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Prevalence of Some Spore Forming Food Poisoning Bacteria in Milk and Some Milk Products

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ABSTRACT

Spore-forming bacteria are a group of bacteria can form spore and they grow aerobically and anaerobically as Bacilli and *Clostridia* spp. This group of bacteria has public health hazards and economic loss significance. This study was designed to determine the prevalence of spore-forming bacteria isolated from marketable milk and some dairy products as pasteurized milk, UHT milk, milk powder, and baby food (30 samples of each). The samples were purchased randomly from different dairy shops, supermarkets, and pharmacies in Assiut Governorate, Egypt. *Bacillus cereus* was detected in 23.33, 13.33, 6.66, 13.33, and 10% from marketable milk, pasteurized milk, UHT milk, milk powder, and baby food, respectively. *Clostridium perfringens* was recovered in 20, 6.66, 0, 3.33 and 0%, respectively. This study concluded that there is a need for hygienic measures must be applied in the milk and dairy products production and manufacture to minimize the possibility of entering the spore-forming bacteria in these products.

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Introduction

Spore-forming bacteria are Gram-positive microorganisms, aerobic or anaerobic, ubiquitous in nature, and belong to phylum Firmicutes, which include many classes of bacteria as Bacilli and *Clostridia*, they are the most dominant classes within these family (Gopal *et al*., 2015).

Spore-forming bacteria have the greatest spoilage effect in dairy products, lead to severe economic losses. Also, these bacteria have the ability to form biofilms on the surface of processing equipment in dairy factories. Moreover, *Bacillus* spp. cause many defects in milk and milk products as bitty or broken cream, sweat curdling, off-flavor, flat sour, bitterness, ropiness, and cheese blowing (Ternström *et al*., 1993; Heyndrickx and Scheldeman, 2002; Quiberoni *et al*., 2008; Burgess *et al*., 2010). While *Clostridium* spp. are responsible for the late blowing defect of cheeses (Garde *et al*., 2013).

On the other hand, *Bacillus cereus* and *Clostridium perfringens* are implicated in the poisoning of milk and milk products. In last years, the European Food Safety Authority Control has recorded that *Bacillus* and *Clostridium* toxins were implicated in 5.5 and 6.0 % (the mean values from 2010 and 2014, respectively) of total strong evidence foodborne outbreaks (EFSA 2012; 2013; 2014; 2015 a,b).

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Mainly two types of foodborne disease syndromes are caused by *B. cereus*, the first one is a diarrheal syndrome, due to the production of heat-labile enterotoxins during the growth of vegetative cells in the small intestine of the host. The second is the emetic syndrome due to a heat-stable toxin performed in the food (Tewari and Abdullah, 2015). Also, the foodborne illness caused by *C. perfringens* can take two forms include gastroenteritis form, which has symptoms like abdominal cramps and watery diarrhea and the second form is enteritis necroticans (pig-bel disease), this is much more severe and fatal, but it is rare. The symptoms of this form include pain and gassy bloating in the abdomen, diarrhea, and vomiting (Grass *et al*., 2013 and Kiu and Hall, 2018).

Considering all the public health significance of spore-forming bacteria as well as economic losses, the present study was planned to deal with enumeration, isolation, and identification of *B. cereus* and *C. perfringens* from marketable milk, pasteurized milk, ultra-high temperature (UHT) milk, milk powder, and baby foods sold in Assiut city, Egypt.

Materials and methods

Samples and study area

A total of 150 random samples of milk and milk products were collected from different dairy shops, supermarkets, and pharmacies in Assiut City, Egypt. The samples were represented as market milk, pasteurized milk, UHT milk, milk pow-

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der, and baby food. Each sample was obtained as sold to the public and dispatched directly to the laboratory with a minimum of delay.

Preparation of samples

11 ml or grams (according to the type of sample) from each sample were thoroughly mixed before being examined according to A.P.H.A. (2004).

