance of *B. subtilis* group members as FP organisms was being increasingly recognized[30]. *B. licheniformis* and *B. subtilis* have been identified in several examined dairy products in current study.These bacilli have been recovered from similar dairy products in previous studies[12,25]. Members of *B. amyloliquefaciens* and *B. subtilis group*, of FP origin have also showed to produce heat-stable compounds, that are toxic to mammalian cells, and extremely stable despite pH alterations[23]. Unfortunately, all members of *B. subtilis* are generally recognized as safe (GRAS) and granted with Qualified Presumption of Safety (QPS) by several Food Safety Authorities.

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