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RESEARCH ARTICLE

Physiological and Molecular Studies on Thermotolerance of *Bacillus cereus* Isolated from Some Dairy Products and Fast Foods

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Abstract

Several members of *Bacillus cereus* (*B. cereus*) have been recognized as significant agents of food and health problems. Thermotolerance of *B. cereus* after pre-exposure to temperature shifts or other stress may influence its ability to grow in minimally processed foods. This study was proposed to determine the recovery rate of *B. cereus* group from dairy products and fast foods. Moreover, the responses of a recovered isolate to different stresses were evaluated to stand upon its thermotolerance against lethal temperature. In total, 200 different samples from milk and milk products (164) and fast food (36) were examined for the presence of *B. cereus* group. The effects of heat, cold, acid and salt stresses on *B. cereus* heat resistance were physiologically and molecularly assessed through examining its viability and measuring the gene expression of its heat induced proteins (GroEL and DnaJ) by reverse transcription quantitative polymerase chain reaction (RT-qPCR), respectively. Overall, 35 (17.5%) samples were positive for *B. cereus* group isolates. The higher *B. cereus* count (> 10^4 CFU/mL or g) was found in 7% of milk and fast food samples. Three *B. cereus* group species (*B. cereus, B. thuringiensis,* and *B. pseudomycoides* or *B. mycoides*) were identified. After time intervals of mild heat pre-exposure at 42°C or exposure to other stresses, *B. cereus* developed an increased thermotolerance at 50°C/20 min. This thermotolerance was pronounced after exposure of *B. cereus* cells to 42°C /1 h and 2.5 % salt/30°C/ 40 min with a 3.9 CFU log increase compared to those exposed to lethal treatment only. RT-qPCR results revealed up-regulation of *dna*J gene expression in pretreated cells compared to the lethal only treated cells. Overall, these results confirmed the heat resistance occurs in *B.cereus* during food processing, which results in its survival in the food products.
Keywords: *B. cereus*, Viability, Thermotolerance, *dna*J gene, RT-qPCR

Introduction

A well effective and an efficiently administered national food control system is necessary to provide the assurances to consumers for preventing or minimizing the health and safety risks from food [1]. *Bacillus cereus* (*B. cereus*) has been reported as a significant agent of foodborne outbreaks and it was identified as the causative agent of 19% of foodborne outbreaks between 1998 and 2008 in the United States [2]. These outbreaks were not only confined to rice and meat, but *B. cereus* group isolates have also been detected in milk [3, 4].

Inadequate cooling or storage of food at temperatures below 60°C can support *B. cereus* spores survival in different products after the thermal treatment due to their survival at high temperature exposure resulting in food decay and a danger for their consumers [5,6].

The *B. cereus* group includes eight bacterial species; *B. cereus* sensustricto, *B. thuringensis, B. anthracis, B. mycoides, B. pseudomycoides, B. cytotoxicus, B. weihenstephanensis,* and *B. toyanensis* [7]. *B. cereus* group was referred to as *B. cereus* sensulato and this group includes closed related species those cannot be phenotypically

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or biochemically differentiated. In the Bergey's manual of determinative bacteriology, some phenotypic characteristics were used to differentiate the species within the *B. cereus* group; a combination of capsule formation, inability to cause hemolysis and non-motility is specific for *B. anthracis*, the production of crystal protein is specific for *B. thuringienesis*,rhizoid colony shape is specific for *B. mycoides* and *B. pseudomycoides* and finally the ability to grow at 7°C, but not at 43°C is specific for *B. weihenstephansis* [8].

The heat shock response in a large group of bacteria is a protective mechanism occurs after a prior exposure to the non-lethal heat shock or other stresses by the production of a wide range of proteins known as heat shock proteins (HSPs). The mechanisms of production of HSPs and heat adaptation have been previously discussed for *B. cereus* [9-11]. The heat adaptation of *B. cereus* involved proteins (chaperones and proteases) those play a significant role in a variety of cellular processes including protein folding as a protective processes, assembly and aggregation prevention and repair under stress and non-stress conditions. *Bacillus cereus* was adapted to heat exposure at the lethal temperature of 50°C after 42°C pre-exposure (maximum protection occurs after 15 min to 1 h of pre-exposure to 42°C) as a result of de novo protein synthesis [10].Thirty-one heat-induced proteins were observed using gel electrophoresis, grouped into 3 groups depending upon the time points of highest production, and the N-terminal sequences of a subset of these proteins were then detected. This showed the induction of proteins responsible for sporulation (AldA and SpoVG), stress proteins (SodA, CspB andCspE), metabolic enzymes (Dra and FolD), identified heat-induced proteins in related organisms (DnaK, HSP16.4, GroEL, RsbV, ClpP, ClpP, YflT, PpiB, and TrxA) and other proteins (MreB, YloH, and YbbT) [10]. Moreover, the up-regulation of several stress proteins was confirmed by using antibodies specific for the HSPs of *B. subtilis* except GroES. A Pre-exposure to 4% ethanol, pH 5, or 2.5% NaCl for 30 min also resulted in improved thermotolerance with the induction of some HSPs [10], suggesting that during the mild processing, a cross-protection from heating occurred in pathogenic *B. cereus*, which may result in increased its survival in foods. In Egypt, no studies have been conducted to investigate the physiological and molecular responses of *B. cereus* in terms of heat tolerance. To the best of our knowledge, there was no worldwide trial for measuring the *B. cereus* heat induced protein genes expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay. So, the main aim of this work was to investigate the occurrence of *B. cereus* group strains in different types of foodstuffs collected at Sharkia Governorate, Egypt and to verify whether the pretreatment with heat, cold, acid, and salt could influence the *B. cereus* heat tolerance physiologically as well as molecularly.

