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INCIDENCE, SURVIVAL AND BIOCONTROL OF PSYCHROTROPHIC *Bacillus cereus* AND ITS POTENTIAL FOR TOXIN PRODUCTION IN MILK AND TALLAGA CHEESE

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Key words: *B. cereus*, toxin production, dairy products, antimicrobial activity

The incidence of *Bacillus cereus*, psychrotrophic character and the ability of isolates to produce haemolysin were investigated to evaluate their health potential in some dairy products. In total 125 samples (skim milk powder, white soft cheese, processed cheese, Kareish cheese and rice with milk) were analysed. Of these (39.2%) contained *B. cereus*. The viability of (reference and isolated strains) *B. cereus* and toxin production in sterilized milk was examined during storage at 10°C for 7 days. The two tested strains, when inoculated in milk with 10^5 cfu/mL, were shown to be capable of producing toxin at the end of the storage period. The antimicrobial activity of 7 strains of lactic acid bacteria against *B. cereus* was tested to select the effective starter to control the pathogen. *Lactobacillus reuteri* followed by *Lb. rhamnosus* were the most effective probiotic cultures. The choice was a mixed culture of *Lactococcus lactis* ssp. *diacetylactis* as a starter culture and *Lb. rhamnosus* as a probiotic culture (1:1) to use in manufacture of Tallaga cheese. The use of this starter resulted in reduction of viable count of *B. cereus* and so, no toxin was detected in these cheeses. In contrast, in the control cheese (inoculated with 10^5 cfu isolated strain of *B. cereus*), the viable counts of *B. cereus* increased and released detectable amount of enterotoxin at the end of refrigerated storage.

INTRODUCTION

Bacillus cereus is a common contaminant frequently isolated from foods and dairy products and the fact that the bacterium has a remarkable ability to survive different environmental stresses makes it difficult to separate it from dairy products. The spores survive pasteurization and transfer from milk to pasteurized milk products. The psychrotrophic strains often limit the keeping quality of pasteurized milk and its products [Giffel *et al.*, 1996]. Cases of *B. cereus* food poisoning in milk-based products have been reported [Johnson, 1984; Meer *et al.,* 1991; Andersson *et al.,* 1995; Schoeni & Wang, 2005] and as many as 69-85% psychrotrophic *B. cereus* isolates from milk and milk products have been found to be enterotoxigenic [Griffiths, 1990]. *B. cereus* has been shown to cause two different forms of human gastroenteritis, as well as to be capable of causing mastitis, systemic infection and gangrene [Johnson, 1984]. One toxin is responsible for emetic outbreaks characterised by nausea and vomiting within 0.5 to 6 h after ingestion of contaminated food. The other causes the onset of watery diarrhea and abdominal cramps [Meer *et al.*, 1991].

The organism should be considered as a hazardous in the dairy products due to its heat resistance, psychrotrophic growth, its potential pathogenic character and the capability to grow in milk.

There has been an increased activity in the development of concepts for production of safe food to protect the consumer. The incorporation of some lactic acid bacteria, which show high antimicrobial activity against *B. cereus,* arrest spore outgrowth and enterotoxin production, may be an efficient way to prevent *B. cereus* food poisoning. During growth in fermented products, dairy starter including lactobacilli, lactococci, leuconostocs and streptococci produce inhibitory metabolites against some pathogenic bacteria [Daeschel, 1989; Barefoot & Nettles, 1993; Abriouel *et al.,* 2002; Carrasco *et al.*, 2002]. These antagonists may lead to targeted biocontrol of spoilage flora and foodborne pathogens.

The aim of the present study was to investigate the incidence of *B. cereus* in some dairy products and to determine the growth of *B. cereus* and enterotoxin production in milk and white soft cheese (Tallaga cheese) as well as to validate the effectiveness of antibacterial activity of some LAB against this bacterium to select suitable starter to suppress the growth of *B. cereus* and subsequently to increase the safety of Tallaga chesse.

MATERIALS AND METHODS

Samples. A total of 125 samples of skim milk powder, white soft cheese, Kareish cheese (25 samples each), processed cheese (20) and rice with milk (30) were collected from different markets in Cairo and Giza and analysed for the presence of *Bacillus cereus*.