Isolation and enumeration of B. cereus

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

For enrichment of *B. cereus*, 10 ml of each sample was transferred to 90 ml of tryptone soy broth (TSB) supplemented with polymyxin β, the broth was incubated at 30°C for 24-30 h. To confirm purity, streaked onto mannitol egg yolk polymyxin β (MYP) agar. The plates were incubated for 18-24 h at 30 °C, pink lecithinase-positive colonies were observed.

Enumeration of *B. cereus* using Most Probable Number (MPN) technique (Tallent *et al*., 2012)

Three tubes MPN series in trypticase soy-polymyxin both inoculated, using 1 ml inoculum from previously prepared dilutions of the sample with 3 tubes at each dilution. The tubes were incubated for 48h at 30 °C and observed for turbid growth, which was typical of *B. cereus*. Cultures from turbid, positive tubes were streaked on MYP plates and incubated for 18-24 h at 30 °C. The lecithinase-positive colonies were transferred at nutrient agar slants and incubated at 30 °C for 24 h for further identification of *B. cereus*. Typical colonies grown on MYP were confirmed with biochemical testing. The calculation of MPN of *B. cereus* cells/ml or g of sample based on the number of tubes at each dilution in which the presence of *B. cereus* was confirmed.

Identification of B. cereus

Morphological character and Biochemical tests

Microscopical identification was done by Staining. (Cruickshank *et al*., 1975), Rhizoid growth (Tallent *et al*., 2012), catalase test (Macfaddin, 1976), Egg yolk lecithinase (Collins and Lyne, 1984), and Sugar fermentation (Aruwa and Olatope, 2015).

Polymerase chain reaction (PCR)

DNA extraction of the suspected bacterial colonies (5 strains) and the reference strain were subcultured onto nutrient broth and incubated overnight at 37 °C for DNA extraction using Patho Gene-spin TM DNA/RNA Extraction kit (ISO 9001/14001) according to manufacturer instruction. The extracted DNA was stored at -20 °C.

The 16S rRNA gene was amplified using primers BcAPPRI [C T T (C / T) TT GGC CTT CTT CTAA] and BcFF2 [GAG ATT TAA ATG AGC TGT AA] (Applied Biosystem, USA) (Altayar and Sutherland, 2006).

DNA amplification was performed in final volume 25 µl (12.5 µl of 2X PCR master mix (Green Master, Promega, USA), 5 µl of the DNA template, 1 µl (0.5 µM) of each primer and Nuclease free water were added up to 25 µl in a PCR tube). The amplification was performed in a programmable Thermal cycler (Gradient Thermal Cycler, Veriti Applied Biosystem, USA) at 95 °C for 10 min, followed by 40 cycles were run–under the following conditions: denaturation at 95 °C for 15s, 50 °C for the 30s and 72°C for 30s, followed by final extension step at 72 °C for 10 min.

Gel Electrophoresis

PCR products were electrophoresed in 1% agarose gel (GX 040.90, Gen AGarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno–Train Diagnostic, D–61476, Kronberg/Taunus) containing Ethidium bromide as 1µl /ml electrophoresis buffer at 100 volts for 60 min. Using 100 bp DNA–ladder in (SCiE–PLAS, HU 10, 5636, UK). The result was obtained through a High-performance ultraviolet Transilluminator, (UV, INC, UK). The image of the PCR products containing the positive DNA sequence of 284 bp for 16S rRNA gene was amplified using DOC–It ® LS, Image acquisition–software, (Biodoc Analyzer, Biometra, Germany).

