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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(1): 550-559. **OPEN ACCESS**

Incidence of *Bacillus cereus* in Egyptian foods and its control by probiotics

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There is currently a high incidence of the enterotoxigenic *Bacillus cereus (B.cereus)* in Egyptian foods. One hundred food samples were collected; 20 samples from each food type such as corn snacks (CS), Koshary (K), beef Lancheon (BL), Karish cheese (KC) and beef burger (BB). After microbiological investigation, about 36 bacterial isolates were obtained with incidence percentage of about 10%, 10%; 7%, 4%; 5% in CS; K; BL; KC; BB respectively. These bacterial isolates were characterized and identified as *B.cereus* except for isolates CS1; CS14; K18, BL7 which showed to be other species. All the *B.cereus* strains produced protease, amylase and gelatinase, but most of them produced both hemolysin and lecithinase as virulent factors. Cell free supernatant fluids (CFS) of some probiotic bacteria inhibited the *B.cereus* bacteria *in vitro*.

Keywords: *Bacillus cereus*, Egyptian foods, Identification, Probiotics

INTRODUCTION

Bacillus cereus is Gram positive, rod-shaped, facultative anaerobic, motile (flagellated), beta-hemolytic bacterium; commonly found in nature and proliferates in different habitats like soil, decaying organic matter, vegetation, fresh and marine water and the invertebrate gut. Among these habitats is "food" such as beef luncheon, raw cow milk, row cow meat and karish cheese (Abdou et al. 2012) It is spore forming bacterium which is able to grow at a wide range of temperature (8 – 55ºC), pH (4.3 to 9.3) and water activity (0.912 to 0.450) (Adams and Moss, 1995)

Bacillus cereus is the causative agent of two distinct food poisoning syndromes: the diarrheal and emetic illnesses. The emetic food poisoning syndrome is mediated by a small temperature stable, preformed peptide called cereulide (Horwood et al. 2004). Cereulide is a cyclic peptide and its small size and non-antigenicity makes it difficult to produce a reliable toxin detection method (Horwood et al. 2004). Cereulide is produced by a non-ribosomal peptide synthetase complex (Horwood et al. 2004).

Production of the emetic toxin has been shown to occur in skim milk within the temperature range of 12ºC–37°C, with more toxin produced at 12ºC and 15°C compared to higher temperatures (Finlay et al. 2000). The emetic toxin is highly resistant to environmental factors, showing stability from pH 2–11 and during heating to 100°C for 150 minutes (pH 8.7–10.6) (Jenson and Moir, 2003).

The diarrheal illness is caused by three types of enterotoxins which are haemolysin BL, non-haemolysin enterotoxin and cytotoxin K. After consumption of food contaminated with spore-forming *B. cereus*, the spores pass the stomach, reach the small intestine where they germinate

and grow. Also potentially some vegetative cells survive in the stomach and grow in the small intestine. During growth in the small intestine, enterotoxins are produced (Wijnands et al. 2009).

The bicontrol of the *B.cereus* pathogen is of interest and there is a need to continue research to find out novel effective protocols to inhibit such pathogen *in vitro* and in food. In this regard, probiotics showed promising results in controlling *B.cereus* growth. (Enan et al.2015). The inhibitory activity of the probiotic lactic acid bacteria is due to the metabolites of such probiotics such as lactic acid and other organic acids, diacetyl acetaldehyde, ethanol and bacteriocins (Sahar-Eissa et al. 2016).

The present work was undertaken to study the incidence of *B.cereus* in Egyptian foods and to control the growth of this pathogen by probiotic lactic acid bacteria.

MATERIALS AND METHODS

Media and media preparation:

B.cereus selective agar, (Oxoid) was used for selective isolation of the suspected *B.cereus* bacterium. The *Bacillus cereus* selective agar is composed of: agar, 2.0 g; D-mannitol, 1.0 g; (NH4)2PO4, 0.1 g; KCl, 0.02 g; MgSO 4. 7H 2 O, 0.02 g; Yeast extract, 0.02 g; Bromocresol purple, 0.004 g and 10 mI of 20% egg yolk emulsion (Ranald, 1996). All these components were dissolved in 100 ml of distilled water, (Enan et al., 2012).