Bacterial strains. The strain of *B. cereus* (reference) was provided by Northern Regional Research Laboratory, Illinois, USA (NRRL). In addition, one strain of *B. cereus* (local) was isolated from the tested dairy products and selected for its psychrotrophic growth and high production of toxin.

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Lactic acid bacteria. *Lactococcus lactis* and *Lactobacillus casei* were obtained from the Dairy Department, National Research Center, *Lactococcus lactis* ssp. *diacetylactis* from Chr. Hansen's Lab., Denmark. *Lactobacillus gasseri* B-14168, *Lactobacillus rhamnosus* B-445, *Lactobacillus reuteri* B-14171 and *Pediococcus acidilactici* B-1153 were supplied by the Northern Regional Research Laboratory, Illinois, USA (NRRL).

Isolation and identification of *B. cereus*. *B. cereus* was determined using surface plating technique on polymyxin-pyruvate-eggyolk-mannitol-bromothymol blue agar (PEMBA) (Oxoid). *B. cereus* appears as peacock blue-coloured colonies surrounded by a zone of precipitation of egg yolk. The isolates from typical colonies were identified according to Varadaraj [1993].

Determination of psychrotrophic growth at refrigerated temperature of isolated *B. cereus* strains. *B. cereus* isolates were grown for 24 h in tryptone soy broth (T.S.B.), then streaked onto tryptone soy agar (TSA) plates and incubated at 7°C for 10 days [Abdel-Khalek & El-Sherbini, 1996].

Detection of haemolysin activity. Haemolysin activity of the isolates was assayed on sheep blood agar plates (5% v/v) by plating a loopful of 24-h old culture of the isolates. The plates were then incubated at 30°C for 24 h and checked for haemolysis surrounding the growth [Nour *et al.*, 2002].

Screening for antagonistic activity of some lactic acid bacteria. Strains of lactic acid bacteria were cultivated in MRS broth. The cell-free supernatant fluids were obtained by centrifuging the cultures (grown at 37°C for 24 h) at 4000 rpm for 15 min at 4°C. Supernatant was filter-sterilised through 0.22 μm Millex-GV membranes (Millipore) and pH was adjusted to 6 to exclude the organic acid effects. The activity of the resulting solutions was tested against reference strain of *B. cereus* using agar well diffusion assay as described by Lyon & Glatz [1993].

EXPERIMENTAL PROCEDURES

Production of *B. cereus* toxin during growth in milk. Two strains of *B. cereus* (reference and isolated strain (local)) were tested for their ability to synthesize toxin in milk. Sterile reconstituted skim milk powder was inoculated with cells of each of the *B. cereus* strains to an initial concentration of about 10^3 and 10^5 cfu/mL. The inoculated milk was refrigerated at 10°C for 7 days and examined every day for the *B. cereus* count and toxin production.

Production of toxin during storage of Tallaga cheese. Cow's milk was pasteurized at 73°C for 15 s. Salt was added to the milk at the rate of 2%. The milk was divided into four equal portions; the first two portions were inoculated with about 10^3 and 10^5 cfu/mL of *B. cereus* reference strain. The second two portions were inoculated with about 10^3 and 10^5 cfu/mL of isolated *B. cereus* strain. Each portion from the previous portions were divided into two parts, one part was inoculated with 2% of starter culture (*Lac. lactis* ssp. *diacetylactis* and *Lb. rhamnosus* 1:1) and incubated for 2 h at 30°C and the other part was used to serve as a control. All milk portions were manufactured into Tallaga cheese followed the conventional method of Domiati cheese [Fahmi & Sharara, 1950], cheese samples were stored under refrigerator temperature and examined for *B. cereus*, lactic acid bacteria counts, pH and production of *B. cereus* toxin when fresh and after 3, 7, 15, 21 and 30 days.

Bacteriological analysis. *B. cereus* counts were carried out by spreading 0.1 of the appropriate dilution onto PEMBA medium (Oxoid). The incubation temperature was 37°C for 18-24 h. Lactic acid bacteria were enumerated according to Elliker *et al.* [1956].

