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Enterotoxigenic Profiles of Virulent *Bacillus cereus* Isolated from Dairy Environments: Antimicrobials Resistant Pattern and Sporicidal Disinfectants Efficacy

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Abstract | The study was aimed to characterize *Bacillus cereus* using culture-based and PCR techniques among 390 samples including milk, dairy products, water and environmental specimens. Antimicrobial activities of disinfectants and bioactive peptides were performed using European Suspension Test and liquid-broth method. Overall of 29.5 and 67.4% among dairy and environmental samples were positive for *B. cereus*. The prevalence of *B. cereus* in household milk, rice milk, water and street vendor milk was 37.5, 36.4, 33.3, and 30%, respectively. Moreover, the prevalence in the rinse water, used bedding material, pasture grass and pasture soil were 38.5, 37.5, 37.5 and 33.3%, respectively. *HblA*, *hblD*, *hblC* were detected in 42.2, 26.7, 24.4% and the *nheA*, *nheB*, *nheC* occurred in 31.1, 33.3, 24.4%, respectively. *B. cereus* isolates showed resistance to Amoxicillin, Ampicillin, Penicillin, Cephalexin and Tetracycline with 68.9, 62.2, 62.2, 57.8, and 51.1%, respectively. Hyperox and CIDEX disinfectants have extremely rapid bactericidal activity at concentrations 2 and 2.4% after five minutes of exposure times under clean condition resulting in 10.3 and 9.7 log reductions, respectively. Adding organic substances results in a marked decline of the bactericidal activity for both disinfectants. CIDEX exhibited lower Sporicidal efficacy than Hyperox under clean conditions at various exposure times/temperatures. Under the dirty condition, Cidex showed similar or slightly higher sporicidal activity than Hyperox. At 4 hrs/ 20 & 40 °C under dirty conditions, 4.8, 5.6 and 4.6, 5.2 log reductions for CIDEX and Hyperox, respectively were observed. P-CWP and P-CCP showed effective antimicrobial action resulting in 10 and 8 log reductions at a concentration of 2 mg/ml, respectively against *B. cereus*. However, CCP and CWP exhibited little to moderate antimicrobial activity. The prevalence of Eneterotoxigenic virulent *B. cereus* and their drug resistance profiles diversity suggest that the examined dairy environments in villages of Sohag governorates, Egypt poses animal and public health threats.

Keywords | *Bacillus cereus*; Milk & Milk products; Water; Disinfectants; Antimicrobial biopeptides

INTRODUCTION

The genus *Bacillus* is a Gram-positive, aerobic-to facultative, spore-forming rod-shaped bacterium. It is ubiquitous in nature and constituted the majority of aerobic spore-formers flora that likely to inhabit water-damaged indoor environments and foods (Mohamed et al., 2016). Those thermotolerant spore formers constitute a considerable risk towards keeping quality of both pasteurized and refrigerated dairy products (Caamaño-Antelo et al., 2015; Singh et al., 2015).

There are different species of *Bacillus*, of which the *Bacillus cereus* is the most important species since its classifi-

cation as a common food contaminant involved in both food spoilage Yusuf et al. (2018) as well as food poisoning that usually occurs in two types of illness: the emetic and diarrheal syndromes.

The diarrheal syndrome is due to five enterotoxins have been detected. Hemolysin BL (*Hbl*) and nonhemolytic enterotoxin (*Nhe*) have been identified as the major virulence factor among the various enterotoxins of *B. cereus* (Cui et al., 2016). Hemolysin BL consists of binding protein B, lysing proteins L1, and L2 encoded by *hblA*, *hblC* and *hblD* genes, respectively. While nonhemolytic enterotoxin has three proteins moieties B, L1 and L2 encoded by the three genes *nheA*, *nheB* and *nheC*, respectively (Ombui et al., 2008).

Antibiotic therapy is still the most important treatment method for *B. cereus* infections. However, antibiotic resistance profiles of *B. cereus* isolates are of concern for public health (Cui et al., 2016). Acquired resistance to antibiotics is mainly due to antibiotic misuse or acquisition of transferable multidrug resistance genes (Gao et al., 2018). However, there is no report on the antimicrobial susceptibilities of *B. cereus* isolates from milk, milk products, water and farm environment in Egypt.

Many sporicidal disinfectants, including peroxygens glutaraldehyde, and chlorine-releasing agents (hypochlorite) are commercially available and effective; however, most require long contact times (Russell, 1990). Peracetic acid is a mixture of acetic acid and hydrogen peroxide. It is a very strong oxidizing agent and is considered bactericidal, and sporicidal. It also retains its activity in the presence of organic matter. Peracetic acid is used in food processing and handling as a sanitizer and disinfectant for food contact surfaces (Marquis et al., 1995). Glutaraldehyde was an effective sporicide. CIDEX Activated Dialdehyde Solution is a 2.4% activated glutaraldehyde solution available commercially and has been in use for many years (Lane et al., 1996; Tennen et al., 2000).

Quaternary Ammonium Compounds (QAC) disinfectants are effective against gram-positive and gram-negative bacteria but less or not effective against bacterial spores. Their efficiency is reduced markedly in the presence of organic matter (Anonymous, 2005).

In recent years, the role of milk proteins as the main source of a wide range of biologically active peptides has been acknowledged worldwide (Sindayikengera and Xia, 2005). The antimicrobial activities of milk protein-derived peptides are very diverse, ranging from those with a prebiotic effect, peptides with the ability to prevent the attachment or invasion of pathogen microorganisms, to peptides kill-ing or inhibiting the growth of microorganisms (Hernández-Ledesma et al., 2013). Many peptides with antimicrobial activity have been released from bovine milk proteins, either during fermentation of milk or through enzymatic hydrolysis; these antimicrobial peptides have been found to have activity against many gram-positive and gram-negative bacteria, as reviewed by (Akalın, 2014).

The present study aimed to investigate the prevalence of *Bacillus* species especially *B. cereus* in milk, milk products, water and dairy cattle macro-environmental samples in some villages of Sohag, Egypt, in addition to detection the *hbl* and *nhe* toxin genes in *B. cereus* isolates by polymerase chain reaction (PCR). The antibiotic susceptibility profiles of the isolated strains for 10 antimicrobial agents were assessed. The antimicrobial activity of disinfectants and milk proteins hydrolysates against isolated and identified *B. cereus* strains will be evaluated in vitro. Innovative approaches are necessary to address the health needs of those targeted villages communities. The findings and recommendations of the current study will notify relevant health decision-makers to take the necessary counteract measures.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Rationale of the study: The current study was designed to target the household rearing of cattle in Sohag governorate, Egypt to determine the prevalence and monitor the main source of *Bacillus* species especially *B. cereus* in cow's house environment and local dairy shops. An initial survey was conducted to establish the bacteriological status of milk (household and street vendor milk), milk products (Baladi yoghurt, Kareish cheese, Butter and rice milk), water (drinking and rinse water) and environmental samples (bedding materials, pasture grass, pasture soil and air) with optimum treatment using milk proteins hydrolysates and some disinfectants against the recovered virulent *B. cereus* strains.

The surveyed localities do not rely on supplementary feeding or routine antibiotics use. There was also no record of *B. cereus* infection among these localities. Economic, environmental and societal factors are coexisted in those selected villages. The Economic aspects is leading factor resulted in rearing of dairy cattle inside the farmers house to use their raw milk as source of income. Moreover, lacking of the basic requirements for housing of dairy cattle and primitive sanitary measures including selling of raw milk without any heat treatment created threats to animal and public health. *B. cereus* infections remain a major public health issue in those selected sites to which the public health concept principles represent a promising pathway.

COLLECTION OF SAMPLES

A total of 390 samples out of milk and milk products (180 samples), water (30 samples), environmental samples (150 samples) and air (30 samples) were collected from dairy cattle rural house and local dairy vendors.

From local dairy houses and vendors, a total of 60 raw milk samples (household and street vendor milk) were collected in clean sterilized 50 ml Falcon™ conical tubes. Household and street vendor milk are considered the most important raw milk samples supplied to the public in Sohag governorate, Egypt. Additionally, 30 milk product samples were also collected from each of the following categories, including; Baladi yoghurt, Kareish cheese, Butter and rice milk. All samples were transported to the laboratory and were stored at -20 °C until further analysis.

Sixty drinking and rinse water samples were collected out of local water sources inside framer houses in clean sterile transparent 500 ml capacity glass bottles fitted tightly with ground glass stoppers. The samples were immediately placed in a lightproof insulated box containing melting ice or ice-packs with water to ensure rapid cooling. The box used to carry samples was cleaned and disinfected after each use to avoid contaminating the surfaces of the bottles and the sampler's hands (WHO, 1971).

A total of 60 bedding materials (new & used) and 30 (pasture grass) were collected according to Rendos et al. (1975). The total amount of material (approximately 250 g) collected in sterile plastic bags. Ten grams were chopped into fine particles with sterile scissors then added to 90 ml of sterile 0.1% peptone solution in a sterile glass bottle. The prepared bottles were then mixed thoroughly by manual agitation for 5 min. The mixture then aseptically strained through sterile gauze; the filtrate was collected in a sterile flask for further analysis.