Materials and methods

Samples collection

A number of 200 food samples were randomly collected from different restaurants, supermarkets and private companies in Sharkia Governorate, Egypt. These samples represented 164 milk and milk products including marketed milk (51), cheese (38), yoghurt (54) and ice cream (21), and 36 fast food samples. The collected samples were transported in a cool transport container to the laboratory of Microbiology, Faculty of Veterinary Medicine, Zagazig University on the same day of collection for bacteriological analysis under complete aseptic conditions.

Isolation and enumeration of B. cereus group bacteria

Ten grams of each food sample were homogenized for a minute with 90 mL of buffered peptone water (Oxoid, UK) [12] in a stomacher (Stomacher lab-Blender 400; AEs Laboratory, Combourg, France). After preparation of tenfold serial dilution, 0.1 mLof each diluted sample was streaked onto polymyxin egg yolk mannitol bromothymol blue agar (PEMBA, Oxoid, UK) medium. This medium was prepared by mixing sterile *B. cereus* selective agar base (CM0617, Oxoid, UK) with Polymyxin B Supplement (SR0099, Oxoid, UK) and sterile Egg Yolk Emulsion (SR0047, Oxoid, UK) at 50°C. The agar plates were incubated for 24-48 h at 30°C. The typical colonial appearance of *B. cereus* group isolates is crenated with a distinctive turquoise

to peacock blue color and they are surrounded by a good egg yolk precipitate of the same colour with a failure to utilize mannitol (Nagler's reaction) [3]. The total viable counts of presumptive colonies were obtained and calculated as $\log_{10}$ colony forming unit (CFU)/g or mL of the sample [8]. Three independent experiments for each sample were performed. Furthermore, an ideal colony of the supposed *B. cereus* group was subcultured onto brain heart infusion (BHI) agar (Sigma-Aldrich, USA) and incubated at 30°C for 24 h. Each colony was then cultured into Eppendorf tubes filled with trypticsoy broth (TSB, Oxoid, UK) and the tubes were further incubated at 30°C for 24 h. The cultures were preserved at −20°C after addition of 30% glycerol (Sigma-Aldrich, USA).

Species confirmation

The isolates were subjected for the conventional identification procedures of the *B. cereus* group including Gram staining, testing haemolysis, motility, rhizoid growth and psychrotolerance, and some biochemical tests including catalase, gelatin liquefaction, starch hydrolysis,Voges-Proskauer, sugar fermentation, and citrate utilization as stated previously [3,12- 14].

The physiological and molecular effects of heat, cold, acid, and salt stress factors on the heat tolerance of B. cereus

One *B. cereus* isolate of fast food origin was selected as a base strain for physiological and molecular evaluation of *B. cereus* adaptation against different stress conditions depending on its typical identification criteria as *B. cereus* in addition to its high count (10^6 CFU/g) in the fast food sample.

I-Physiological effects of a pretreatment with low levels of different stresses on stimulation of B. cereus survival at 50°C

For monitoring the capability of *B. cereus* of food origin to survive during the food products minimal processing, the thermotolerance of cells pre-exposed to the following stresses: an adaptive non-lethal heat treatment at 42°C for different time points, low temperature for 2 h at 7 °C, salt shock using 2.5% NaCl for 40 min at 30 °C and pH 5 at 30°C for 30 min was analyzed as described previously [10, 15]. Briefly, *B. cereus* cells were grown in sterile BHI broth and the broth was incubated at 30 °C with aeration for 4 h (at mid-exponential phase). After that, the culture was diluted 1/100 into fresh BHI broth till the optical density reach 0.15 at 550 nm. Centrifugation to harvest cells was made at 3000 rpm/10 min and the sediments were then diluted 1/10 in preheated BHI at 42ºC as a mild adaptive heat treatment for 30, 60, 120, and 240 min. The cells at different time points were then exposed consecutively to the lethal heat treatment at 50ºC / 20 min and then 0.1 mL aliquot for each time point was plated onto TSA culture medium and the plates were incubated at 30ºC/24 h, where viable cell counts were measured. Three separate experiments were done during heat exposure treatments and all prepared cultures were plated, twice, for each time point. The viability percentage was detected relative to the untreated control culture, which was considered to have 100% survival. The degree of protection afforded by a pretreatment is the percentage of viability after lethal treatment following adaptation divided by the percentage of viability after lethal treatment only. During adaptation, the protection range of 10-10000 folds is better than in absence of pretreatment. Protection values are expressed as a range of protection in log values e.g. 100-1000 fold protection is expressed as 2-3 logs of protection.The heat tolerance of cells adapted to the other different stresses of cold temperature, salt shock and acidic pH was analyzed as mentioned above. The pre-exposure to all different stresses and heat was performed in the occurrence of chloramphenicol (100цg/mL) that stops synthesizing of new proteins.

II- RT-qPCR assay for studying effects of low levels of stresses on enhancing B. cereus survival at 50°C

The RT-qPCR assay was used for studying the relative expression of *B. cereus* heat shock chaperones genes, *groEl* and *dnaJ* upon exposure to different stress conditions mentioned in the physiological tests as a first trial. The choice of *groEl* gene was as a housekeeping gene for the purpose of calibrating the expression of the other gene in *B. cereus* [16]. Moreover, GroEl is considered one of the *B. cereus* heat induced proteins.

RNA extraction

Total RNA from bulk bacterial cells was extracted and purified using RNeasy Mini Kit (Qiagen-GmbH, Germany) according to the manufacturer`s instructions.