To prepare the egg yolk emulsion, 12 eggs were soaked in 1:100 dilution of saturated mercuric chloride solution for 1 min. Eleven eggs were cracked and yolks were separated from whites. The egg yolks were then mixed with the 12th egg (white and yolk) and mixed. Twenty milliliters of egg yolk mixture was added to 80 mL of 0.9% NaCl solution. This was then mixed thoroughly. The emulsion was warmed to 45 – 50^{o}C. The medium components except egg yolk emulsion were added to 100 mL of distilled water. The mixture was autoclaved at 121^{o}C for 15 min and then cooled to 45 – 50^{o}C. Ten milliliters of sterile egg yolk emulsion (20%) was added aseptically and mixed thoroughly. The medium was then poured into sterile Petri dishes and incubated for 24 h at 37^{o}C to ensure sterility, (Enan et al. 2012).

Sample Collection:

Different food samples such as corn snaks (CS), Koshary (K), beef luncheon (BL), Karsh cheese (KC) and beef burger (BB) were collected from local marks from Egypt; food samples including 20 ones from each food type were collected. These samples were wrapped in sterile white polythene bags as soon as they were bought and brought to the laboratory. They were used immediately .

Isolation of bacteria:

After the samples were collected and brought to the laboratory, one gram of each sample was ground in a sterile mortar and re-suspended in 5mL of sterile distilled water. *Bacillus cereus* strains were isolated onto *Bacillus cereus* selective medium by inoculating 0.1 mL of the food suspension onto *Bacillus cereus* agar and incubating for 24 h at 37^{o}C. Resulting colonies were purified on the same medium and the pure colonies were streaked onto slope cultures of nutrient agar medium (Oxoid).

Identification of bacterial isolates:

The obtained bacterial isolates were subjected to identification tests regarding Gram staining, cell morphology, spore staining, oxidase and catalase tests according to Krieg and Holt (1984). The identification at the species level was completed by API *Bacillus* cereus kits (Biomereux Company, Montalieu, France).

Extracellular enzyme production:

The identified isolates were tested for extracellular enzyme production according to the following: gelatinase activity, pectiolytic activity, amylase, and catalase and oxidase (Krieg and Holt, 1984).

Test for virulence factor:

The ability of *B.cereus* isolates to produce virulence factors (hemolysin, lecithinase and protease) was determined using an agar well diffusion assay according to Misra and Kuila (1992). About 24h old bacterial cultures were centrifuged at 3000 rpm for 15 to 20 min, neutralized with 5 mol//L NaOH to the final pH-7.0 and then filler sterilized (pore size 0.45µm). Wells of 5.0 mm diameter were made on solidified blood agar, egg ylk agar, and casein agar plates and filled with 50µl aliquots of filter-sterilized bacterial culture filtrates. Plates were incubated at 37^{o}C for 24 h and the diameters of clear zones around the wells were measured.

Inhibition of B.cereus by CFS from some probiotic bacteria:

Probiotic cultures which were previously identified were used such as *Lact. plantarum* LPS10 isolated from pickles (Abdel-Haliem et al., 2016), *Ent.Bacium* NM_2 isolated from urine of healthy man (Enan et al., 2014a,b), *L. lactisssp. lactis* Z_1 isolated from yoghurt (Enan et al., 2013a) and *Lact. delbreukisspbulgaricus* Z_{11} isolated from yoghurt (Enan et al., 2013b) were used as they showed to produce inhibitory substances such as bacteriocins and organic acids (Abdel-Shafi et al. 2014).

CFS fluids were collected from cultures grown for 18h at 30^{o}C in MRS broth (De Man et al., 1960) by centrifuging cultures at 10.000 rpm for 10 minutes. These CFS fluids were sterilized by filtration using Millipore filters (0.25µm) and added at 2% v/v to broths containing either *B.cereus* K20 or *B.cereus* BB15 (2x10^{4}CFU/mL). Samples and uninoculated controls were incubated at 30^{o}C. After appropriate time intervals, aliquots (1mL) of the inoculated samples were withdrawn, serially diluted and CFU/mL of *B.cereus* were counted onto *B.cereus* selective agar plates.