A total of thirty pasture soil samples were collected according to Clegg et al. (1983). Each sample was removed at a depth of 5 cm from different places of the house and transferred to a sterile glass bottle, fitted with a sterile ground glass stopper. After thorough mixing of each soil sample, one gram was crushed well in a sterile mortar with 99 ml of the sterile normal saline solution then aseptically strained through sterile gauze; the filtrate was collected in a sterile flask for further analysis.

Thirty air samples were collected from the examined animal enclosures by liquid impinger air sampler according to Cown et al. (1957). About 50 ml of sterile saline solution was used for collecting the airborne bacteria. The liquid impinger was adjusted at a rate of 5 liters/min. During sampling, the liquid impinger was moved inside the animal house to trap all suspended dust particles. The collected samples were carried in the icebox to the laboratory for bacteriological examination.

ISOLATION AND IDENTIFICATION OF THE *BACILLUS* SPECIES

The isolation of *Bacillus* species is adopted according to (Harrigan, 1998; Banykó and Vyletělová, 2009). About 25 ml/g of each sample was aseptically homogenized in 225 ml of nutrient broth for primary enrichment and incubated at 34 °C for 24 h. Then cultures were streaked on the Mannitol egg yolk-polymyxin (MYP) agar plate supplemented with egg yolk, mannitol, and Polymyxin B and incubated at 34 °C for 24-48 h. Biochemical identification according to Varadaraj (1993); Banykó and Vyletělová (2009); Bottone (2010).

DETECTION OF *B. CEREUS* VIRULENCE GENES

The PCR-technique was applied for the detection of six virulence genes using six sets of primers. Those genes were hemolysin BL (*HBL*) *hblA*, *hblC*, *hblD* and nonhemolytic enterotoxin (*NHE*) *nheA*, *nheB*, *nheC* genes. Primer sequences and amplicon size used in this study were presented in Table 1 as described by Yang et al. (2005) and Ombui et al. (2008). PCR was applied following QIA amp DNA mini kit instructions (Catalogue no. 51304); Dream Taq Green PCR Master Mix (2X) (Thermo Scientific) Cat No. K1081 (Sambrook et al., 1989). Positive PCR products for each gene were detected and visualized in single 1.5% agarose gel electrophoreses stained with ethidium bromide, viewed by U.V. light.

ANTIBIOGRAM PATTERN OF *B. CEREUS*

All *B. cereus* isolates were examined for their antibiogram pattern by disc diffusion technique as described by Bauer et al. (1966) against a panel of 10 antibiotics. The selection of antibiotics (10) was based on their common use in human and animals in Egypt which included amoxycillin (20 µg), ampicillin (10 µg), cephalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 unit), streptomycin (10 µg), tetracycline (30 µg). All antibiotics used were obtained from Oxoid, UK. The results were interpreted as sensitive or resistant based on CLSI interpretive standards (2007).

ANTIMICROBIAL ACTIVITY OF DISINFECTANTS AND MILK PROTEINS HYDROLYSATES AGAINST *B. CEREUS*

Microorganisms: Recovered *B. cereus* virulent strains were enriched in tryptic soy broth before cultivation on MYP agar for 24 hrs at 37 °C before assay.

Disinfectants: The study aimed to evaluate the bactericidal and sporicidal activity of four disinfectants on *B. cereus* and its spores: CIDEX® is an activated dialdehyde solution,

Table 1: PCR protocol including primer sequences, Amplicon size and amplification reactions [a].

Target gene	Primers sequences	Amplified segment (bp)
hblA	ATTAATACAGGGGATGGAGAAACTT	237
	TGATCCTAATACTTCTTCTAGACGCTT	
hblC	CCTATCAATACTCTCGCAACACCAAT	386
	TTTTCTTGATTCGTCATAGCCATTTCT	
hblD	AGATGCTACAAGACTTCAAAGGGAAACTAT	436
	TGATTAGCACGATCTGCTTTCATACTT	
nheA	ATTACAGGGTTATTGGTTACAGCAGT	475
	AATCTTGCTCCATACTCTCTTGGATGCT	
nheB	GTGCAGCAGCTGTAGGCGGT	328
	ATGTTTTTCCAGCTATCTTTCGCAAT	
nheC	GCGGATATTGTAAAGAATCAAAATGAGGT	557
	TTTCCAGCTATCTTTCGCTGTATGTAAAT	

[a] Primer design and amplification process steps as published by yang *et al.*, 2005 and Ombui *et al.*, 2008.

2.4% glutaraldehyde (Johnson & Johnson Medical Egypt, Cairo, Egypt), Hyperox®, is a colorless aqueous formulation of peracetic acid, hydrogen peroxide, acetic acid and surfactant (Antec International Limited, United Kingdom), Evans Hypochlorite contains a blend of sodium hypochlorite solution (Evans Vanodine International plc, United Kingdom) and BioSentry® 904, quaternary ammonium compounds base (HACCO, Inc., USA). All disinfectants were handled according to the manufacturer's instructions.

Evaluation of the bactericidal activity of disinfectant: The test method is based on the suspension test according to European Norms (EN) 1276 (BS EN 1276:1997) which specifies a quantitative suspension test for the evaluation of the bactericidal activity of chemical disinfectant used in the food industry. The bacterial suspension was prepared from fresh *B. cereus* strains isolated during the current study. The suspension was used to simulate clean condition without interfering substance and dirty dairy environment condition through adding milk 4% to form a bacterial-interfering substance mixture. One and five minutes exposure times were chosen. The log count of *B. cereus* was determined after plate counting on the Mannitol egg yolk-polymyxin (MYP) agar plate.

Evaluation of Sporicidal activity of disinfectant: For the preparation of spore suspensions, wash the growth from a fresh *B. cereus* culture with sterile physiological saline onto the surface of a Roux bottle containing 300 ml of AK Agar #2 (Sporulating Agar). Incubate the bottles for 5 days at 35 ± 2 °C and wash off the resulting growth into 50 ml of sterile physiological saline. Centrifuge the suspension and decant and discard the supernatant fluid. Resuspend the sediment in sterile saline and heat shock the suspension at 70 °C for 30 minutes (Wehr and Frank, 2004). To represent the in-use situation in the dairy industry concern-

ing exposure time, temperature and interfering substances, tests were carried out in the presence of milk (4% v/v) as an organic interfering substance (Te Giffel et al., 1995).

The disinfectant quantitative suspension test for the evaluation of sporicidal activity was performed using the approach outlined in (BS EN 13704:2002) and according to European Norms (EN) suspension sporicidal tests (BS EN 14885:2006). Spores in suspension with/without interfering substance were exposed to disinfectants for specified exposure times: 0.5, 1, 2, 3, and 4 hrs at 20 °C and 40 °C; respectively. The neutralizers used in this study were according (Russell, 1981; Espigares et al., 2003).

Hydrolysis of cow milk proteins with pepsin enzyme: Separation of caseins and whey proteins were performed according to (Ahmed et al., 2015); briefly fat was removed from 250 ml raw milk by centrifugation and then the skimmed milk was passed through three layers of gauze. The defatted milk was adjusted to pH 4.6 with 10% acetic acid, followed by centrifugation. The fractions cow casein proteins (CCP) and cow whey proteins (CWP) were lyophilized.

***In vitro* pepsin digestion of CCP ad CWP**: The CCP and CWP were dissolved in Milli-Q water and adjusted to pH 3.0 with HCl. Pepsin in 1 mM HCl was added to the protein solution at enzyme-to-substrate (E/S) ratio of 1:50 (w/w) and incubated for 2 h at 37 °C with mild shaking. Pepsin inactivation was achieved by heating at 85 °C for 5 min and immediately cooled in ice for 5 min. Insoluble solids were removed by centrifugation. The resulting supernatants were adjusted to pH 7.0, to fully inactivate pepsin, and then lyophilized (Ahmed et al., 2015). The CCP and CWP, as well as their hydrolysates (P-CCP and P-CWP), were tested for antimicrobial activity against *B*.

Table 2: Distribution of *Bacillus* spp. in examined samples (N=30).