RT-qPCR amplification

The RT-qPCR amplification reaction was carried out, in triplicates, using QuantiTect SYBR green RT-PCR kit (Qiagen-GmbH, Germany) in a total reaction volume of 25 µL containing12.5 µL of 2x Quantitect SYBR Green PCR Master Mix (Qiagen, Germany), 0.25 µL of reverse transcriptase, 0.5 µL of each primer (20 pmoL) (Metabion, Germany), 3 µL of template RNA and 8.25µL of RNase free water. Gene specific primer sequences were selected according to the previously published papers; BCF: GCGTATCGTCGTTTGGCT and BCR: CTATCATCCTGCTCTTCTTG for *dnaJ* gene [17] and balF: TGCAACTG TATTAG CACA AGCT and balR: TACCAC GAAGT TTGTT C ACTACT for *groEL* gene [16]. In the Stratagene Mx3005P RT-PCR machine (Thermo Fisher, CA, USA), reverse transcription was performed at 50°C for 30 min. After a preliminary denaturation step at 94°C for 15 min, the reaction mixture was subjected to 40 cycles of 94°C for 15 sec, 55°C for 30 sec (*dnaJ* gene) or 40 sec (*groEL* gene) and 72°C for 40 sec and finally one cycle at 72ºC for 5 min. Dissociation curves were generated by a cycle of 94°C for 1 min, 55°C for 1 min and 94°C for 1 min. Amplification curve and cycle threshold (C_t) values were determined by Stratagene MX3005P software. The relative gene expression in *B. cereus* cells exposed to different stress conditions compared to the non-treated (control) cells were obtained using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method [18].

Results

Isolation, enumeration and identification of the recovered B. cereus group isolates

The bacteriological analysis of the collected samples revealed that *B. cereus* group species were detected in 17.5% (35/200) of different food and milk samples. Only 23 (65.7%) of 35 positive isolates showed CFU values ≥ 10^3 with the highest counts (1.58 X 10^6 CFU/g) in the fast food samples (Table 1). Fourteen out of 35 isolates which were depicted mainly in 8 milk product and 6 fast food samples had CFU values more than 10^4 with a total ratio of 7% (14/200). Meanwhile, 9 (4.5%; 9/200) isolates showed low count ratios of 10^3-10^4 CFU/g or mL (data not shown). The concentrations of *B. cereus*-group isolates were below10^3CFU/g or mL in the rest of the examined samples.Of note, all milk and milk byproducts contaminated with *B. cereus*-like bacteria (5.6 %, 8/143) were not considered acceptable (counts >10^4 CFU/mL or g). The distribution of 35 (17.5%) recovered *B. cereus* group isolates was from 25% of diverse fast foods types, 24.1 % of yoghurt, 13.2% of cheese, 11.8% of milk and 9.5% of ice cream samples. Only 3 species of *B. cereus* group were currently identified including *B. cereus*, *B. thuringiensis*, and *B. pseudomycoides* or *B. mycoides* (Table 2). The highest percentage of the isolates (71.4%, 25/35) was related to *B. cereus* with a total recovery rate of 12.5% (25/200), followed by *B. mycoides* (3%) and *B. thuringiensis* (2%). *Bacillus cereus* isolates were distributed as 19.4% (7/36) in fast foods and11% (18/164) in dairy products; mainly yoghurt (20.4%).

Table (1): Mean and Log_{10} of *Bacillus cereus* group CFU/g or mL of various milk products and food samples

Sample	No. of positive isolates*	CFU/g		
		Range	Mean	Log_{10}
		Min - Max		
Milk	5	$3x10^3$ - $1x10^6$	5.01 x 10^5	5.7
Cheese	3	$1x10^3$ – $3.4x10^5$	1.7 x 10^5	5.2
Yoghurt	6	$6.1x10^4$– $4.5x10^5$	2.5 X 10^5	5.4
Fast food	9	$6.5x10^4$ – $3.1x10^6$	1.58 X 10^6	6.2

* The isolates that had CFU ≥10^3 were selected. Min: Minimum count and Max: Maximum count.

Table (2): Occurrence of *Bacillus cereus* group species in different dairy products and fast food samples

Sample type	No. of samples	No. (%) of positive isolates	No.(%) of identified *Bacillus cereus* group isolates		
			B. cereus	*B. mycoides or B. pseudomycoides*	*B. thuringiensis*
Milk					
Raw milk	6	2 (33.3)	1 (16.7)	0.00	1 (16.7)
Fat milk	29	4 (13.8)	3 (10.3)	0.00	1 (3.4)
Mixed milk	10	0.00	0.00	0.00	0.00
Powdered milk	6	0.00	0.00	0.00	0.00
Sum	51	6 (11.8)	4 (7.8)	0.00	2 (3.9)
Cheese					
Kareesh cheese	12	3 (25)	0.00	2 (16.7)	1 (8.3)
Feta creamy cheese	11	2 (18.2)	1 (9.1)	1 (9.1)	0.00
Edam flamink cheese	3	0.00	0.00	0.00	0.00
Blue cheese	3	0.00	0.00	0.00	0.00
Mozzarella cheese	3	0.00	0.00	0.00	0.00
Cooked cheese	3	0.00	0.00	0.00	0.00
Chedder cheese	3	0.00	0.00	0.00	0.00
Sum	38	5 (13.2)	1 (2.6)	3 (7.9)	1 (2.6)
Yoghurt					
Baladi yoghurt	22	4 (18.2)	3 (13.6)	1 (4.5)	0.00
Processed yoghurt	24	9 (37.5)	8 (33.3)	0.00	1 (4.2)
Yoghurt mixed with fruits	8	0.00	0.00	0.00	0.00
Sum	54	13 (24.1)	11 (20.4)	1 (1.9)	1 (1.8)
Ice-cream					
Processed ice cream	11	2 (18.2)	2 (18.2)	0.00	0.00
Baladi ice cream	10	0.00	0.00	0.00	0.00
Sum	21	2 (9.5)	2 (9.5)	0.00	0.00
Fast foods					
Koshary	6	5 (83.3)	5 (83.3)	0.00	0.00
Katchub	5	1 (20)	0.00	1 (20)	0.00
Hot katchub	5	1 (20)	1 (20)	0.00	0.00
Pasta	5	0.00	0.00	0.00	0.00
Potato	5	2 (40)	1 (20)	1 (20)	0.00
Cooked rice	5	0.00	0.00	0.00	0.00
Coleslaw	5	0.00	0.00	0.00	0.00
Sum	36	9 (25)	7 (19.4)	2 (5.6)	0.00
Total	200	35 (17.5)	25 (12.5)	6 (3)	4 (2)