RESULTS

Collection of food samples and isolation of bacteria suspected to be *B .cereus*:

About four food types of Egyptian foods were choosedviz, corn snacks (CS), Koshary (k), beef Lancheon (BL), Karish cheese (KC) and beef burger (BB).

Twenty samples from each food types were investigated for incidence of the suspected *B .cereus* bacteria. After collection of food samples from local markets of Gharbia Governorate (Egypt) in sterile plastic bags (100 g for each); these food samples were transported to Microbiology Laboratory, Tanta University Hospitals for microbiological investigations. The results given in Table, (1) shows that all the food types tested were contaminant with the bacteria *B .cereus* by varying degrees as out of 20 samples tested from each food type about 10; 10; 7; 4; 5 were positive for the suspected *B.cereus*bacteria in corn snacks; Koshary; Beef luncheon; Karish cheese; beef burger respectively (Table 1).

Biological characterization and identification of the suspected *B.cereus* bacteria:

All the bacteria isolated were subjected to identification experiments. All the 36 bacterial isolates were Gram positive motile rods and showed positive oxidase and catalase tests; the spore stain showed an obvious endospores in cells of all bacterial isolates obtained (Table,2).

For identification of bacteria at the species level, all the bacterial isolates were identified by Vitek–2 equipment. Except for the bacterial isolates CS1; CS14 and BL7 which were identified as *B .amyloliquefaciens*; *B.amyloliquefaciens* and *B .mycoides* respectively. All the bacterial isolates were identified as belonging to B .Cereus (Table 2).

Incidence of *B .cereus* in Egyptian foods:

The general incidence of the *B .cereus* bacteria among Egyptian food was about 36 %. The incidence of *B.cereus* in each food type such as corn snacks; Koshary; Beef luncheon; Karish cheese; beef burger was 50 % (10/20) ; 35 % (7/20) ; 20% (4/20) ; 25 % (5/20) respectively (Table 1) .

Virulence of the isolated bacteria:

Both *B .amyloliquefaciens* (CS1, CS14) and *B.mycoides* (BL7) showed positive reaction regarding hemolysin, Lecithinase, protease, amylase and gelatinase (Table, 3). In view of the other 33 strains of *B .cereus*, all of them showed positive results regarding protease, amylase and gelatinase (Table 3). Out of the 33 *B.cereus* investigated, about 15 and 27 strains were positive regarding *hemolysin* and *lecithinase* production respectively (Table, 3)

Inhibition of B. cereus by CFS from some probiotic bacteria:

Both *B.cereus* K20 and *B.cereus* BB15 were chose in this investigation as they were isolated from both Koshary and Beef Luncheom; two traditionally used food types in Egypt. CFS from *Lact.plantarum* LPS10 was collected and added to aliquots (10-mL) of BHI broths inoculated by 2×10^4 CFU/mL final concentrations of *B.cereus.* Results are given in (Figure, 1). The counts of *B.cereus* bacteria in control samples were increased by 4 log cycles within 96h, however in treated samples the counts remained fixed or decreased by one log cycle in the case of *B.cereus* BB15 after 24 h of incubation.

CFS from *Ent.faecium* NM_2 showed distinctive inhibition of both *B. cereus* k20 and *B.cereus* BB15. When CFS of *Ent.faecuim* NM_2 was added to *B.cereus*, no viable bacteria were detected after 24- 48h of incubation. (Figure, 2)

Table 1.Incidence of *B.cereus* in Egyptian Foods

Food Product	No. of Food Samples	No.of positive samples	Incidence (%)
Corn Snacks	20	10	50
Koshary	20	10	50
Beef Lancheon	20	7	35
Kareesh Cheese	20	4	20
Beef Burger	20	5	25
Total	100	36	36

Table 2.Identification of bacterial isolates obtained from Egyptian Foods by Vitek.2.