Determination of pH. pH values were measured using a digital pH meter model Hanna HT 4817.

Assessment of enterotoxin production. The production of *B. cereus* enterotoxin either in culture supernatant or in milk and cheese was determined by the reversed passive latex agglutination test kit (BCET-RPLA, Oxoid) following the manufactures instructions. The sensitivity of this assay procedure is 4 ng enterotoxin per g food.

RESULTS AND DISCUSSION

Prevalence and distribution of *B. cereus* in some dairy products

The presence of *B. cereus* in 125 samples of dairy products is recorded in Table 1. *B. cereus* was detected in 11 (44%), 8 (32%), 5 (25%), 7 (28%) and 18 (60%) of the samples of skim milk powder, white soft cheese processed cheese, Kareish cheese and rice with milk, respectively. *B. cereus* was previously isolated by other investigators. It has been isolated from dried milk samples (37.5% and 43.6%) [Shinagawa, 1993], from raw and pasteurized milk [Meer *et al.,* 1991; Abdel-Khalek & El-Sherbini, 1996; Giffel *et al.,* 1996 & Larsen and Jorgensen, 1997], also Van Netten *et al.,* [1990] isolated *B. cereus* from soft cheese (2%), custard (19%), cream pastry (11%), infant foods and dried milk products [Becker *et al.,* 1994].

The frequency of *B. cereus* occurrence in rice with milk (60%) was comparable with that reported by Andersson *et al.* [1995] who reported that rice containing dishes have been known to cause *B. cereus* food poisoning of the emetic kind. Also, Agata *et al.* [2002] mentioned that in boiled rice, *B. cereus* count rapidly increased and produced emetic toxin.

From the aforementioned results, it could be noticed that

TABLE 1. Prevalence and distribution of *B. cereus* in some dairy products.

Samples	No. of samples	Positive samples	
		No.	%
Skim milk powder	25	11	44
White soft cheese	25	8	32
Processed cheese	20	5	25
Kareish cheese	25	7	28
Rice with milk	30	18	60
Total	125	49	39.2

the tested dairy products constituted the major sources of *B. cereus*. Also, there is an exceedingly high incidence of *B. cereus* was observed in rice with milk, dried milk and white soft cheese compared to processed and Kareish cheese.

Psychrotrophic properties of *B. cereus* isolates

A total of 38 isolates out of 69 isolates (55%) from different tested samples (Table 2), showed visible growth at 7°C within 10 days and thus fitted the generally accepted definition of psychrotrophs. In this respect, Van Netten *et al.* [1990] found that among 155 strains isolated from pasteurized milk 54% were capable to grow at 7°C. Also, psychrotrophic sporeformers were isolated from nearly 70% of freshly pasteurized milk [Meer *et al.,* 1991]. Moreover, Vaisainin *et al.* [1991] reported that the majority of the strains isolated from dairy products were able to grow at a temperature below 10°C. In contrast, the lower incidence of sporeforming psychrotrophs amounting to 28–35%, 31% was reported by Coghill [1982] and Andersson *et al.* [1995], respectively.

TABLE 2. Psychrotrophic properties of isolated *B. cereus* strains incubated at 7°C for 10 days.