The physiological and molecular effects of stress factors on the heat responses of B. cereus

I- Physiological effects of a pretreatment with low levels of stresses on stimulation of B. cereus survival at 50°C (a high level of stress)

A- ***B.cereus*** **thermotolerance that pre-exposed to heat stress at 42°C**

During exposure of mid logarithmic stage growth of control *B. cereus* cells at 30°C to 50°C directly without a pre-treatment, a four - log decreasing in viable cells count was observed after 20 min, where the count of the control mid- exponential phase cells at 30°C was 3.5 x 10^8 CFU/mL (log 8.5) and became 1.5x10^4 /mL (log 4.2) after exposure to 50°C (lethal only treated cells without a pre-treatment at 42°C) (Table 3).

Viable cell counts were detected for pre-exposed *B. cereus* at 42°C for various time intervals (30, 60, 120, and 240 min) and subsequently exposed to the lethal temperature of 50°C for 20 min. The results revealed increased thermotolerance of pre-treated cells at 50°C compared to that in the lethal only treated cells as illustrated in Table 3. The highest increase in the heat survival was detected after pre-exposure at 42°C for 30 and 60 min (3.4 and 3.9 CFU log increase, respectively) compared to that in the lethal only treated cells. Remarkably, in the occurrence of chloramphenicol (that stop the new protein induction) during *B. cereus* cells pre-exposure at 42°C for 30, 60, 120, and 240 min, no enhancement in their thermotolerances was detected compared to that of the pre-treated cells without chloramphenicol (Table 3).

Table (3): *Bacillus cereus* heat survival and protection afforded by pretreatment with low levels of stresses

Pretreatment stress/ time	CFU/mL at 50°C/20min after pretreatment with stresses		CFU Log_{10}		Viability %	Protection range (Log value)
	without ch.	with ch.	without ch.	with ch.		
Lethal only treated cells	$1.5x10^4$	-	4.2	-		
42°C/ 30 min	$5x10^7$	$1.5x10^4$	7.6	4.2	$0.14 x10^2$	3.5
42°C/ 60 min	$1.2x10^8$	$9x10^4$	8.1	4.9	$0.34 x10^2$	3.9
42°C/ 120 min	$3x10^6$	$3x10^3$	6.4	3.5	$0.009 x10^2$	2.4
42°C/ 240 min	$1.1x10^6$	$1x10^3$	6	3.0	$0.003 x10^2$	1.9
NaCl 2.5 %/40 min at 30°C	$1.2 x10^8$	$1x10^6$	8.1	6	$0.34 x10^2$	3.9
pH 5/30 min at3 0°C	$3.1x10^6$	$3.5x10^5$	6.5	5.5	0.9	2.4
Cold temperature at 7°C/2h	$3.4x10^5$	$1.5x10^4$	5.4	4.2	0.09	1.4

ch.: chloramephenicol NB.

a) Control untreated cells at 30°C had a count of $3.5x10^8$CFU/mL (log 8.5)

b) Lethal only treated cells (exposed to 50°C without pretreatments)

c) Adaptive non-lethal pretreatment at 42°C, followed by a lethal treatment

The colony counts from control cells (a) represent 100% survival and the survival percentages following treatments (b) and (c) were estimated relative to the viable counts of the untreated cells.

A-Thermotolerance of *B. cereus* pre-exposed to other stress factors

To confirm whether different stresses other than heat could affect thermotolerance, *B. cereus* cells were exposed to 7°C/2h, 2.5% NaCl/40min at 30°C, or pH 5/30 min at 30°C before exposure to 50°C of lethal heat treatment. All of these pre-exposures caused an increase in thermotolerance with some variations compared to lethal only treated cells. The salt stress showed the highest protection with a slight cell number decrease detected after 20 min of incubation at 50°C than that of the control untreated cells, where the count of the salt treated cells was $1.2x10^8$ CFU/mL compared to $1.5x10^4$ CFU/mL of the lethal only treated cells (a 4-log decrease in viable cell counts) and that count nearly reached to the count of the control untreated cells ($3.5x10^8$ CFU/mL). However after 20 min at 50°C, nearly 2- and 3-log decreases in viable counts were observed after pre-exposure of *B. cereus* cells to the low-pH and the low temperature, respectively comparing with the control untreated cells (log 8.5). During the stress pretreatment and in the occurrence of chloramphenicol, some thermotolerance of *B. cereus* cells was developed compared to those exposed to the lethal temperature only, but this tolerance was not equal to that acquired without chloramphenicol. About 1.8 and 1.3 log increases in thermotolerance of salt and pH stressed cells, respectively were observed when compared to the cells exposed to the lethal temperature only, while no enhancement in thermotolerance was noticed in cold temperature stressed cells in the occurrence of chloramphenicol after 20 min at 50°C as presented in Table 3. This shows that the enhancement in thermotolerance attained upon these treatments is not reliant mainly on new protein induction, but may be other factors.

The results of physiological effects of pre-exposure with low levels of stresses in stimulating *B. cereus* survival at 50°C indicated that the pre-exposure with low levels of heat, salt and pH adapted the cells, so they showed a heat survival in the presence of these treatments better than in the absence of them.