SN	Code	The Identified Strain
1	CS1	*B.amyloliquefaciens*
2	CS3	*B.cereus*
3	CS5	*B.cereus*
4	CS6	*B.cereus*
5	CS7	*B.cereus*
6	CS9	*B.cereus*
7	CS11	*B.cereus*
8	CS14	*B.amyloliquefaciens*
9	CS16	*B.cereus*
10	CS18	*B.cereus*
11	K3	*B.cereus*
12	K6	*B.cereus*
13	K8	*B.cereus*
14	K10	*B.cereus*
15	K12	*B.cereus*
16	K14	*B.cereus*
17	K16	*B.cereus*
18	K17	*B.cereus*
19	K18	*B.mycoides*
20	K20	*B.cereus*
21	BL2	*B.cereus*
22	BL4	*B.cereus*
23	BL7	*B.mycoides*
24	BL8	*B.cereus*
25	BL11	*B.cereus*
26	BL13	*B. cereus*
27	BL16	*B. cereus*
28	KC4	*B. cereus*
29	KC7	*B. cereus*
30	KC15	*B. cereus*
31	KC19	*B. cereus*
32	BB4	*B. cereus*
33	BB12	*B. cereus*
34	BB15	*B.subtilis*
35	BB18	*B. cereus*
36	BB20	*B. cereus*

Table 3.Production of enzymes as a virulence factors by the obtained *B.cereus* strains

SN	Code	Hemolysin	lecithinase	Protease	Amylase	Gelatinase
1	CS1	+	+	+	+	+
2	CS3	+	+	+	+	+
3	CS5	+	+	+	+	+
4	CS6	-	+	+	+	+
5	CS7	+	+	+	+	+
6	CS9	-	+	+	+	+
7	CS11	+	+	+	+	+
8	CS14	+	±	+	+	+
9	CS16	+	+	+	+	+
10	CS18	-	+	+	+	+
11	K3	+	±	+	+	+
12	K6	-	-	+	+	+
13	K8	+	-	+	+	+
14	K10	-	+	+	+	+
15	K12	-	+	+	+	+
16	K14	-	+	+	+	+
17	K16	+	+	+	+	+
18	K17	+	+	+	+	+
19	K18	-	+	+	+	+
20	K20	-	+	+	+	+
21	BL2	-	+	+	+	+
22	BL4	-	-	+	+	+
23	BL7	+	+	+	+	+
24	BL8	-	+	+	+	+
25	BL11	+	+	+	+	+
26	BL13	+	-	+	+	+
27	BL16	-	+	+	+	+
28	KC4	-	+	+	+	+
29	KC7	+	+	+	+	+
30	KC15	-	+	+	+	+
31	KC19	+	+	+	+	+
32	BB4	-	+	+	+	+
33	BB12	+	+	+	+	+
34	BB15	+	-	+	+	+
35	BB18	-	+	+	+	+
36	BB20	-	-	+	+	+