Type of sample		Total No. of *Bacillus* spp.		*B. cereus*		*B. subtilis*		*B. mycoides*		*B. licheniformis*	
		No.	%	No.	%	No.	%	No.	%	No.	%
Milk and milk products samples	Household milk	8	26.6	3	37.5	2	25	1	12.5	2	25
	Street vendor milk	10	33.3	3	30	1	10	3	30	3	30
	Baladi yoghurt	5	16.7	1	20	2	40	1	20	1	20
	Kareish cheese	7	23.3	2	28.6	2	28.6	3	42.8	0	0
	Butter	3	10	0	0	3	100	0	0	0	0
	Rice milk	11	36.6	4	36.4	4	36.4	1	9.1	2	18.1
Dairy cattle macroenvironment	Drinking water	6	20.0	2	33.3	0	0	1	16.7	3	50
	Rinse water	13	43.3	5	38.5	4	30.7	3	23.1	1	7.7
	New bedding material	11	30.6	3	27.3	2	18.2	4	36.3	2	18.2
	Used bedding material	24	80.0	9	37.5	7	29.2	5	20.8	3	12.5
	Pasture grass	16	53.0	6	37.4	3	18.8	3	18.8	4	25
	Pasture soil	21	70.0	7	33.4	5	23.8	5	23.8	4	19
	Air	3	10.0	0	0	0	0	2	66.7	1	33.3
Total (%)		138	100%	45	32.6%	35	25.4%	32	23.2%	26	18.8%

Antimicrobial assay: The liquid-broth method (Ahmed et al., 2020) was used to assess the bactericidal activity of milk proteins hydrolysates against *B. cereus* at different concentrations. Mid-logarithmic phase cells, grown in brain heart infusion (BHI) broth, were washed and resuspended (to give 6-7 log10 CFU/ml) in 1% trypticase soya broth (TSB), pH 7.3. An aliquot (100 μl) of bacterial suspension was mixed with an equal volume of series of hydrolysates. After incubation at 37 °C for 24 h, a 30-μl portion was serially diluted in physiological saline and spotted onto a nutrient agar plate. The colony-forming units (CFU) were obtained after incubation at 37 °C for 18 h.

RESULT

BACTERIOLOGICAL ANALYSIS OF SAMPLES

A total of 180, 30 and 180 milk & milk products, water and environmental samples were screened for the presence of *B. cereus*, overall 44(24.4%), 6(20%) and 88(48.9%) samples yielded *Bacillus* spp., respectively. Of these 13(29.5%), 2(33.3%) and 30(34.1%) samples were positive for *B. cereus*, respectively (Table 2). The highest occurrence% of *B. cereus* was detected in household milk (37.5%), followed in order by rice milk (36.4%), water (33.3%) and street vendor milk (30%). Moreover, the highest occurrence% of *B. cereus* in environmental samples was detected in rinse water (38.5%), used bedding material (37.5%), pasture grass (37.5%) and pasture soil (33.3%). Meanwhile, all butter and air samples were devoid of *B. cereus* (Table 2).

As shown in Table 2, isolates of *Bacillus* spp. were studied for morphological, biochemical properties and API 50 CHB (Biomerieux, France). These tests were recom-

mended for the identification of *Bacillus* spp. according to (Varadaraj, 1993; Banykó and Vyletělová, 2009; Bottone, 2010). The identification with classical methods based on morphological and biochemical criteria as well as API techniques showed that *Bacillus* spp. isolates were identified as *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*), *Bacillus mycoides* (*B. mycoides*) and *Bacillus licheniformis* (*B. licheniformis*). Among these isolates, it can be noted that *B. cereus* was the most frequently occurring *Bacillus* spp. with an incidence of 32.6% of isolates followed by *B. subtilis* (25.4%), *B. mycoides* (23.2%), *B. licheniformis* (18.8%) (Table 2).

POLYMERASE CHAIN REACTION

PCR assay was performed on identified *B. cereus* isolates using primers designed for virulence genes (Table 3). The *hblA*, *hblD* and *hblC* genes coding for Hemolysin BL (*HBL*) A, D and C enterotoxigenic gene were demonstrated 42.2, 26.7 and 24.4%, respectively among *B. cereus* isolates from examined samples. Moreover the *nheA*, *nheB* and *nheC* genes coding for Nonhemolytic (*NHE*) A, B and C enterotoxigenic gene occurred in 31.1, 33.3 and 24.4%, respectively. A representative gel electrophoresis profile of amplified products of the investigated pathogenic genes was shown in Figure 1.

A total of 45 *B. cereus* isolates were recovered. Among of them, 33 strains (73.3%) exhibited enterotoxigenic virulent profiles while 12 (26.7%) were non-toxigenic isolates. Six toxin gene profiles were detected in our survey, which covered a total of 45 isolates (Table 4). The most common toxin profile found among the latter isolates was toxin profile 5 (*hbl+/nhe+*), where strains isolated from most types

Table 3: Haemolytic BL complex (A) and Non-haemolytic enterotoxin complex (B) genes profile of 45 *B. cereus* strains screened by PCR. ᵃ

Genes profile		Milk and milk products samples							Dairy cattle macroenvironment					
		Household milk (N=3)	Street vendor milk (N=3)	Baladi yoghurt (N=1)	Kareish cheese (N=2)	Butter milk (N=0)	Rice milk (N=4)	Drinking water (N=2)	Rinse water (N=5)	New bedding material (N=3)	Used bedding material (N=9)	Pasture grass (N=6)	Pasture soil (N=7)	Air (N=0)
A	*hblaA*	2	0	1	0	2	2	2	0	4	3	3	0	0
	hblaC	1	1	0	1	0	0	1	1	2	2	2	0	0
	hblaD	0	0	1	0	1	1	0	1	3	2	2	0	0
B	*nheA*	1	0	1	0	1	1	2	1	3	2	2	0	0
	nheB	0	2	1	2	0	2	1	2	2	2	2	0	0
	nheC	1	1	0	1	0	1	1	0	3	1	2	0	0

ᵃ All butter and air samples were devoid of *B. cereus.*

Table 4: Toxigenicity profiles of 45 *B. cereus* isolates obtained from screened samples.

Toxin profile	Haemolytic BL complex (hblaA, C and D)	Non-haemolytic enterotoxin complex (nheA, B and C)	Milk and milk products samples							Dairy cattle macroenvironment					
			Household milk	Street vendor yoghurt	Baladi cheese	Kareish Butter milk	Rice water	Drinking water	Rinse water	New bedding material	Used bedding material	Pasture grass	Pasture soil	Air	
1	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	
2	++/-	++/-	-	-	-	1	-	-	1	-	-	1	1	-	
3	++/-	+/--	-	-	1	-	-	-	-	-	1	2	1	-	
4	+/--	++/-	1	-	-	-	-	1	-	-	-	-	-	-	
5	+/--	+/--	1	2	1	-	-	2	1	5	1	3	-	-	
6	---	---	1	1	-	1	-	1	2	-	2	2	2	-	

Table 5: Antibiogram resistance pattern of *B. cereus* isolates.

Samples	No. of B. cereus	Amoxicillin		Ampicillin		Cephalexin		Chloramphenicol		Ciprofloxacin		Erythromycin		Gentamicin		Penicillin		Streptomycin		Tetracycline	
	No.	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Household milk	3	2	66.7	2	66.7	1	33.3	0	0	0	0	1	33.3	0	0	2	66.7	0	0.0	2	66.7
Street vendor milk	3	2	66.7	1	33.3	1	33.3	1	33.3	0	0	1	33.3	0	0	1	33.3	0	0	1	33.3
Baladi yoghurt	1	1	100	1	100	1	100	0	0	0	0	1	100	0	0	0	0.0	1	100	1	100
Kareish cheese	2	2	100	1	50	1	50	0	0	0	0	1	50	1	50	1	50	0	0.0	1	50
Rice milk	4	3	75	2	50	3	75	2	50	1	25	2	50	1	25	3	75	1	25	2	50
Drinking water	2	1	50	1	50	1	50	1	50	1	50	1	50	1	50	2	100	1	50	1	50
Rinse water	5	3	60	3	60	4	80	2	40	2	40	2	40	2	40	3	60	2	40	2	40
New bedding material	3	2	66.7	2	66.7	2	66.7	1	33.3	1	33.3	1	33.3	1	33.3	2	66.7	1	33.3	2	66.7
Used bedding material	9	5	55.6	7	77.8	6	66.7	4	44.4	3	33.3	3	33.3	2	22.2	6	66.7	3	33.3	4	44.4
Pasture grass	6	4	66.7	3	50	3	50.0	2	33.3	2	33.3	2	33.3	1	16.7	4	66.7	2	33.3	3	50
Pasture soil	7	6	85.7	5	71.4	3	42.9	2	28.6	2	28.6	2	28.6	2	28.6	4	57.1	3	42.9	4	57.1
Resistance	45	31	68.9	28	62.2	26	57.8	15	33.3	12	26.7	17	37.8	11	24.4	28	62.2	14	31.1	23	51.1
Susceptible		14	31.1	17	37.8	19	42.2	30	66.7	33	73.3	28	62.2	34	75.6	17	37.8	31	68.9	22	48.9

Table 6: Log Reduction Values of Disinfectants-Treated *Bacillus cereus* Spores in suspension at various contact times and temperatures under clean /dirty environment dairy condition.