II-Molecular effects of a pretreatment with low levels of stresses on enhancing B. cereus survival at 50 °C (a high level of stress)

The mRNA expression levels of *groEL* and *dnaJ* genes of selected *B. cereus* isolate, which was isolated from fast food origin and displayed a high count (10^6 CFU/g), was determined after 20 min exposure to lethal heat at 50°C upon pretreatments with mild levels of different stresses. The results revealed up-regulation of both heat induced genes in the treated cells compared to the lethal only treated cells with variable ranges for different stress types confirming a strong thermotolerance (Figure 1).

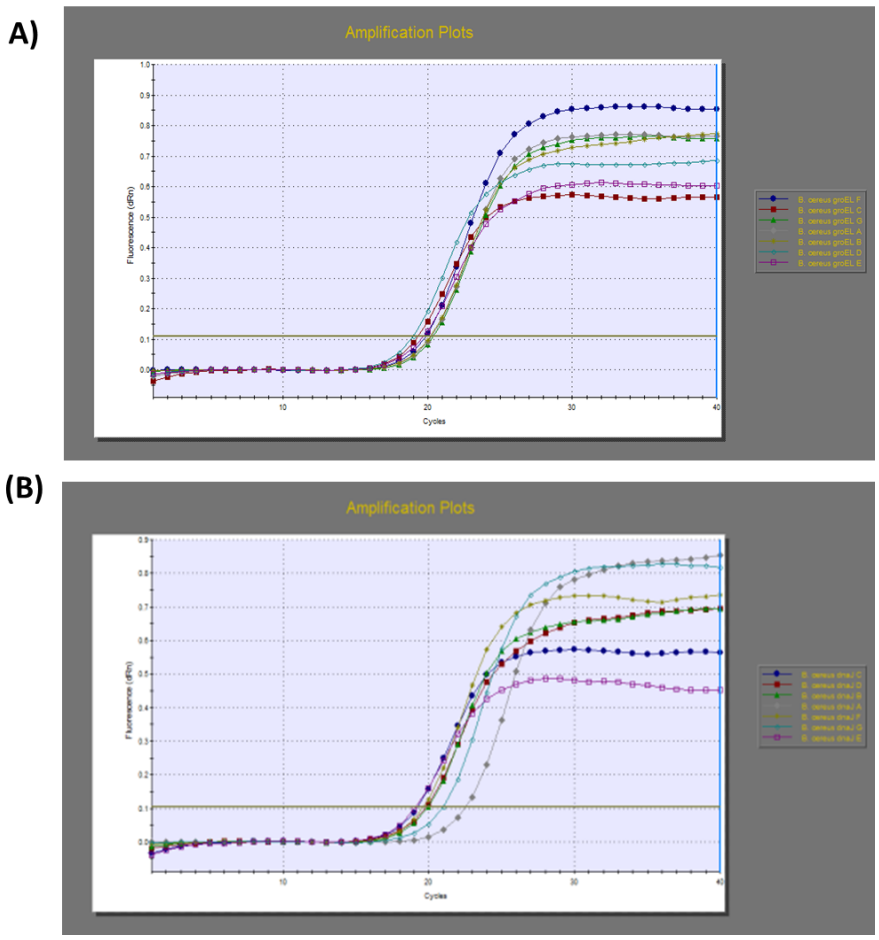

Figure (1): Amplification plots of Real Time ; RT-qPCR assay for detection of *B. cereus groEL* (A) and *dnaJ* (B) genes` expressions after lethal heat stress following mild treatment. The *groEL* was mainly used as a housekeeping gene. Positive control: The curve appeared at Ct 20.53 and 22.86 for *groEL* and *dnaJ* genes, respectively. Negative control: It appeared under the threshold line.

From Figure 1, increased expression levels of *dnaJ* gene were observed after pretreatment with NaCl 2.5% /40 min at 30°C (9.2535 fold-change), followed by cold temperature at 7°C / 2h (7.6211-fold change) and then mild heat at 42°C/ 60 min (6.7740 fold-change). Meanwhile, *groEL* gene expression was the highest in pretreatment with mild heat at 42°C/ 60 min, followed by NaCl 2.5% /40 min at 30°C. The presence of chloramphenicol during exposure of the isolate to 42°C for 60 min for example adversely affected the development of the thermotolerance with a down-regulation of *dnaJ* gene expression to reach a 2.7702 -fold change.

Discussion

Really, it is important to note that the different food products could be simply contaminated as a result of the storage

conditions, handling or due to ineffective sanitation and cleaning of the all used tools. Hence, the concentration of bacteria may rapidly increase and reach the hazardous scale (till 10^4 CFU/mL or g). Based on the permitted levels of *B. cereus* in food, the amounts of *B. cereus* those were considered unsafe and risky for consumption were $>10^5$ CFU/mL or g in UK [19] and $\geq 10^4$ CFU/mL or g in Food Standards Australia New Zealand [20]. Our data showed that in only 7% of the examined milk and fast foods samples, the *B. cereus*-like isolates count was $> 10^4$ CFU/mL or g, which was as the Australia New Zealand's hazardous level [20]. Meanwhile, in only 4.5% of the samples, the counts were $10^3 – 10^4$ CFU/mL or g and the rest of the samples showed counts less than 10^3 CFU/mL or g, which were lower than the UK's permissible level [19].The high counts depicted in fast foods samples; mainly koshary, ketchup as well as potato were parallel to those reported previously in fast and cooked food in Tunisia [21] and Denmark [22]. The detected high counts in fast foods samples may be resulted from using additives of the contaminated spices in food that may denote a danger in the case of insufficient heat treatment. Higher counts have been reported on spices in a Turkish study [23]. However, in Vienna high *B. cereus* counts (10^5CFU/g) were detected in some spices [24].