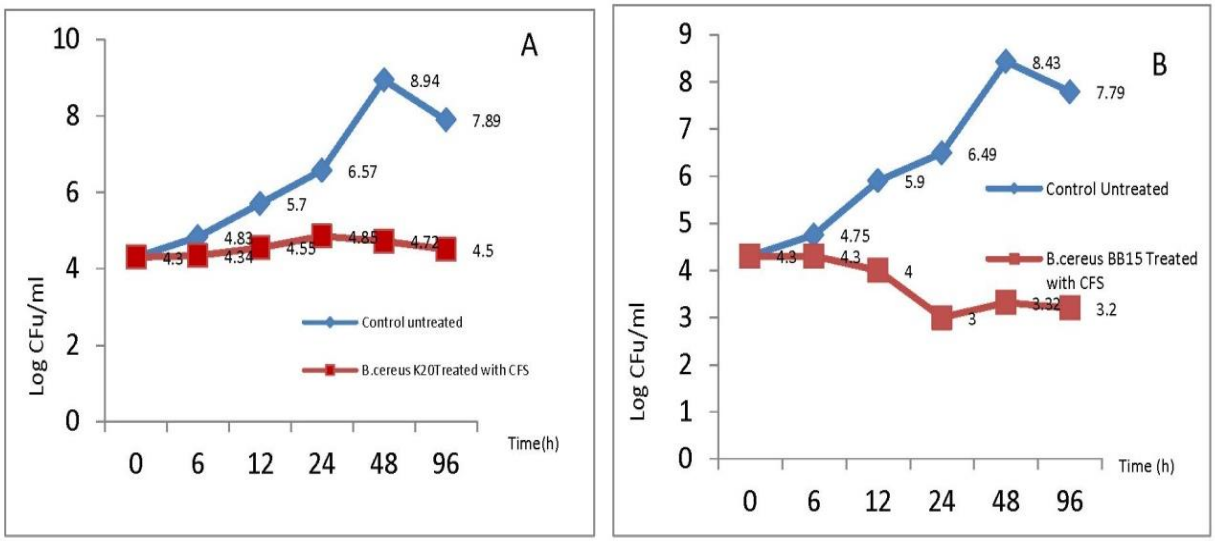

Figure 1: Inhibition of both *B.cereus* K20 (A) and *B.cerus* BB 15 (B) by CFS from *Lact. plantarum* LPS10 at 30ºC

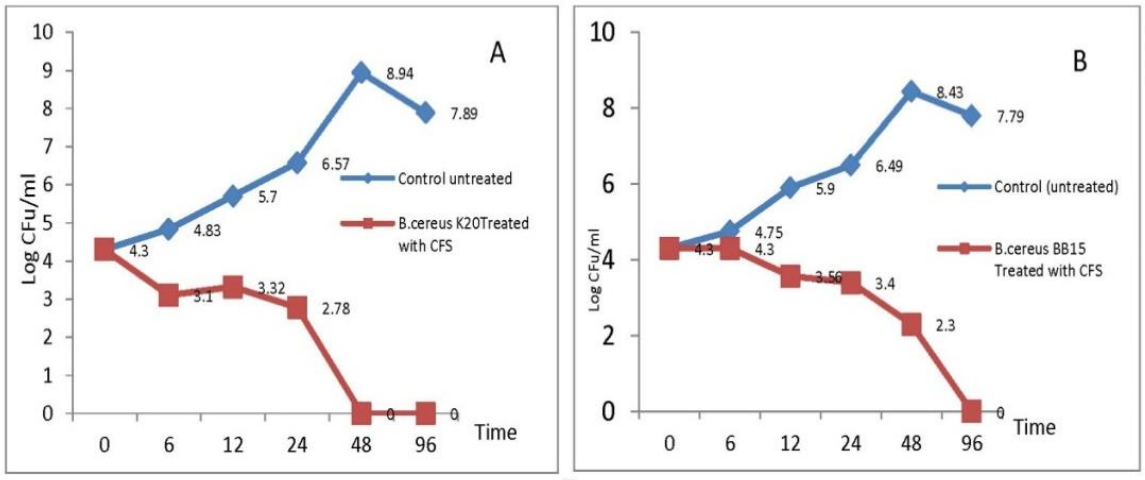

Figure 2: Inhibition of both *B.cereus* K20 (A) and *B.cerus* BB 15 (B) by CFS from *Ent. Faecium*NM_2.

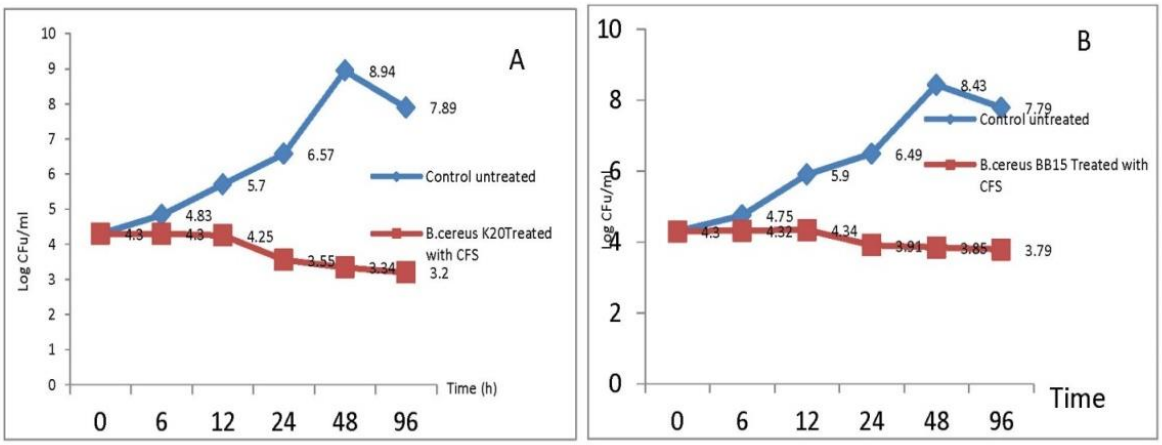

Figure :3 Inhibition of both *B.cereus* K20 (A) and *B.cerus* BB 15 (B) by CFS from *Lact. delbrenki*sspbulgaricus Z_{11}