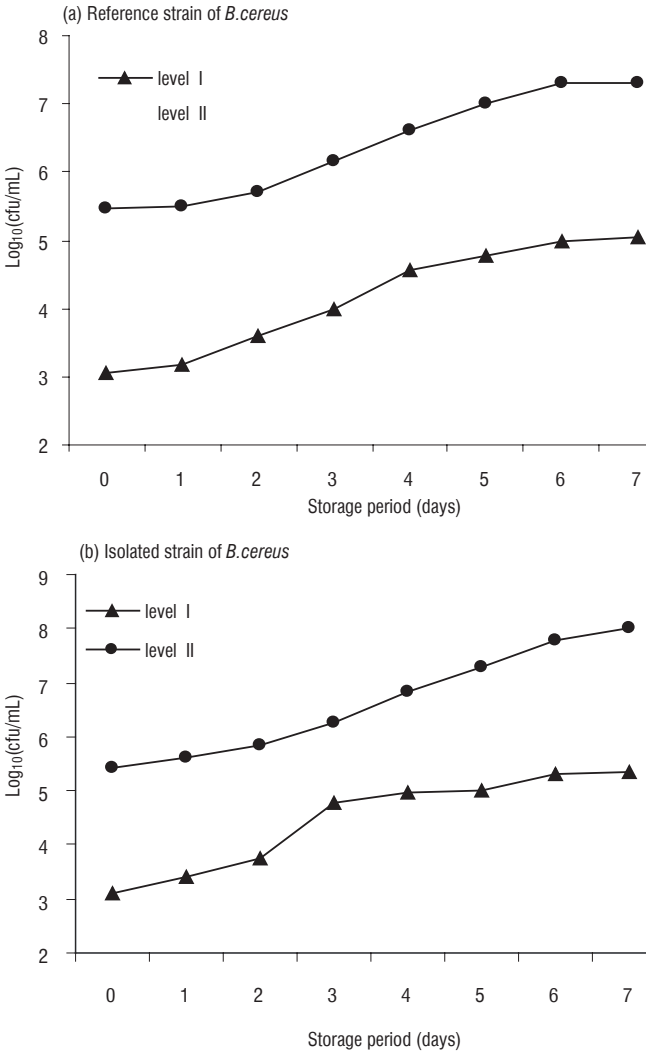
Samples	No. of *B. cereus* isolates	Psychrotrophic growth	
		No.	%
Skim milk powder	17	7	41.1
White soft cheese	15	11	73.3
Processed cheese	8	2	25
Kareish cheese	9	4	44.4
Rice with milk	20	15	75
Total	69	38	55

Hemolytic activity of the isolates

B. cereus produce a large number of potentially virulent factors, including tripartite hemolysin [Schoeni & Wang, 2005]. Results reveal that 61 out of 69 isolates (88.4%) show hemolytic activity. This result is not far from those obtained by Nour *et al.* [2002] who recorded that 89.74% and 86.29% show hemolysis surrounding the growth of the isolates from market dairy products and farm samples, respectively.

Production of *B. cereus* toxin during growth in milk

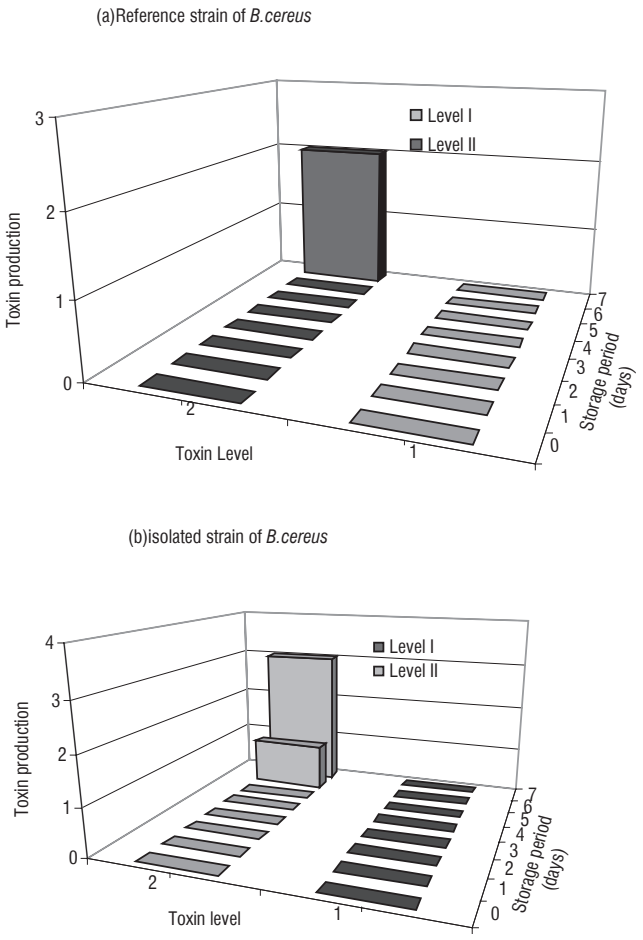
Results of behaviour and toxin production during growth of the *B. cereus* reference and the isolated strains in sterilized milk at 10°C are shown in Figures 1 and 2. Both of the local isolated and reference strain, when inoculated with 10^3 and 10^5 cfu/mL, grew exponentially from the second day of storage at 10°C, and by day 7, counts reached 10^5 and 10^7 cfu/mL with the two levels of contamination (10^3 and 10^5 cfu/mL), respectively (Figure 1 a, b). But the viable count of the local isolated strain was higher than the reference strain. Several reports have shown that the organism can proliferate rapidly in milk, depending on the storage temperature [Christiansson *et al.*, 1989; Notermans *et al.,* 1997]. Moreover, some studies revealed that the organism would grow at a temperature as low as 5°C [Meer *et al.,* 1991]. On the other hand, there was no measurable toxin when the milk was inoculated with 10^3 cfu/mL with the both strains. The failure



Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.

FIGURE 1. Growth of *B. cereus* during storage of inoculated milk at 10°C.

of the strains to produce toxin may be due to low bacterial counts (10^5 cfu/mL after 7 days) hence inability to produce detectable amount of toxin. But, these samples could not be considered safe because the two tested strains may grow and increase in count even while refrigerated, so the toxin would be produced if stored for longer periods. The two tested strains, when inoculated in the milk with 10^5 cfu/mL, were shown to be capable of producing toxin in sterilized milk at the end of the storage period, but the isolated strain could produce the toxin from day 6, and the reference strain from the seventh day (Figure 2). These data indicate that there are strain differences in the growth characteristics of *B. cereus.* Beattie & Williams [1999] reported that there were pronounced strain differences in the amount of toxin produced by *B. cereus* isolates. These results agree with those reported by Baker & Griffiths [1995] who mentioned that psychrotrophic strains of *B. cereus* could produce enterotoxin during growth in milk at refrigeration temperature, but high bacterial numbers ($>1\times10^7$ cfu/mL) were required before detectable levels of toxin were synthesized. Also, Griffiths [1990] stated that a majority of *B. cereus* isolated from a variety of dairy products including pasteurized milk, ice cream and milk powder were capable of producing toxins. Production of



Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.

0 = Negative 1 = + 2 = ++ 3 = +++

FIGURE 2. Toxin production of *B. cereus* during storage of milk at 10°C.

toxin by *B. cereus* at low temperature in dairy products has been reported previously [Sutherland, 1993; Andersson *et al.,* 1995; Finlay *et al.,* 2000; Jaaskelainen *et al.,* 2003].

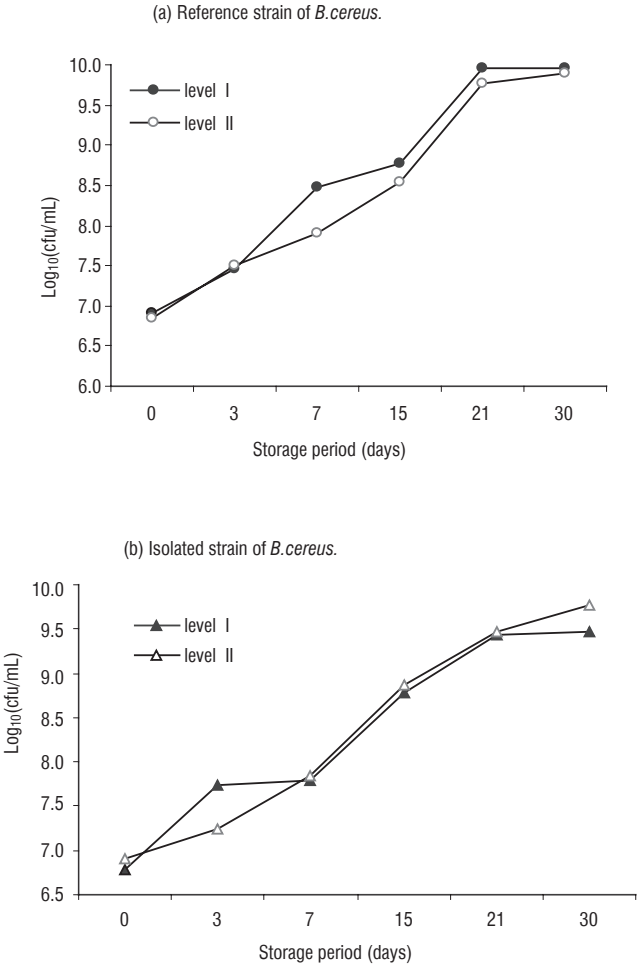
As a consequence of all the above-mentioned aspects, the presence of *B. cereus* in milk should be considered as hazardous etiology.