| A	Exposure duration /temperature	zero	0.5 hr/20 °C		1 hr/20 °C		2 hrs/20 °C		3 hrs/20 °C		4 hrs/20 °C	
	Disinfectant	log count cfu/ml	log reduction cfu/ml									
			clean	dirty	clean	dirty	clean	dirty	clean	dirty	clean	**dirty**
	Hyperox	9 log$_{10}$	4	2.5	5.7	3.2	6.4	4.3	6.8	4.5	7.3	4.6
	Cidex		3.0	2.7	5.3	3.6	6	4.5	6.4	4.7	6.8	4.8
	Evans Hypochorite		2.8	1.4	4	2.3	4.5	2.8	4.9	3.2	5.3	3.5
	Bio Sentry 904		1.5	1	2.7	1.3	2.9	1.7	3.4	1.9	3.7	2.3
B	Exposure duration /temperature	zero	0.5 hr/40 °C		1 hr/40 °C		2 hrs/40 °C		3 hrs/40 °C		4 hrs/40 °C	
	Disinfectant	log count cfu/ml	log reduction cfu/ml									
			clean	dirty	clean	dirty	clean	dirty	clean	dirty	clean	dirty
	Hyperox	9 log$_{10}$	4.8	2.8	6.5	3.8	6.9	4.6	7.5	4.9	7.8	5.2
	Cidex		4.6	3.0	6.3	4.0	6.5	4.8	7.3	5.2	7.6	5.6
	Evans Hypochorite		3.5	2.0	4.8	2.7	5.3	3.2	5.6	3.6	5.9	3.9
	Bio Sentry 904		1.9	1.3	2.9	1.8	3.3	2.3	3.8	2.7	4.3	3.4
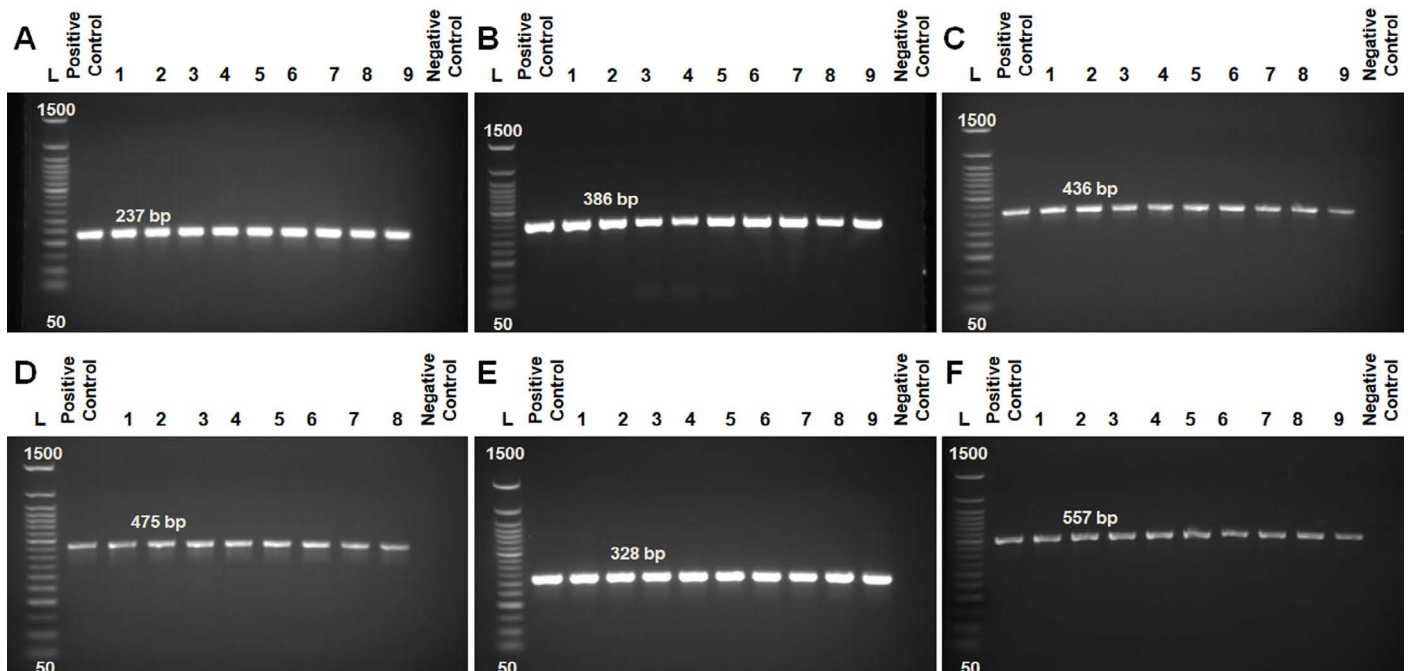
Figure 1: PCR products of amplified of hemolytic non-hemolytic enterotoxin virulent genes identified in *B. cereus* visualized on agarose gel electrophoresis. The expected molecular size of amplified DNA: 237 bp for *hblA* gene (A), 386 bp for *hblC* gene (B), 436 bp for *hblD* gene (C), 475 bp for *nheA* gene (D), 328 bp for *nheB* gene (E) and 557 bp for *nheC* gene (F). Lane 1-9: samples and Lane (L) DNA ladder 100 bp.

of examined samples were found. Other profiles of less common toxigenic genes were 2 (*hbl*++/*nhe*++), 3 (*hbl*++/*nhe*+) and 4 (*hbl*+/*nhe*++) with four, five and three strains, respectively. The toxigenic gene profile 6 included negative strains for all genes detected (*hbl*-/*nhe*-). In almost all profiles, isolated strains of water and milk-related samples were involved.

ANTIBIOGRAM PATTERN OF *B. CEREUS* ISOLATES

Antimicrobial susceptibilities for 10 antimicrobial agents commonly used in veterinary clinics and farms were assessed in 45 *B. cereus* isolates (Table 5 and Figure 2A and B). Irrespective of the origin (milk or milk products, water sources, environmental samples) of the isolates, they were generally resistant to Amoxicillin (68.9%), Ampicillin (62.2%), Penicillin (62.2%), Cephalexin (57.8%) and Tetracycline (51.1%). They were, however, susceptible to other

antimicrobials such as Gentamicin (75.6%), Ciprofloxacin (73.3%), Streptomycin (68.9%), Chloramphenicol (66.7%) and Erythromycin (62.2%). The rates of isolates that were resistant to five or more antibiotics from different origins were 61.5% (8/13) of milk-related samples, 50% (1/2) of water sources, 46.7% (14/30) of environmental samples, respectively.
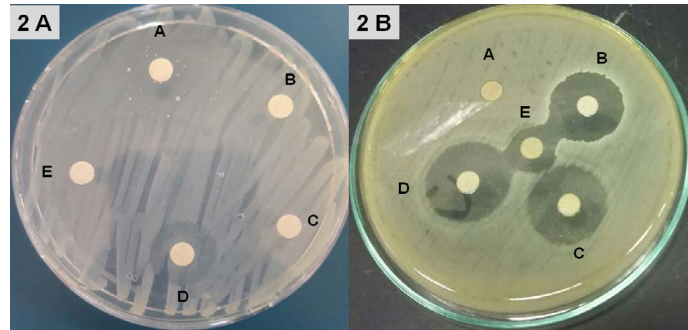

Figure 2: Fig. 2 A from A-E samples of disc diffusion test for Amoxicillin, Ampicillin, Cephalexin, Chloramphenicol, Penicillin; 2 B from A-E samples of disc diffusion test for Tetracycline, Ciprofloxacin, Gentamicin, Streptomycin, Erythromycin.

ANTIMICROBIAL ACTIVITY

Disinfectants: Upon exposure to Hyperox, *B. cereus* showed variable log reduction at various contact times under the clean /dirty dairy environment condition. The highest log reduction was 9.32, 10.3 after one /five minutes under clean condition, but an average log reduction of only 5.83, 6.87 after one /five minutes under dirty condition (Figure 3).
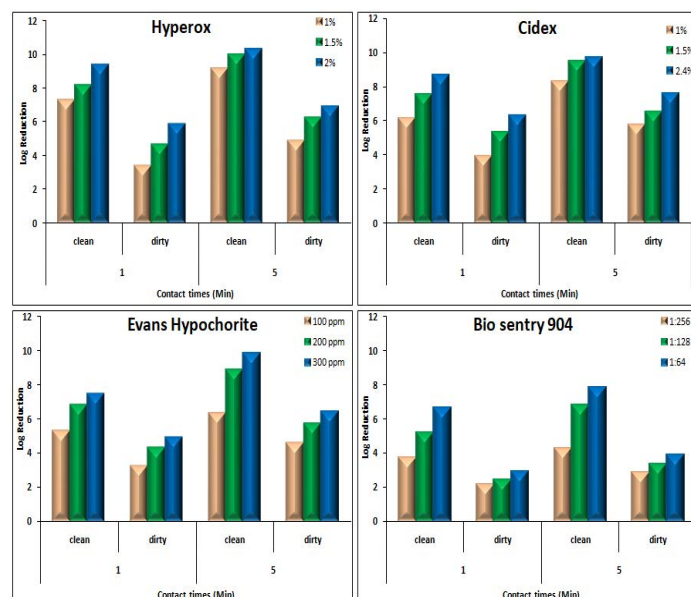

Figure 3: Log Reduction Values of Disinfectants-Treated *B. cereus* in suspension at various concentrations and contact times under clean /dirty dairy environment condition.