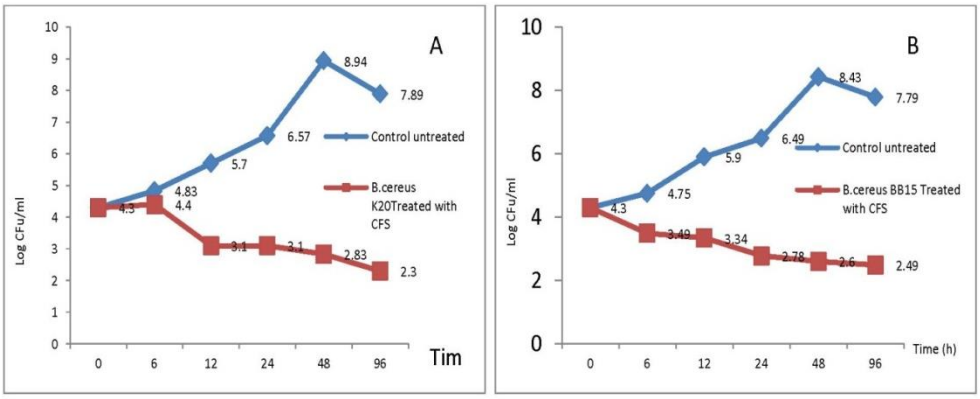

Figure :4 Inhibition of both *B.cereus* K20 (A) and *B.cerus* BB 15 (B) by CFS from *Lactis ssp. Lactis*Z_1

The inhibition of the experimental *B.cereus* strains by CFS of *L.lactis* ssp. *lactis* Z_{11} was studied. Results are given in (Figure, 3). About 50 % decline of initial *B.cereus* was showed in both treatments after almost 24 – 48h of incubation.

Finally CFS from *Lact.delbreukeii* ssp. *bulgaricus* was obtained by addition of this CFS to both experimental *B. cereus* strains at their initial concentration (2×10^4 CFU/mL). Cells of *B.cereus* did not multiply as initial counts were continued constant for the first 12h of incubation and rather decrease of colony counts by 1 log cycle was showed by further incubation (Figure, 4).

DISCUSSION

There is a recent interest in studies focusing on *B. cereus* and its incidence in foods and its virulence (Enan et al. 2012). Many worldwide studies have reported the importance of *B. cereus* as a cause of food poisoning outbreaks (Kotiranta et al., 2000). *B cereus* has been listed as one of the food – borne microorganisms of public health significance (Kramar and Giebert, 1489).

The food types studied were chose as they were reported to be polluted by *B cereus* in Egypt (Enan et al. 2012). About 100 food samples were tested microbiologically for incidence of *B. cereus*; 20 samples from each food type such as corn snacks, Koshary, beef Lancheon, Kareesh cheese and beef burger. The incidence of *B.cereus* in these foods varied; the highest prevalence (50%) was detected in both corn snacks and Koshary followed by beef lansheon and beef burger. This

is coupled with the results obtained by Abdou et al. (2012). Who stated that corn flour and rice media are suitable environments for germination of *B. cereus* spores. The 35 % prevalence of *B.cereus* might be possible due to decreasing of health instruction and precaution and/or bad processing.

The lower incidence of *B.cereus* (20 %) was found in Kareesh cheese. This is because Kareesh cheese contains a wide varaiety of lactic acid bacteria such as *Lactococcus lactis*, *Lacto bacillus bulgarians* which decrease the pH value of Kareesh cheese towards acidity (pH 6.0) where *B. cereus* and other pathogenic bacteria cannot grow (Enan et al. 2013b).