Antagonistic activity of some lactic acid bacteria against *B. cereus*

Results in Table 3 show the antibacterial activity of 7 strains of lactic acid bacteria (LAB) against *B. cereus* by agar well diffusion technique. Results recorded reveal that the entire tested LAB has variable antibacterial activity. *Lb. reuteri* was the most effective probiotic culture among all tested cultures, followed by *Lb. rhamnosus*. These results are in agreement with findings of Lewus *et al.* [1991] and Alak *et al.* [1997] who observed that *Lb. reuteri* inhibit the growth of potential pathogens by the secretion of inhibitory products, including reuterin. Also, *Lb. rhamnosus* produces a substance having a potent inhibitory effect on a wide range of bacterial species. Also, *P. acidilactici* show antibacterial activity towards *B. cereus* [Bhunia *et al.,* 1988]. This result may be due to the inhibitor substances. Moreover, the starter culture *Lac. lactis* ssp. *diacetylactis*, which was the preferable starter for soft cheese, also affected the growth of *B. cereus* with zone diameter of 6 mm (Table 3). This result is due to the production of inhibitory flavour metabolite, diacetyl. Diacetyl may be a minor contributor to broad-spectrum antagonism [Barefoot & Nettles, 1993]. Thus, the starter of a mixed culture of *Lac. lactis* ssp. *diacetylactis* and *Lb. rhamnosus* was further applied in Tallaga cheese to study its effect on the growth of

TABLE 3. Antimicrobial activity of some lactic bacteria against *B. cereus* reference strain.

Lactic acid bacteria	Diameter of inhibition zone (mm)
Lac. lactis spp. *diacetylactis*	6.0
Lac. lactis spp. *lactis*	4.0
P. acidilactici	7.5
Lb. gasseri	7.0
Lb. casei	5.0
Lb. rhamnosus	8.0
Lb. reuteri	9.0



Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.

FIGURE 3. Changes in lactic acid bacterial count in Tallaga cheese during refrigerated storage for 30 days.

B cereus. Results obtained by El-Shafei *et al*. [2004] proved that *Lb. reuteri* inhibited the growth of *Lac. lactis* ssp. *diacetylactis*, thus *Lb. reuteri* was excluded out of work.

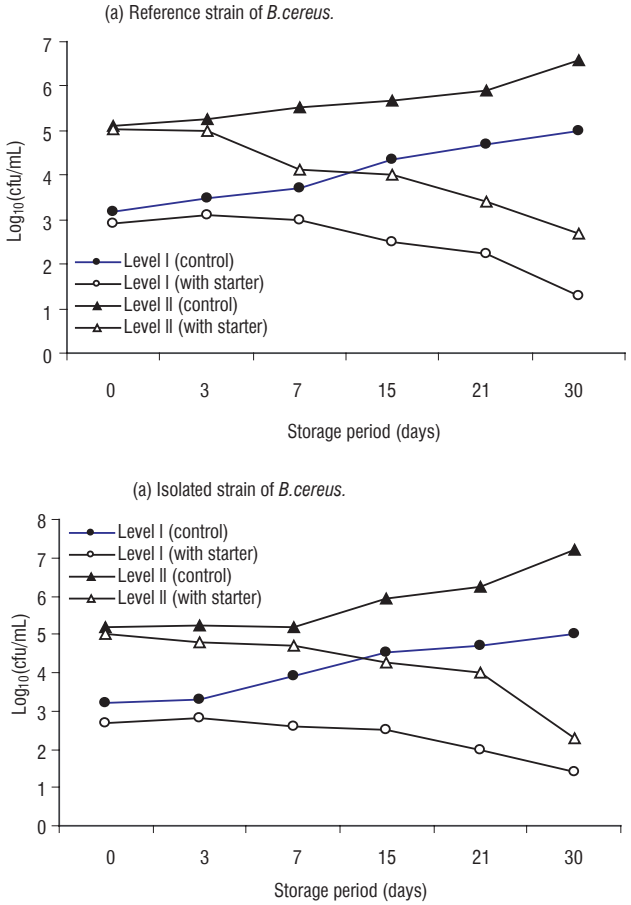
Behavior of lactic acid bacteria, *B. cereus* and production of enterotoxin during storage of Tallaga cheese