After exposure to CIDEX, *B. cereus* showed the lowest log

reduction at conc.1% under both clean conditions and dirty conditions at the two contact times. The highest log reduction was 9.7 which achieved at conc. 2.4% after 5 minutes exposure under clean condition while log reduction was sharply decreased to 7.56 under the dirty condition at the same concentration.

Hypochlorite showed log reduction of 7.43, 4.89 after 1 min, with an additional average log reduction of 2.41 1.49 following increasing contact times to 5 min under clean and dirty condition respectively at conc. 300 ppm.

In the presence of 4% milk, the efficacy of BioSentry as quaternary ammonium-based disinfectants was mostly affected. Even at the highest con. 1:64, it only reduced the bacterial load by 2.91, 3.88 after 1, 5 minutes respectively. However, it still keeps good activity against *B. cereus* in the absence of organic matter with log reduction 6.63, 7.84 at 1, 5 minutes using conc. 1:64.

The various disinfectants were more sporicidal in clean than on dirty conditions and in higher temperatures (40 ℃) than lowers (20 ℃) at various contact times. Results showed that Hyperox and Cidex had good sporicidal activity under clean condition with SE values ranging from 7.3-7.8 and 6.8 to 7.6 upon exposure time for 4 hrs at 20 & 40 ℃, respectively while adding milk as organic interfering substance resulted in declining the SE at all the contact times and temperatures with reduction log ranged from 5.2-5.6 and 4.6 to 4.8 (Table 6).

After exposure to Hypochlorite under clean condition, *B. cereus* spores showed log reduction of 2.8, 4, 4.5, 4.9 and 5.3 /20 ℃, while log reduction increased upon elevating the temperatures to 40 ℃ to be 3.5, 4.8, 5.3, 5.6, and 5.9 at exposure times 0.5, 1, 2, 3, 4 hr, respectively. In the presence of organic matter, the Sporicidal efficacy of Hypochlorite was markedly decreased at all contact times at both temperatures. Under the dirty condition, the lowest SE value was 1.4/0.5 hr/ 20 ℃ while the highest was 3.9/4hrs/40 ℃.

BioSentry disinfectants exhibited diminished sporicidal activity with SE ranged from 1.0 to 3.4 at 0.5-4hrs hr/20-40 ℃ under dirty condition while SE was slightly improved in the absence of organic load to be 1.5 to 4.3 at 0.5-4hrs hr/20-40 ℃.

The kills' kinetics of Hyperox, CIDEX, Hypochlorite, and BioSentry on *B. cereus* were shown in Table 6 and Figure 3.

Cow milk proteins hydrolysates: Cow milk proteins and its pepsin hydrolysates were evaluated for their antimicrobial activity against the isolates of recovered *B. cereus* using a bactericidal assay method as recorded in Figure 4. CCP,

CWP and pepsin hydrolysates (P-CCP and P-CWP) were potentially effective in inhibiting *B. cereus* growth with variable potency and in a dose-dependent manner. Their activity was expressed as log CFU/ml as a function of tested samples concentration incubated with *B. cereus* for 24 hrs. The intact proteins (CCP and CWP) exhibited little to moderate antimicrobial activity. While P-CWP and P-CCP were very effective against *B. cereus* resulting in a severe reduction in the CFU of bacteria (ten and eight log10 orders of killing) at a concentration of 2 mg/ml of P-CWP and P-CCP, respectively (Figure 4A and B).

Figure 4: Antibacterial activity of CCP and CWP and its pepsin hydrolysates against *B. cereus* at different concentrations. The data is presented as log CFU/ml. (Inset) Killing power of algae extracts (2000, 1000 and 500 μg/ml) against *Bacillus cereus*. The assays were performed in triplicate.

DISCUSSION

The current study revealed the ubiquitous distribution of *B. cereus* among the investigated dairy environment. Overall of 29.5 and 67% among dairy and environmental samples were positive for *B. cereus*. Our results are comparable to a similar study that was aimed to isolate *B. cereus* from a range of farm environments samples include milk, tap water, milking equipment rinse water, grass, and soil. They indicated that the incidence of *B. cereus* strains in environmental samples of 71.3% was much higher than the found values of 9.8% for raw milk samples (Cui et al., 2016). We agreed with their conclusion that *B. cereus* was widely distributed within the dairy farm environment with multiple sources for contamination of raw milk.

Our results highlighted that the prevalence of *B. cereus* in household raw milk was 37.5%. Comparable findings were reported by several studies performed in Ethiopia, Irish, Tunisia, Turkey and Egypt found that the prevalence of *B. cereus* in raw milk reached 12.86, 23, 47.5, 90.0 and 32%, respectively (Aouadhi et al., 2014; Gundogan and Avci,

2014; Naguib et al., 2014; Garedew et al., 2015; O'Connell et al., 2016).

The occurrence of *B. cereus* in dairy products including rice milk and street vendor milk were 36.4% and 30%, respectively which agreed well with other studies in Egypt and globally in different studies in USA, Turkey and Brazil reached 17.64, 20 and 24.23%, respectively (Gundogan and Avci, 2014; Montanhini and dos Santos Bersot, 2013; Reis et al., 2013).

B. cereus is a common soil bacterium that is often present in raw milk (Griffiths and Phillips, 1990). The results indicated the occurrences of *B. cereus* in used bedding material, pasture grass, and pasture soil were 37.5, 37.5, and 33.3%, respectively. Those finding are consistence with the previous study in which the prevalence of *B. cereus* isolates in bedding, feces, feed, and liquid manure were 93.3, 78.9, 41.2, and 100%, respectively (Cui et al., 2016).

The problem with the presence of *B. cereus* spores in raw milk is most prominent during the grazing period (Slaghuis et al., 1997) when there is a risk of the teats being contaminated by soil. The spore content of milk was found to be strongly associated with the degree of contamination of the teats with soil (Christiansson et al., 1999). It is very important to prevent the contamination of teats with soil during grazing because *B. cereus* spore concentrations in soil are approximately 100-fold higher than concentrations in other environmental carriers (feeds, feces, and bedding material) (Vissers et al., 2007).

The earlier study investigated spores of *B. cereus* in the grass, soil, milking equipment, and raw milk and found a significant positive correlation between the contents of spores in soil, feed, and feces were judged as being important possible sources of raw milk contamination (Labots et al., 1965). Large numbers of *Bacillus* spores were found in used bedding material (McKinnon and Pettipher, 1983) and bedding was suggested as a participant in the contamination route for *B. cereus* (Te Giffel et al., 1996; Slaghuis et al., 1997). The soil-milk route is dominant during the grazing of cows, whereas the feed-milk route is dominant during the housing of cows (Slaghuis et al., 1997; Christiansson et al., 1999).

Milking equipment was considered to be an important source of contamination in earlier investigations (Labots et al., 1965; McKinnon et al., 1990). Our findings cleared that the prevalence of *B. cereus* in milking equipment rinse water was 38.5%. Those results agreed with the former study which mentioned that elevated *Bacillus* spore contents in the rinse water from the milking equipment were observed and they found a positive correlation of *Bacillus* spore content between the bedding material and residual
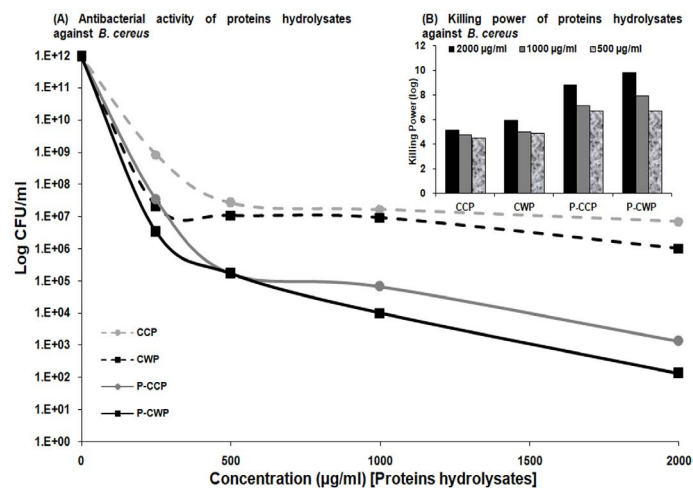
milk equipment rinse water (Magnusson et al., 2007).

Overall, the possible sources of raw milk contamination that were considered are soil, feces, bedding, feed, air, and milking equipment (Van Heddeghem and Vlaemynck, 1992). The isolation rates of *B. cereus* from different sources were high; particularly the detection rate in bedding samples even reached 93.3% (Cui et al., 2016). The most important contamination source found was the bedding material, which contained large numbers of spores and contaminated the milk via contaminated teats. A positive correlation was found between the *B. cereus* spore content in used bedding material and milk (Magnusson et al., 2007).

According to our results, the air in the barn is not considered a source of raw milk contamination with *B. cereus*, which agreed well with previous studies which have shown that the number of *B. cereus* spores in the air was too small to be of major importance source (Te Giffel et al., 1996; Christiansson et al., 1999; Magnusson et al., 2007).