It was necessary to identify the suspected *B.cereus* isolates. Preliminary experiments for identification were carried out such as Gram staining, cell morphology, catalase and oxidase tests (Krig and Holts, 1984). All bacterial isolates were Gram positive motile rods and showed positive catalase reaction. Such results coincided with preliminary identification of bacterial isolates as belonging to Genus: *Bacillus* (Krieg and Holts, 1984).

To identify bacterial isolates at the species level Vitek-2 equipment was used. Except for the bacterial isolates CS4; CS14; BL7 which were identified as *B. amyloliquefaciens; B.amyloliquefaciens*; *B. mycoides* respectively, all other bacterial isolates were identified as belonging to *B. cereus.* Such identification procedure using Vitek.2 showed successful identification as reported previously (Abdel-SHafi et al. 2014).

Extracellular enzyme production by microorganisms causes deterioration and food spoilage (Enan et al., 2012). *B. cereus* strains were found to produce extracellular enzymes: gelatinase (83%), lecithinase (83%), protease (100%), cellulase (0%), amylase (83%) and pectinase (0%) (Lund et al. 2000). The ability of *B. cereus* strains (83%) to ferment lactose which is uncommon carbon source reflect their ability to grow on milk and milk products (Lund et al., 2000). Similar study by Kramer and Gilbert, (1989) observed that 20% of 134 *B. cereus* strains isolated from pasteurized milk samples could ferment lactose. Eid et al. (2008) reported that 23% of the 48 *B.cereus* isolates were capable of producing protease, amylase and lipase enzymes which indicates their potentially for food spoilage in many foods.

Lactic acid bacteria are used currently as starter cultures for food fermentations with inhibition of many food borne pathogens such as *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* and clostridia (Enan et al. 2014 a, b). The antimicrobial potentiality of lactic acid bacteria is due to their extracellular metabolites such as lactic acid, diacetyl, acetaldehyde, ethanol and bacteriocins (Enan et al. 1996). *Lactobacillus plantarum* LPS10 was isolated from pickles and produced planarian LPS10 (Abdel-Shafi et al. 2014); *Enterococcus faecium* NM_2 isolated from urine produced enterocin NM_2 (Enan et al. 2014a,b); *Lactococcus lactic* ssp. *lactis* Z_1 isolated from Zabady (a dairy yogurt) produce the lacticin Z_1 (Enan et al. 2013a); *Lactobacillus delbreukii*spp. bulgaricus Z_{11} produce bulgaricin Z_{11}(Abdel-Shafi et al. 2014). Consequently, the bacteriocin producing strains including LPS10, NM_2, Z_1 and Z_{11} were used for inhibition of both *B. cereus* k20 and *B. cereus* BB15 *in vitro*. CFS values were collected from the above mentioned bacteriocin producer strains and their antimicrobial activity against both BB 15 and K20 strains was tested. It was showed that the CFS of the four bacteriocin producer strains resulted inhabited both *B.cerues* K20 and *B.cereus* BB15. The inhibition of *B.cereus* by CFS of lactic acid bacteria might be due to both lactic acid and bacteriocins produced by lactic acid bacteria cultures. This is parallel to latter studies in this respect (Enan et al. 2013a).

Further studies will be necessary to study the inhibition of both viable *B.cereus* cells and endo spores in foods by these lactic acid bacteria producing bacteriocins.

CONCLUSION

The B. cereus bacterial strains were isolated fron some Egyptian foods with an incidence percentage of about 5 to 10 %. The suspected isolates were characterized and showed to be virulent strains. Probiotics inhibited some representative Bacillus cereus strains.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest

ACKNOWLEGEMENT

Authors thank Zagazig University, Egypt for facilities to carry out this work, and thank A. Almohammady for paying the publication fees.

AUTHOR CONTRIBUTIONS

Gamal Enan, Fifi Mohamed Reda and Nashwa El Gazzar proposed the research topic and supervised the work. Nermin Abdel Aziz carried

out the isolation of bacteria and testing the efficacy of probiotics. Nashwa El Gazzar, Gamal Enan identified the organisms by API kits, provided the probiotics and assessed the production of enzymes. Abdul Raouf Almohammadi provided the API kits for identification, standard strains and funded the publication fees. All authors read and approved the final version.

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