Lactic acid bacteria

Data illustrated in Figure 3 show a gradual increase in the lactic acid bacteria counts during the first three weeks, then the increase was slight, at subsequent weeks of the storage period (30 days) in all cheesees manufactured with the starter culture (Figure 4). These results are in line with those reported by Grace & Bernie [2001].

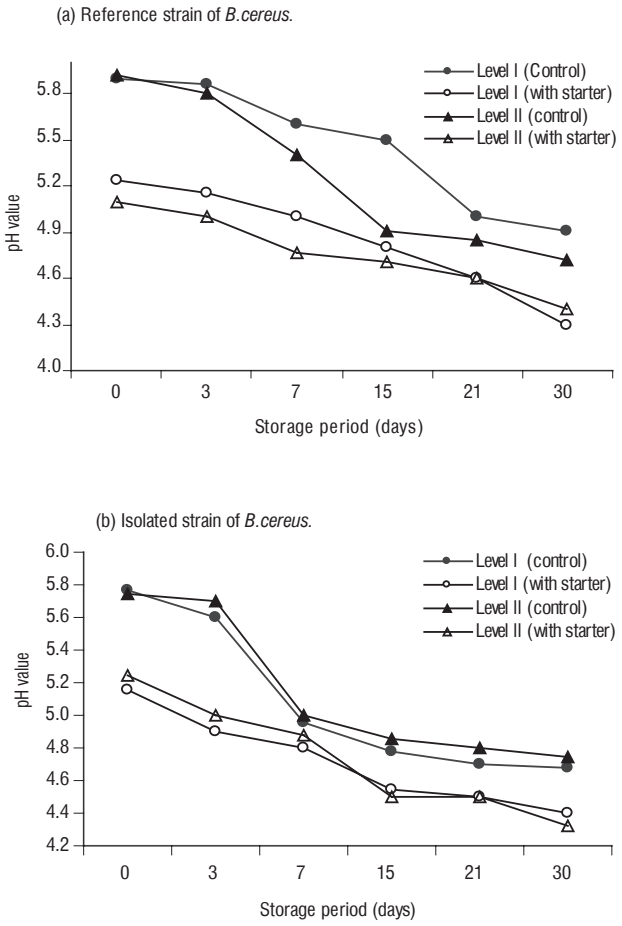
B. cereus

Growth and survival of *B. cereus* in Tallaga cheese with and without starter culture are shown in Figure 4 a, b. Results show that *B. cereus* (the reference & isolated strains), both initiated with 10^3 and 10^5 cfu/mL *B. cereus*, grew well in control cheese and reached the maximum growth rate at the end of the storage period. On the other hand, in cheeses manufactured by inoculation of starter culture (about 6 $\log_{10}$ cfu/mL) and contaminated with B. *cereus* (reference strain) with (about 3 $\log_{10}$ cfu/mL), the counts of *B. cereus* decreased by



Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.

FIGURE 4. Growth of *B. cereus* during refrigerated storage of Tallaga cheese at 10°C.