The results clarified that *hblA*, *hblC* and *hblD* were detected in 42.2, 26.7 and 24.4%, respectively and the *nheA*, *nheB* and *nheC* occurred in 31.1, 33.3 and 24.4%, respectively among *B. cereus* isolates from examined samples. The most common toxin profile found among the latter isolates was toxin profile 5 (*hbl+*/*nhe+*), where strains isolated from most types of examined samples were found. The occurrence of *hblACD nheABC*, observed in this study is consistent with previous studies (Jeßberger et al. 2014; Arslan et al., 2014). Also, the positive rates for *nhe* (100%) and *hbl* (55%) in raw milk samples were higher than those found for 54 pasteurized full fat milk samples in China, where the positive rates of *nhe* and *hbl* were approximately 62.0 and 37.0%, respectively (Zhou et al. 2008).

The antibiotic resistance profiles of *B. cereus* isolates are of concern for public health (Cui et al., 2016). All isolates were tested for antimicrobial resistance against 10 antibiotics. *B. cereus* isolates showed resistance to Amoxicillin, Ampicillin, Penicillin, Cephalexin and Tetracycline with 68.9, 62.2, 62.2, 57.8 and 51.1%, respectively. Multidrug resistance was detected among isolates in 8 milk-related samples, one of the water sources and 14 of environmental samples, respectively. The results of antimicrobials' susceptibility tests were generally consistent with the previous studies from other countries (Luna et al., 2007; Chaves et al., 2011). *B. cereus* group strains were found to be sensitive to chloramphenicol, ciprofloxacin, gentamicin, rifampicin, and vancomycin (Luna et al., 2007), and clinical and environmental *B. cereus* isolates showed a high probability of resistance to erythromycin and tetracycline (Fernandez-Fuentes et al., 2014; Merzougui et al., 2014).

The results indicated that Hyperox and CIDEX have extremely rapid bactericidal activity at higher concentrations 2 and 2.4% after five minutes of exposure times under clean condition resulting in 10.3 and 9.7 log reduction, respectively. Decreasing concentrations and adding organic substances results in the marked decline of the bactericidal activity for both disinfectants. Hypochlorite was still keeping good bactericidal activity against *B. cereus* at 300 ppm/5minutes under clean condition resulted in 9.82 log reduction, however, after adding 4% milk, the activity decreased which showed 6.38 log reduction.

BioSentry exhibited less potent activity against *B. cereus*. Even at higher concentrations 1:64/5 minutes, the organic matter sharply hinders its activity which only resulted in 3.88 log reduction. This agreed well with other studies reported that the efficacy of a quaternary ammonium compound (QAC) disinfectant was reduced by two to three logs in the presence of feces as compared to water (Berchieri and Barrow, 1996). Organic matter is one of the most important factors which interfere with the activity of a disinfectant. Disinfectants should be used after thoroughly removing the organic matter as it provides a physical barrier and protects microorganisms from contact with disinfectants (Stringfellow et al., 2009).

B. cereus spores have been isolated from dairy farm sources, including rinse water from the milking equipments, used bedding (Magnusson et al., 2007), teats contaminated with soil, dirty alleys (Christiansson et al., 1999), feces and silage (Vissers et al., 2007). When cows are kept indoors, spores in used bedding are a major source of contamination of milk via contaminated teat and udder surfaces (Magnusson et al., 2007). However, when cows are grazing outdoors, contamination of teats with soil is the main route of milk contamination (Christiansson et al., 1999).

Poor cleaning and disinfection may lead to greater numbers of spores present in the milk (Magnusson et al., 2007). The inactivation of *Bacillus* spores remains a significant challenge for the food industry (Soni et al., 2016). *B. cereus* spores are highly resistant to dehydrating and heating treatments and during food preparation, spores may germinate, producing food spoilage or food poisoning (Logan, 2012).

Disinfection of surfaces contaminated with bacterial endospores is not only an issue of bio-security but also an issue faced repeatedly in clinical settings (Rutala et al., 1998). The data highlighted that Hyperox peracetic acid-based disinfectants exhibited high sporicidal activity against *B. cereus* spores under clean conditions at various contact times at both tested temperatures. The Highest SE value was 7.8 which achieved at 4hrs/40 ℃ while the lowest was 4 at 0.5 hr/20 ℃. Even, when 4% of milk was added to the

Hyperox solutions, it still showed a satisfactory sporicidal activity at longer contact times with elevated temperatures. At 4hrs/ 20 & 40 ℃ under dirty condition, SE values were 4.6 and 5.2, respectively. However, at shorter contact times even at higher temperatures, SE values still low. After 30 minutes of contact time at 20 & 40 ℃, the spores load was reduced only by 2.50 and 2.8 logs, respectively.

CIDEX glutaraldehyde-based disinfectants had a comparable activity to Hyperox on spores of *B. Ceruse*. CIDEX exhibited lower SE values than Hyperox under clean conditions at various exposure times/temperatures. Under the dirty condition, Cidex showed similar or slightly higher SE values than Hyperox. At 4hrs/ 20 & 40 ℃ under dirty condition, SE values were 4.8, 5.6 and 4.6, 5.2 for CIDEX and Hyperox respectively.

In the presence of 4% milk, the efficacy of Sodium Hypochlorite was affected to some extent. It was unable to achieve good SE after 0.5hr /20 ℃ under both clean and dirty conditions. Increasing the exposure times to 1, 2, 3 and 4hrs under clean condition/20 ℃, the kill rate got better and the spores load was reduced by 4, 4.5, 4.9 and 5.3 logs respectively. Under dirty condition the efficacy was markedly reduced after 1, 2, 3 and 4hrs, The SE was 2.3, 2.8, 3.2, and 3.5 log kill was observed. Elevating temperatures to 40 ℃ resulted in improving the efficacy at all the various contact times under both clean and dirty conditions. However, organic load still represents an obstacle even after rising the temperatures /40 ℃, the efficacy was still low after 1, 2, 3 and 4hrs, with SE was 2.7, 3.2, 3.6, and 3.9 log reductions which highlighted the importance of cleaning and sanitation procedures to eliminate the organic load before the application of the chemical disinfectants. Quaternary ammonium-based BioSentry disinfectants showed the lowest sporicidal efficacy against *B. cereus* spores at all contact times under both clean and dirty conditions. Even after increasing the exposure times and elevating the temperatures, an only slight improvement in the efficacy with minor addition log reduction was observed. We assumed that *B. cereus* spores had a more resistant ability to BioSentry than other disinfectants.

Protecting human health from the harmful effects of pathogenic *B. cereus* has become extremely challenging. The adverse effects of chemical preservatives on human health, their limited application, susceptibility, toxicity, and microbial resistance increase the demand to search for potentially effective, healthy, safer and natural antimicrobial agents with a unique mechanism of action against the dreadful pathogens. Thus, antimicrobial activity of cow milk proteins and their pepsin hydrolysates can provide a key aspect of treatment of *B. cereus* infections than traditional antimicrobial agents cannot provide, and be used as natural preservatives to ensure healthy and safe food with-

out the unpleasant side effects of chemical preservatives. We have shown that pepsin hydrolysates of cow milk proteins exerted strong bactericidal activity, dose-dependently, against *B. cereus*. Notably, *B. cereus* killing by P-CWP and P-CCP were observed at concentrations as low as 2 mg/ml. P-CWP and P-CCP were potentially effective against tested pathogens with variable potency (ten and eight log10 orders of killing).

Various studies evaluated the antimicrobial activities of the cow milk proteins hydrolysates viz., *P*-CWP and P-CCP. Different enzymes viz., pepsin, trypsin, α-chymotrypsin, enzymes of animal, microbial, or plant origin (papain, alcalase, flavourzyme, pronase, ficin, thermolysin and neutrase) are used to break large polypeptides into specific small peptides to investigate its antimicrobial activity against both Gram-positive and Gram-negative bacteria including *B. cereus* and *B. subtilis* (Benkerroum, 2010; Abd El-Fattah et al., 2017; El-Sayed and Awad, 2019). Antimicrobial activity of pepsin hydrolysates of milk proteins has been reported with clear inhibitory effects on various pathogenic cultures especially *B. cereus* (Almaas et al., 2011; Mohanty et al., 2014; Kumari and Vij, 2015).

CONCLUSION

The milking environment in the household rearing of cattle in Sohag governorate, Egypt was deemed to be unsafe. High humidity, mud and animal excretion were present in all milking areas. Before, during and after milking, the skin of the udder and milkers' hands were not cleaned or disinfected. There is lacking of the basic requirements and sanitary measures. The milk was transported and distributed from farmers' houses to local dairy shops or consumers without cooling or heat treatment. The practices described could lead to contamination of raw milk and milk products on the farm with *B. cereus*. Other sources of contamination are contaminated water, udder infections and environmental contamination in cow's house environment and local dairy shops. Strict sanitary measures and disinfection practices should be followed to reduce the initial raw water-milk contamination level to avoid subsequent recontamination of dairy products. Close monitoring and adhering to the national and international regulation of antibiotic usage is crucial to minimize the amplification and dissemination of resistant strains.