Isolation and enumeration of C. perfringens

Isolation of *C. perfringens* (Rhodehamel and Harmon, 1991)

For enrichment of *C. perfringens*, a 25 ml or g portion of each sample was aseptically added to 225 ml of Perfringens Enrichment Medium [PEM; Fluid Thioglycollate Medium, supplemented with supplement tryptose sulphite cycloserine (TSC) supplement] and homogenized by a stomacher for approx. 1 min and then incubated at 46 °C for 20 h in an anaerobic condition. After the samples were enriched in PEM, one loopful from the enrichment was streaked onto TSC agar containing egg yolk and incubated at 35 °C for 24 h. After incubation, the plates were removed from the anaerobic jar and selected colonies were black with a 2-4 mm opaque white zone surrounding the colony as a result of lecithinase activity. To confirm presumptive *C. perfringens*, suspected colonies from each positive TSC agar plate were purified and identified biochemically.

Enumeration of *C. perfringens*

C. perfringens was enumerated using Most Probable Number (MPN) technique using lactose sulphite broth (LSB) (Beerens *et al*., 1980). From each dilution, 1 ml was inoculated into 3 replicate tubes of LS broth supplied with inverted Durham's tubes and incubated anaerobically using a Gas-Pack anaerobic jar at 46 °C for 18-24 h. The positive tubes were confirmed by clouding of the broth with iron sulphide (Fe ppt) and gas production was visible in the inverted Durham tubes within the incubation period and the counts of *C. perfringens* were recorded using MPN Tables.

Identification of C. perfringens

Morphological characters and Biochemical tests

Microscopical identification by staining (Rhodehamel and Harmon, 1991), Iron-milk presumptive test (Rhodehamel and Harmon, 1991), Egg yolk agar plate test (Dar *et al*., 2017), Sugar fermentation (Krieg and Holt, 1984), and Indol production (Krieg and Holt, 1984).

Results and Discussion

Data summarized in Table 1 showed that the prevalence of *B. cereus* was from 150 samples of marketable milk and some milk products. The highest prevalence was recorded in the examined marketable milk (23.33%) followed by pasteurized milk and milk powder samples. While the lowest prevalence was found in baby food and UHT milk samples. These

results are nearly similar to the results obtained by El-Leboudy (1985); Vyletelová *et al*. (2001) and Mohamed *et al*. (2016), they isolated *B. cereus* in the same percentages from raw milk, pasteurized, milk powder, and baby food samples. On the other side, the results of the examined UHT milk samples are lower than those obtained by Rezende-Lago (2002) and Vidal *et al*. (2016). Most counts of *B. cereus* lied in the range 3 ≤ 10 cfu/ ml or g in the examined milk and milk products samples as shown in Table 2.

Survival and growth of *B. cereus* in a wide variety of foods could be traced to several reasons as high resistance of the endospores and the toxins to heat preservation processes. Also, *B. cereus* has a great ability to resist several commercial disinfectants (Nieminen *et al*., 2007). Moreover, the unsuitable storage of baby food and milk powder could be another reason for the growth of *B. cereus* in these products (El-Karamani, 2017).

The data illustrated in Table 1 revealed that 6 (20%) of the examined marketable milk samples were contaminated *C. perfringens*. These results with agreement with results obtained by Osama *et al*. (2015) and Haque *et al*. (2018). While, in the examined pasteurized milk and milk powder the prevalence of *C. perfringens* was 6.66 and 3.33%, respectively. The obtained result of the examined pasteurized milk samples is higher than the result carried by Gurmu *et al*. (2013), they couldn't isolate *C. perfringens* from the examined pasteurized milk samples. On the other side, the obtained results for the examined milk powder samples was nearly similar to the results obtained by El-Leboudy (1985). On the other hand, *C. perfringens* couldn't be detected in the examined UHT milk and baby food samples. These findings agreed with the results of Osama *et al*. (2015) and Sezer *et al*. (2015), they couldn't isolate *C. perfringens* from UHT and baby food samples, respectively. Five samples of the examined marketable milk samples had counts of *C. perfringens* more than 10^3 cfu/ml, while two and one samples of the examined pasteurized and powdered milk samples respectively were lied in range between 3 ≤ 10 cfu/ ml or g, (Table 3).