All milk and milk byproducts contaminated with *B. cereus*-like bacteria (5.6 %, 8/143) were not considered acceptable (counts $>10^4$ CFU/mL or g). In the same way, a previous study conducted in India stated much higher *B. cereus* counts ($> 10^5$ CFU/g) in 10% of dairy food samples [25]. The high bacterial pollution level of processed foods may be due to uncooked materials contamination and the subsequent spore resistance to heat or other industrial processes as well as unhurried cooling and prolonged storage at the room temperature permit the spores to germinate [26,27]. *Bacillus cereus* bacterial group biofilm existed on the surface of the tubes, utensils and other processing tools as storage containers can be a basis of the processed food contamination [28].

The obtained results recorded that the distribution of 35 (17.5%) recovered *B. cereus* group isolates was from 25% of diverse fast foods types, 24.1 % of yoghurt, 13.2% of cheese, 11.8% of milk and 9.5% of ice cream samples. The current high *B. cereus* group occurrence (25%) in fast and cooked foods samples mainly koshary, ketchup as well as potato is concurring with previous findings, where the *B. cereus* group was detected in heat-treated food such as cooked products in Denmark [22], pizza in Australia [29], pastry products and desserts in Netherlands [30], and canned products in Nigeria [31]. Moreover, a higher occurrence of *B. cereus* was previously found in 46.3% of the pastry products and 40.8% of the cooked food samples in Tunisia [21].This variation may be due to the differences in the hygienic practices followed in the shops and restaurants in the recent times.

The overall rating of species recovery among various samples was 12.5% (25/200) for *B. cereus*. *B. cereus* isolates were recovered from 19.4% of fast food samples and 11% of dairy products; mainly yoghurt (20.4%), followed by 3% for *B. mycoides* and 2% for *B.thuringiensis*.

Higher occurrence rates of *B. cereus* in milk and dairy products were reported previously in several studies in Brazil, 58.3% [32] and 24.23 % [33] and in Italy (18.6%) [34].The variability of *B. cereus* prevalence in dairy products may be attributed to the practices of the manufacturers and also to the storage conditions of these products [35].

Herein, physiologically, after different time intervals of mild heat pre exposure at 42°C and upon a shift to 50°C, *B. cereus* cells developed an improved thermotolerance to the lethal heat exposure and the major increase was produced after 1 h at 42°C. The increase in *B. cereus* thermotolerance was not observed at the investigated time intervals in the presence of chloramphenicol compared to that for cells without chloramphenicol.

Similarly, several authors demonstrated that the pervious exposure at 42°C adapted *B. cereus* to lethal heat at 50°C and the protein synthesis was required to obtain this increase in thermotolerance with an extreme protection occurred after 15 min to 1 h of pervious exposure to 42 °C [10, 15]. It was proved that the increased levels of HSP and

thermotolerance were reliant on new protein induction, where they were not increased in the presence of chloramphenicol [10].The present research examined the first responses of *B. cereus* to other stresses prior to exposure to 50°C/20 min. the results showed an increase in thermotolerance with some variations and the highest protection was delivered by the salt stress, where salt stressed *B. cereus* count was $1.2x10^8$CFU/mL with a 3.9 CFU log increase in the viable cell counts compared to the lethal only treated cells those had a count of $1.5x10^4$ CFU/mL. The increase in thermotolerance was also obtained in the presence of chloramphenicol. In line with the obtained findings, Periago and his colleagues [10] ascertained that the pretreatment with a salt stress, followed by acidic pH then low temperature stresses led to an increase in thermotolerance of *B. cereus* upon exposure to 50°C/25 min. The highest protection was provided by the salt stress with no cell number reduction compared to 4-log reduction in the control cells. The increase in thermotolerance obtained in the presence of chloramphenicol upon these processes is not mainly depended on new protein synthesis and may be related to regulator factors.

The RT-qPCR analysis revealed that the expression levels of *dn*aJ gene increased after pretreatment with NaCl 2.5%/40 min at 30°C, followed by cold temperature at 7°C/2h. Meanwhile, *groEL* gene expression was the highest in pretreatment with mild heat at 42°C/60 min, followed by NaCl 2.5%/40 min at 30°C. Compared to our results, Periago *et al.* [10] mentioned that after a pre-exposure at 42°C, *B. cereus* cells showed a strong heat tolerance at 50°C compared to control cells. This is depended on HSPs (GroES, GroEL, Dna K, DnaJ, CLPC, CLPP and CLPX) and the genes encoding these HSPs could be recognized by western blotting in the *B. cereus* genome sequence. Moreover, upon exposure to NaCl, the induction of DnaJ, DnaK, ClpC, and ClpX was increased; while under low pH and cold temperature, a slight production of only DnaJ and DnaK was found and finally using 2D-E gels, GroEL was increased only after the heat and salts pretreatments.

Conclusion

From our results, it is essential to retain in mind that *B. cereus* can be more tolerant to aggressive conditions after a previous stress exposure and therefore tolerate and live on normally lethal conditions that present upon food processing. *Bacillus cereus* spore can tolerate high temperature exposure; thus inadequate cooling or storage of food at temperatures under 60°C can protect their growth in food following thermal exposure. Reliant on the strain type, growth to great limits may cause food spoilage and constitute a risk for foodborne illness after ingestion.

Conflict of interest

The authors have no conflict of interest to declare.

References

[1] Whitehead, A.J. (1995): Elements of an effective national food control system. Food Control, 6: 247-251.

[2] Bennet, S.D.; Walsh, K.A. and Gould, L.H. (2013): Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*-United States, 1998-2008. Clin Infect Dis, 57: 425–433.

[3] Stenfors Arnesen, L.P.; Fagerlund, A. and Granum, P.E. (2008): From soil to gut: *B. cereus* and its food poisoning toxins. FEMS Microbiol Rev, 32: 579–606.

[4] Miller, R.A.; Kent, D.J.; Watterson, M. J.; Boor, K.J.; Nicole, H.; Martin, N.H. and Wiedmann, M. (2015): Spore populations among bulk tank raw milk and dairy powders are significantly different. Journal of dairy science, 98: 8492-8504.