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CONFLICT OF INTEREST

No conflicts of interests are announced by authors.

AUTHORS CONTRIBUTION

All authors contributed in writing of this research article. All authors contributed in collection of samples and traditional identification of *B. cereus*, and contributed in molecular characterization of isolated strains, antimicrobial activity of peptides and disinfectant.

REFERENCES

• Abd El-Fattah AM, Sakr SS, El-Dieb SM, Elkashef HAS (2017). Bioactive peptides with ACE-I and antioxidant activity produced from milk proteolysis. Int. J. Food Prop. 20(12): 3033-3042. https://doi.org/10.1080/10942912.2016.1270963

• Ahmed SA, Tawfik E, Laila M, Hisham RI (2015). Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res. Int. 74: 80-88. https://doi.org/10.1016/j.foodres.2015.04.032

• Ahmed SA, Diab HM, Alkahtani MA, Alshehri MA, Saber H, Badr H, Dandrawy MK, El-Mansi AA, Shati AA, Ahmed AE (2020). Molecular epidemiology of virulent *E. coli* among rural small scale dairy herds and shops: Efficacy of selected marine algal extracts and disinfectants. Int. J. Environ. Health Res. 15: 1-23. https://doi.org/10.1080/09603123.2020.1727422

• Akalın AS (2014). Dairy-derived antimicrobial peptides: Action mechanisms, pharmaceutical uses and production proposals. Trends Food Sci. Technol. 36: 79-95. https://doi.org/10.1016/j.tifs.2014.01.002

• Almaas H, Eriksen E, Sekse C, Comi I, Flengsrud R, Holm H, Vegarud GE (2011). Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. Brit. J. Nutr. 106(6): 896-905. https://doi.org/10.1017/S0007114511001085

• Anonymous. Cleaning and Disinfection of premises (2005). University of Georgia Athen. http://www.agrosecurity.uga.edu/annexes/Annex09_Cleaning.pdf Accessed August, 2012.

• Aouadhi C, Maaroufi A, Mejri S (2014). Incidence and characterisation of aerobic spore-forming bacteria originating from dairy milk in Tunisia. Int. J. Dairy Technol. 67(1): 95-102. https://doi.org/10.1111/1471-0307.12088

• Arslan S, Eyi A, Küçüksarı R (2014). Toxigenic genes, spoilage potential, and antimicrobial resistance of *Bacillus cereus* group strains from ice cream. Anaerobe. 25: 42-46. https://doi.org/10.1016/j.anaerobe.2013.11.006

• Banykó J, Vyletělová M (2009). Determining the source of Bacillus cereus and Bacillus licheniformis isolated from raw milk, pasteurized milk and yoghurt. Lett. Appl. Microbiol. 48(3): 318-323. https://doi.org/10.1111/j.1472-765X.2008.02526.x

• Bauer AW, Kerby WM, Sherris JC, Truck M (1966). Antibiotic susceptibility testing by a standardized single disc method. Am. J. Pathol. 45: 493-496. https://doi.org/10.1093/ajcp/45.4_ts.493

• Benkerroum N (2010). Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. Int. J. Dairy Technol. 63(3): 320-338. https://doi.org/10.1111/j.1471-0307.2010.00584.x

• Berchieri JrA, Barrow PA (1996). The antibacterial effects for *Salmonella Enteritidis* phage type 4 of different chemical disinfectants and cleaning agents tested under different conditions. Avian Pathol. 25(4): 663-673. https://doi.org/10.1080/03079459608419173

• Bottone EJ (2010). *Bacillus cereus*, a volatile human pathogen. Clin. Microbiol. Rev. 23: 382-98. https://doi.org/10.1128/CMR.00073-09

• BSI. BS EN 1276:1997 (1997). Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas — Test method and requirements (phase 2, step 1). British Standards Institute, London, UK 1997.

• BSI. BS EN 13704:2002 (2002). Chemical Disinfectants-Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas-Test method and requirements (phase 2, step 1). British Standards Institute, London, UK 2002.

• BSI. BS EN 14885:2006 (2006). Chemical disinfectants and antiseptics Application of European Standards for chemical disinfectants and antiseptics. British Standards Institute, London, UK 2006.

• Caamaño-Antelo S, Fernández-No IC, Böhme K, Ezzat-Alnakip M, Quintela-Baluja M, Barros-Velázquez J, Calo-Mata P (2015). Genetic discrimination of foodborne pathogenic and spoilage Bacillus spp. based on three housekeeping genes. Food Microbiol. 46: 288-298. https://doi.org/10.1016/j.fm.2014.08.013

• Chaves JQ, Pires ES, Vivoni AM (2011). Genetic diversity, antimicrobial resistance and toxigenic profiles of Bacillus cereus isolated from food in Brazil over three decades. Int. J. Food Microbiol. 147(1): 12-16. https://doi.org/10.1016/j.ijfoodmicro.2011.02.029

• Christiansson A, Bertilsson J, Svensson B (1999). *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. J. Dairy Sci. 82: 305–314. https://doi.org/10.3168/jds.S0022-0302(99)75237-9

• Clegg FG, Chiejina SN, Duncan AL, Kay RN, Wray C (1983). Outbreak of Salmonella newport infection in dairy herds and their relationship to management and contamination of the environment. Vet. Rec. J. 112(25): 580-4. https://doi.org/10.1136/vr.112.25.580

• Clinical and Laboratory Standards Institute (CLSI) (2007). Performance standards for antimicrobial disk susceptibility tests. Vol. 27, Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

• Cown WB, Kethley TW, Fincher EL (1957). The Critical-Orifice Liquid Impinger as sampler for bacterial aerosols. J. Appl. Microbiol. 5(2): 119-124.

• Cui Y, Liu X, Dietrich R, Märtlbauer E, Cao J, Ding S, Zhu K (2016). Characterization of Bacillus cereus isolates from local dairy farms in China. FEMS microbiology letters. 363(12): fnw096 https://doi.org/10.1093/femsle/fnw096

• El-Sayed M, Awad S (2019). Milk Bioactive Peptides: Antioxidant, Antimicrobial and Anti-Diabetic Activities. Adv. Biochem. 7(1): 22. https://doi.org/10.11648/j.ab.20190701.15

• Espigares E, Bueno A, Fernández-Crehuet M, Espigares

M (2003). Efficacy of some neutralizers in suspension tests determining the activity of disinfectants. J. Hosp. Infect. 55(2): 137-140. https://doi.org/10.1016/S0195-6701(03)00238-X

• Fernández-Fuentes MA, Abriouel H, Morente EO, Pulido RP, Gálvez A (2014). Genetic determinants of antimicrobial resistance in Gram positive bacteria from organic foods. Int. J. Food Microbiol. 172: 49-56. https://doi.org/10.1016/j.ijfoodmicro.2013.11.032

• Gao T, Ding Y, Wu Q, Wang J, Zhang J, Yu S, Chen M (2018). Prevalence, virulence genes, antimicrobial susceptibility, and genetic diversity of Bacillus cereus isolated from pasteurized milk in China. Front. Microbiol. 9: 533. https://doi.org/10.3389/fmicb.2018.00533

• Garedew L, Mengesha D, Birhanu A, Mohammed A (2015). Diverse Gram-positive bacteria identified from raw and pasteurized cow milk consumed at Gondar town and its environs, Ethiopia. Ethiopian Vet. J. 19(1): 49-61. http://dx.doi.org/10.4314/evj.v19i1.3

• Griffiths MW, Phillips JD (1990). Incidence, source and some properties of psychrotrophic *Bacillus* found in raw and pasteurized milk. J. Soc. Dairy Technol. 43: 62-66. https://doi.org/10.1111/j.1471-0307.1990.tb02446.x

• Gundogan N, Avci E (2014). Occurrence and antibiotic resistance of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* in raw milk and dairy products in Turkey. Int. J. Dairy Technol. 67(4): 562-569. https://doi.org/10.1111/1471-0307.12149

• Harrigan WF (1998). Laboratory Methods in Food Microbiology, 3rd ed, Academic Press, London, 532 Pages.

• Hernández-Ledesma B, García-Nebot MJ, Fernández-Tomé S, Amigo L, Recio I (2013). Dairy protein hydrolysates: Peptides for health benefits. Int. Dairy J. 1-19. http://dx.doi.org/10.1016/j.idairyj.2013.11.004

• Jeßberger N, Dietrich R, Bock S, Didier A, Märtlbauer E (2014). *Bacillus cereus* enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. Toxicon. 77: 49-57. https://doi.org/10.1016/j.toxicon.2013.10.028

• Kumari S, Vij S (2015). Effect of bioactive peptides derived from fermented whey based drink against food borne pathogens. Int. J. Curr. Microbiol. Appl. Sci. 4(3): 936-941.

• Labots H, Hup G, Galesloot TE (1965). *Bacillus cereus* in raw and pasteurized milk. III. The contamination of raw milk with *B. cereus* spores during its production. Neth. Milk Dairy J. 19: 191–221.