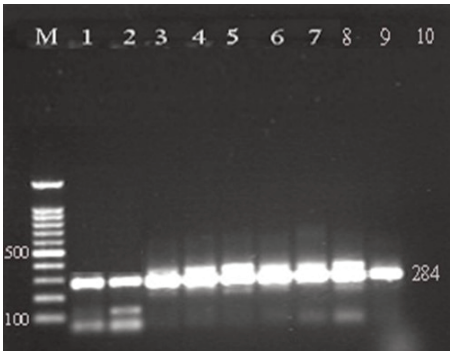

Fig. 1. PCR products of amplified 16S rRNA of *B. cereus* visualized on agarose gel electrophoresis. Lane (M) DNA ladder 100 bp, lanes (1-8) positive strains with specific bands at 284 bp, lane (9) positive control, and lane (10) negative control.

C. perfringens in pasteurized milk and milk powder can resist the drying and pasteurized processing due to the spores of *Clostridium* (Ronimus *et al*., 2006). The source of the spores in these products from the contamination of raw milk used in the manufacture of these products.

Egyptian Standard (2010) stated that milk and milk products must be free from the pathogenic microorganisms and their toxins, so, any sample that contained *B. cereus* or *C. per-*

Table 1. Prevalence of some aerobic and anaerobic spore-forming bacteria (*B. cereus* and *C. perfringens*) in the examined milk and baby food samples.

Types of the examined samples	No. of the examined samples	*B. cereus* positive samples		*C. perfringens* positive samples		Positive samples for spore-forming bacteria		Positive samples for both types of spore-forming bacteria	
		No.	%	No.	%	No.	%	No.	%
Marketable milk	30	7	23.33	6	20	13	43.33	3	10
Pasteurized Milk	30	4	13.33	2	6.66	6	20	-	0
UHT milk	30	2	6.66	-	0	2	6.66	-	0
Milk powder	30	4	13.33	1	3.33	5	16.66	-	0
Baby food	30	3	10	-	20	3	10	-	0
Total	150	20	13.33	9	6	29	19.33	3	2

Table 2. Frequency distribution of the counted *B. cereus* in the examined samples by using MPN technique (MPN/ml or g).

Intervals (CFU)/ml or g	Marketable milk		Past. milk		UHT milk		Milk powder		Baby food	
	No.	%	No.	%	No.	%	No.	%	No.	%
* < 3	-	0	-	0	-	0	-	0	-	0
3 ≤ 10	4	57.14	2	50	2	100	1	25	2	66.6
$10 \leq 10^2$	3	42.85	1	25	-	0	1	25	-	0
$10^2 \leq 10^3$	-	0	-	0	-	0	-	0	-	0
$>10^3$	-	0	1	25	-	0	2	50	1	33.3
Total	7	100	4	100	2	100	4	100	3	100

Table 3. Frequency distribution of the counted *C. perfringens* in the examined samples by using MPN technique (MPN /ml or g).

Intervals (CFU)/ml or g	Marketable milk		Past. milk		UHT milk		Milk powder		Baby food	
	No.	%	No.	%	No.	%	No.	%	No.	%
* < 3	-	0	-	0	-	0	-	0	-	0
3 ≤ 10	1	16.66	2	100	-	0	1	100	-	0
$10 \leq 10^2$	-	0	-	0	-	0	-	0	-	0
$10^2 \leq 10^3$	-	0	-	0	-	0	-	0	-	0
$>10^3$	5	83.33	-	0	-	0	-	0	-	0
Total	6	100	2	100	-	100	1	100	-	100

fringens is considered not in agreement with Egyptian Standard.

Conclusion

B. cereus and *C. perfringens* are established in milk and some dairy products. Therefore, it is recommended to use high-quality raw milk for the manufacture of dairy products. In addition, cleaning and sanitization of equipment, employment health workers with health certificates in the dairy industry. Moreover, sterilization of milk is the best heat treatment of milk.

Conflict of interest

Authors declared that they have no conflict of interest.

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