[5] Schmidt, V.S.; Kaufmann, V.; Kulozik, U.; Scherer, S. and Wenning, M. (2012): Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. Int J Food Microbiol, 154: 1–9.

[6] Lücking, G.; Stoeckel, M.; Atamer, Z.; Hinrichs, J. and Ehling-Schulz, M. (2013): Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments

and product spoilage. Int J Food Microbiol, 166: 270–279.

[7] Kovac, J.; Miller, R.A.; Carroll, L.M.; Kent, D.J.; Jian, J.; Beno, S.M. and Wiedmann, M. (2016): Production of hemolysin BL by *Bacillus cereu*s group isolates of dairy origin is associated with whole-genome phylogenetic clade. BMC Genomics, 17: 581.

[8] Vos, P.; Garrity, G.; Jones, D.; Krieg, N.R.; Ludwig, W.; Rainey, F.A.; Schleifer, K. and Whitman, W.B. (eds., 2009): Bergey's manual of systematic bacteriology, 2nd ed., vol. 3 (The Firmicutes). New York: Springer-Verlag.

[9] Mahakarnchanakul, W. and Beuchat, L.R. (1999): Effect of shift in growth temperature on tolerance of psychrotrophic and mesophilic strains of *Bacillus cereus* to heat and sodium chloride. J Food Prot, 62: 57-64.

[10] Periago, P.M.; van Schaik, W.; Abee, T, and Wouters, J.A. (2002): Identification of proteins involved in the heat stress response of *Bacillus cereus* ATCC 14579. Appl Environ Microbiol, 68: 3486-3495.

[11] Duport, C; _Jobin, M. and Schmitt, P. (2016): Adaptation in *Bacillus cereus*: from stress to disease. Front Microbiol, 7: 1550.

[12] European Food Safety Authority (EFSA, 2009): The community summary report on foodborne outbreaks in the European Union in 2007. EFSA Journal, 7: 271r.

[13] Tallent, S.M.; Rhodehamel, E.J.; Harmon, S.M. and Bennett, R.W. (2012): Data from: Bacteriological analytical manual; methods for specific pathogens. U.S. Food and Drug Administration. Chapter 14, *Bacillus cereus*. https://www.fda.gov/food/laboratory-methods-food/bam-*bacillus-cereus*

[14] Public Health England, PHE (2018): Identification of *Bacillus* species. UK Standards for Microbiology Investigations, ID 9, Issue 3.1. 1-27. https://www.gov.uk/uk-standards-formicrobiology-investigations-smi-quality-and-consistency-in-clinical-laboratories.

[15] Browne, N. and Dowds, B.C.A. (2001): Heat and salt stress in the food pathogen *Bacillus cereus*. Journal of Applied Microbiology, 91: 1085-1094.

[16] Chang, Y.H.; Shangkuan, Y.H.; Lin, H.C. and Liu, H.W. (2003): PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. Appl Environ Microbiol, 69: 4502–4510.

[17] Zhang, J.; Hung, V.; Hayashi, M.; Yoshida, S.; Ohkusu, K. and Ezaki, T. (2011): *DnaJ* sequences of *Bacillus cereus* strains isolated from outbreaks of hospital infection are highly similar to *Bacillus anthracis.* Diagnostic Microbiology and Infectious Disease, 70: 307–315.

[18] Livak, K.J. and Schmittgen, T.D. (2001): Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods, 25: 402–408.

[19] Public Health England, PHE (2009): Guidelines for assessing the microbiological safety of ready-to-eat foods placed on the market, Health Protection Agency Working Group. http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1259151921557.

[20] Food Standards Australia New Zealand, FSANZ (2001): Guidelines for Microbiological Examination of ready-to-eat foods.

[21] Gdoura-Ben Amor, M.; Siala, M.; Zayani, M.; Grosset, N.; Smaoui, S.; Messadi-Akrout, F.; Baron, F.; Jan, S.; Gautier, M. and Gdoura, R. (2018): Isolation, identification, prevalence, and genetic diversity of *Bacillus cereus* group bacteria from different foodstuffs in Tunisia. Front Microbiol, 9: 447.

[22] Rosenquist, H.; Smidt, L.; Andersen, S. R.; Jensen, G.B. and Wilcks, A. (2005): Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in

ready-to-eat food. FEMS Microbiol Lett, 250: 129–136.

[23] Aksu, H.; Bostan, K. and Ergün, O. (2000): Presence of *Bacillus cereus* in packaged some spices and herbs sold in Istanbul. Pak J Biol Sci, 3: 710–712.

[24] Kneifel, W. and Berger. E. (1994): Microbiological criteria of random samples of spices and herbs retailed on the Austrian market. J Food Protect, 57: 893–901.

[25] Bedi, S.K.; Sharma, C.S.; Gill, J.P.S.; Aulakh, R.S. and Sharma, J.K. (2005): Incidence of enterotoxigenic *B. cereus* in milk and milk products. J Food Sci Tech, 42: 272–275.

[26] Borch, E. and Arinder, P. (2002): Bacteriological safety issues in red meat and ready-to-eat meat products, as well as control measures. Meat Sci, 62: 381–390.

[27] Ankolekar, C.; Rahmati, T. and Labbé, R.G. (2009): Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. Int J Food Microbiol, 128: 460–466.

[28] Faille, C.; Bénézech, T.; Midelet-Bourdin, G.; Lequette, Y.; Clarisse, M.; Ronse, G.; Ronse, A. and Slomianny, C. (2014): Sporulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. Food Microbiol, 40: 64–74.

[29] Eglezos, S.; Huang, B.; Dykes, G.A. and Fegan, N. (2010): The prevalence and concentration of *Bacillus cereus* in retail food products in Brisbane, Australia. Foodborne Pathog Dis, 7: 867–870.