• Lane V, McKeever JD, Fallon M (1966). Buffered glutaraldehyde (Cidex). A new disinfectant specially useful in urology. J. Ir. Med. Assoc. 58: 131-2.

• Logan NA (2012). *Bacillus* and relatives in foodborne illness. J. Appl. Microbiol. 112: 417-429. https://doi.org/10.1111/j.1365-2672.2011.05204.x

• Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT, Cattani J (2007). Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. J. Antimicrob. Chemother. 60(3): 555-567. https://doi.org/10.1093/jac/dkm213

• Magnusson M, Christiansson A, Svensson B (2007). *Bacillus cereus* spores during housing of dairy cows: factors affecting contamination of raw milk. J. Dairy Sci. 90: 2745–2754. https://doi.org/10.3168/jds.2006-754

• Marquis RE, Rutherford GC, Faraci MM. Shin SY (1995). Sporicidal action of peracetic acid and protective effects of transition metal ions. J. Ind. Microbiol. 15: 486-92. https://doi.org/10.1007/BF01570019

• McKinnon CH, Pettipher GL (1983). A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-forming bacteria. J. Dairy Res. 50(2): 163-170. https://doi.org/10.1017/S0022029900022962

• McKinnon, CH, Rowlands GJ, Bramley AJ (1990). The effect of udder preparation before milking and contamination from the milking plants on bacterial numbers in bulk milk of eight dairy herds. J. Dairy Res. 57: 307-318. https://doi.org/10.1017/s0022029900026959

• Merzougui S, Lkhider M, Grosset N, Gautier M, Cohen N (2014). Prevalence, PFGE typing, and antibiotic resistance of *Bacillus cereus* group isolated from food in Morocco. Foodborne Pathogens Dis. 11(2): 145-149. . https://doi.org/10.1089/fpd.2013.1615

• Mohamed AS, Alnakip ME, Aal SF (2016). Occurrence of *Bacillus cereus* in raw milk and some dairy products in Egypt. Japanese J. Vet. Res. 64(Supplement 2): S95-S103.

• Mohanty DP, Tripathy P, Mohapatra S, Samantaray DP (2014). Bioactive potential assessment of antibacterial peptide produced by Lactobacillus isolated from milk and milk products. Int. J. Curr. Microbiol. Appl. Sci. 3: 72–80.

• Montanhini MTM, dos Santos Bersot L (2013). Evaluation of psychrotrophic behavior and lipolytic and proteolytic activity of Bacillus cereus isolated from refrigerated dairy products. Acta Scientiar. Technol. 35(1): 163-174. https://doi.org/10.4025/actascitechnol.v35i1.13752

• Naguib, AM, Osman, KM, Ahmed ZAM, Marouf S (2014). Isolation and identification of bacillus species recovered from different milk sources. J. Egypt. Vet. Med. Assoc. 74(5): 939-947.

• O'Connell A, Lawton EM, Leong D, Cotter P, Gleeson D, Guinane CM (2016). Detection of presumptive Bacillus cereus in the Irish dairy farm environment. Irish J. Agric. Food Res. 55(2):145-151. https://doi.org/10.1515/ijafr-2016-0014

• Ombui JN, Gitahi JN, Gicheru M (2008). Direct detection of Bacillus cereus enterotoxin genes in food by multiplex Polymerase Chain Reaction. Int. J. Integrat. Biol. 2(3): 172-81.

• Reis AL, Montanhini M, Bittencourt JV, Destro MT, Bersot LS (2013). Gene detection and toxin production evaluation of hemolysin BL of Bacillus cereus isolated from milk and dairy products marketed in Brazil. Brazilian J. Microbiol. 44(4). 1195-1198. https://doi.org/10.1590/s1517-83822013000400024

• Rendos JJ, Ebehart RJ, Kesler EM (1975). Microbial populations of teat ends of dairy cows and bedding materials. J. Dairy Sci. 58(10): 1495-1500. https://doi.org/10.3168/jds.S0022-0302(75)84740-0

• Russell AD (1981). Neutralization procedures in the evaluation of bactericidal activity. In: Collins CH, Allwood MC, Bloomfield SF, Fox A, eds. Disinfectants: their use and evaluation of effectiveness, London: Academic Press, 45-59.

• Russell AD (1990). Bacterial spores and chemical sporicidal agents. Clin. Microbiol. Rev. 3: 99-119. https://doi.org/10.1128/CMR.3.2.99

• Rutala WA, Gergen MF, Weber, DJ (1998). Comparative evaluation of the sporicidal activity of new low-temperature sterilization technologies: ethylene oxide, 2 plasma

sterilization systems, and liquid peracetic acid. Am. J. Infect. Control. 26: 393-398. https://doi.org/10.1016/S0196-6553(98)70034-3

• Sambrook J, Fritsch EF, Montias T (1989). Molecular Biology. In: Molecular cloning. Laboratory manual, 2nd ed. USA: Cold Spring Harbor Laboratory press; P. 268.

• Sánchez-Chica J, Correa MM, Aceves-Diez AE, Castañeda-Sandoval LM (2020). A novel method for direct detection of *Bacillus cereus* toxin genes in powdered dairy products. International Dairy J. 103: 104625. https://doi.org/10.1016/j.idairyj.2019.104625

• Sindayikengera S, Xia W (2005). Milk Biologically Active Components as Nutraceuticals: Review, Critic. Rev. Food Sci. Nutr. 45(7-8): 645-656. https://doi.org/10.1080/10408690490911756

• Singh VK, Shukla S, Chaturvedi A (2015). Study the incidence of Bacillus cereus isolates from dairy foods. Pharma Innovat. 3(12, Part A): 41.

• Slaghuis BA, Te Giffel MC, Beumer RR, Andre G (1997). Effect of pasturing on the incidence of *Bacillus cereus* spores in raw milk. Int. Dairy J. 7: 201-205. https://doi.org/10.1016/S0958-6946(97)00012-5

• Soni A, Oey I, Silcock P, Bremer P (2016). *Bacillus* spores in the food industry: a review on resistance and response to novel inactivation technologies. Compr. Rev. Food Sci. Food Saf. 15: 1139–48. https://doi.org/10.1111/1541-4337.12231

• Stringfellow K, Anderson P, Caldwell D, Lee J, Byrd J, McReynolds J, ... & Farnell M (2009). Evaluation of disinfectants commonly used by the commercial poultry industry under simulated field conditions. Poult. Sci. 88(6): 1151-1155. 1 https://doi.org/0.3382/ps.2008-00455

• Te Giffel MC, Beumer RR, Slaghuis BA, & Rombouts FM (1996). Occurrence and characterization of (psychrotrophic) Bacillus cereus on farms in the Netherlands. In Symposium on Bacteriological Quality of Raw Milk, Wolfpassing, Austria 13-15 March 1996. Proceedings. (pp. 40-45). International Dairy Federation.

• Te Giffel MC, Beumer RR, Van Dam WF, Slaghuis BA, Rombouts FM (1995). Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment. Int. Biodeteriorat. Biodegrad. 36(3-4): 421-430. https://doi.org/10.1016/0964-8305(95)00104-2

• Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P (2000). Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J. Appl. Microbiol. 89: 330-8. https://doi.org/10.1046/j.1365-2672.2000.01114.x

• Van Heddeghem A, Vlaemynck G (1992). Sources of contamination of milk with Bacillus cereus on the farm and in the factory. Bulletin-Int. Dairy Federation. 19-19.

• Varadaraj MC (1993). Methods for detection and enumeration of food born bacterial pathogens. A critical evaluation. J. Food Sci. Technol. 30: 1-13.

• Vissers MMM, Te Giffel MC, Driehuis F, De Jong P, Lankveld JMG (2007). Predictive modeling of Bacillus cereus spores in farm tank milk during grazing and housing periods. J. Dairy Sci. 90: 281–292. https://doi.org/10.3168/jds.S0022-0302(07)72629-2

• Wehr HM, Frank JF (2004). Standard methods for the examination of dairy products, 17th ed. American Public Health Association. Washington, D.C. ISBN: 978-0-87553-002-4.

• WHO "World Health Organization" (1971). International standards for drinking water, Plais de Nation, 3rd Ed, Geneva.

• Yang IC, Shih DY, Huang TP, Huang PY, Wang JY, Pan TM (2005). Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. J. Food Prot. 68(10): 2123-2130. https://doi.org/10.4315/0362-028x-68.10.2123

• Yusuf U, Kotwal SK, Gupta S, Ahmed T (2018). Identification and antibiogram pattern of Bacillus cereus from the milk and milk products in and around Jammu region. Vet. World. 11(2): 186. https://doi.org/10.14202/vetworld.2018.186-191. Epub 2018

• Zhou G, Liu H, He J, Yuan Y, Yuan Z (2008). The occurrence of *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. Int. J. Food Microbiol. 121(2): 195-200. https://doi.org/10.1016/j.ijfoodmicro.2007.11.028