[30] Wijnands, L.M.; Dufrenne, J.B.; Rombouts, F.M. in 't Veld, P.H. and van Leusden, F.M. (2006): Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Netherlands. J Food Prot, 69: 2587–2594.

[31] Oranusi, U.S.; Braide, W. and Osigwe, G.A. (2012): Investigation on the microbial profile of canned foods. J Biol Food Sci Res, 1: 15–18.

[32] Rezende-Lago, N.C.M.; Rossi Jr., O.D.; Vidal-Martins, A.M.C. and Amaral, L.A. (2007): Ocorrência de Bacillus cereus em leite integral e capacidade enterotoxigênica das cepas isoladas. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 59: 1563-1569.

[33] Reis, A.L.S.; Montanhini, M.T.M.; Bittencourt, J.V.M.; Destro, M.T. and Bersot, L.S. (2013): Gene detection and toxin production evaluation of hemolysin BL of *Bacillus cereus* isolated from milk and dairy products marketed in Brazil. Brazilian Journal of Microbiology, 44: 1195-1198.

[34] Cosentino, S.; Mulagria, A.F.; Pisano, B.; Tuveri, P. and Palmas, F. (1997): Incidence and biochemical characteristics of *Bacillus* flora in Sardinian Dairy products. Int J Food Microbial, 38: 235-238.

[35] Tewari, A. and Abdullah, S. (2015): *Bacillus cereus* food poisoning: international and Indian perspective. J Food Sci Technol, 52: 2500–2511.

الملخص العربي

دراسات فسيولوجية وجزيئيةه علي التحمل الحرارى لميكروب الباسيليس سيريس المعزول من بعض منتجات الألبان والاغذية السريعة

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تعتبر افراد عديدة من عترات الباسيليس سيريس من العوامل المسؤلة عن إحداث المشاكل الغذائية والصحية بدرجة كبيرة في العالم. ربما يؤثرالتحمل الحرارى لبكتيريا الباسيليس سيريس الناتج بعد التعرض المسبق لدرجات حرارة متغيرة أوأى مؤثرات أخرى علي قدرتها لكى تنمو فى الاغذية المحضر' بطريقه متدني' (رديئ') اثناء التخزين والتوزيع. ولذلك قد خصصت هذه الدراس' لتحديد مدى انتشار أنواع من عترات مجموعة الباسيليس سيريس فى منتجات الألبان والأغذية السريعة الجاهزة وايضا لتقييم استجابة احدى العترات المعزولة من هذه المجموعة للمؤثرات الحرارية والملوحة والحموضة المختلفة للوقوف علي مدى تحملها ومقاومتها للحرارة وخاصه ضد درجات الحرارة المميتة.

لقد تم تجميع 200 عينة مختلف من الألبان ومنتجاتها (164) والأغذية السريعة (36) لدراسة مدى تواجد مجموعة الباسيليس سيريس بها وكذلك تم تقييم تاثير كل من الحمض, درجات الحرارة العالية والمنخفضة وكذلك الملح كمؤثرات مختلفة علي المقاومه الحراريه لهذه البكتيريا فسيولوجيا عن طريق اختبار حيويتها وقابليتها للبقاء وجزيئيا عن طريق قياس التعبير الجينيى للبروتينات الناجمه عن الحراره (GroEL and DnaJ) بواسطة اختبارتفاعل انزيم البلمرة المتسلسل العكسي الكمي.

وقد اظهرالتقييم العام للنتائج 35 عترة ايجابية لهذه المجموعه من البكتيريا(17.5٪) وقد أظهرت هذه المعزولات أن العدد الأكبر يزيد عن10^4 مستعمرة/الملليليتر اوالجرام قد وجد فى حوالي 7٪ فقط من عينات الحليب والوجبات السريعة المفحوصة. ولكن حوالي 4,5٪ من العينات اظهرت عددا بين 10^3-10^4مستعمرة/ الملليليتر اوالجرام وقد تم التعرف علي ثلاثة اجناس من هذه المجموعة (الباسيليس سيريس, الباسيليس ثورينجينسيس والباسيليس سيدومايكويديس او مايكويديس) وكانت الباسيليس سيريس أكثر الأجناس انتشارا (71.4%) و بعد التعرض المسبق لدرجة حرارة 42 درجه مئويه لفترات مختلفة وكذلك التعرض للمؤثرات الأخرى فقد أظهرت الباسيليس سيريس زيادة تحملها الحرارى لدرجة 50 درجة مئوية لمدة ثلث ساعة وكانت أعلي زيادة بعد التعرض لدرجة حرارة 42 درجة مئوية لمدة ساعة وكذلك بعد التعرض للملح لمدة 40 دقيقة عند درجة حرارة 30 درجه مئوية وقد اظهر كلاهما عدد خلايا حية $1.2x10^8$مستعمرة/الملليليتر وذلك بمعدل 3.9 زيادة لوغاريتمية فى عدد المستعمرات مقارنة بالخلايا التى تعرضت للحرارة العالية فقط والتى أظهرتعدد خلايا حية $1.5x10^4$مستعمرة/الملليليتر. وعلى المستوى الجزيئي, فقد أظهرت نتائج إختبارتفاعل إنزيم البلمرة المتسلسل العكسي الكمي زيادة فى التعبير الجيني لجين *dnaJ*أيضا فى الخلايا المعرضة مسبقا للمؤثرات مقارنة بالخلايا التى تعرضت للحرارة العالية فقط. وقد أكدت النتائج حقيقة انه اثناء تصنيع ومعالجة الأغذية تحدث المقاومة الحرارية لميكروب الباسيليس سيريس الذى ينتقل عبر الطعام مسببة بقاؤه حيا فى منتجات الأغذية.