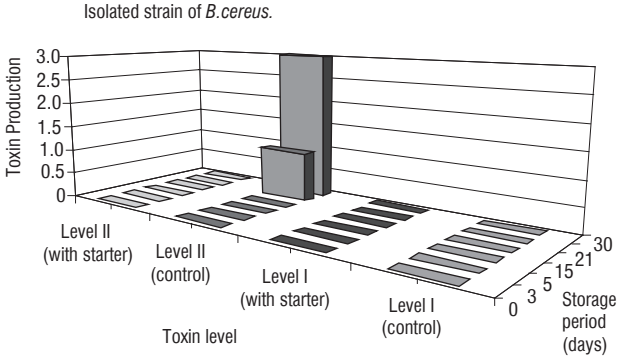


Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.

FIGURE 5. Changes in pH values of Tallaga cheese during refrigerated storage for 30 days.

approximately 1.7 log cycle after 30 days of cold storage and approximately 3.7 log cycle with respect to its control cheese at the end of the storage period. Similar results were obtained with the isolated strain (local) of *B. cereus* and the reduction of count was 3.6 log cycle as compared to the control without starter culture at the end of storage at refrigerator temperature. In cheese manufactured with the used starter and inoculated with about 10^5 cfu/mL reference and isolated strains of *B. cereus*. The reduction in counts of *B.cereus* reached 3.8 and 4.9 log units, respectively, as compared with their control cheese at the end of the storage period (30 days). The reduction of viable count of *B. cereus* could be mainly attributed to the production of antimicrobial substance produced by the starter culture and also to the decrease in pH (Figure 5 a, b), which shows a gradual decrease in pH throughout storage period. Beattie & Williams [2002] reported that growth of *B. cereus* was only possible in the range of pH 4.5–9.5; therefore, *B. cereus* did not represent an immediate hazard in low pH foods.

Concerning the production of the toxin by the two strains of *B. cereus*, enterotoxin was detectable after 21 days of cold storage in the control cheese inoculated with 10^5 cfu/mL of isolated strain of *B. cereus* (Figure 6). This result agreed with that reported by Meer *et al.* [1991] who demonstrated that feta cheese and skim milk powder were implicated in emetic illness. Also, Sutherland [1993] mentioned that toxin formation was possible at 21 and 6°C by a psychrotrophic strains



Level I: Milk inoculated with 10^3 cfu/mL; Level II: Milk inoculated with 10^5 cfu/mL.
0 = Negative 1 = + 2 = ++ 3 = +++

FIGURE 6. Toxin production of *B. cereus* during refrigerated storage of Tallage cheese.

of *B. cereus* in cream and some dairy products. In contrast, in Gouda cheese, Grace & Bernie [2001] contaminated the cheese milk with 10^2 *B. cereus*/mL and found that the viable counts were 10^4 cfu/g of cheese at hooping, then, reduced to less than 10^2 cfu/g after pressing and after brining *B. cereus* was not detected in cheese curd.

On the other hand, the other control (inoculated with 10^5 cfu/mL reference strain) was unable to produce detectable amount of toxin despite high growth level (10^6 cfu/mL). This may be due to the amount of toxin produced by the reference strain under the sensitivity of the RPLA kit or the strain could not produce detectable toxin in cheese. In spite of the production of the enterotoxin by this strain in the milk, it could not produce detectable amount of toxin in cheese. This may be due to the different conditions, particularly the low pH and salt content. On occasions, proliferation of *B. cereus* may not result in the formation and accumulation of toxin within the food system [Sutherland, 1993]. Also, Gabig-Ciminska *et al.* [2004] reported that large differences in the amounts of enterotoxins produced by different strains makes it difficult to give a total infective dose of *B. cereus*, and consumption of food that contains more than 10^6 *B. cereus* cfu/g may result in food poisoning. No toxin was detectable in all cheeses manufactured by the addition of starter; this is due to the reduction of *B. cereus* numbers (Figure 6).

CONCLUSION

Incidence of *B. cereus* is high in the tested dairy products, which indicates an emergency problem in safe food production. Therefore, it is highly recommended that control measures be taken during production, processing and handling of milk and milk products.

Out of these control measures our results support the use of antimicrobial strains (*Lac. lactis* ssp. *diacetylactis* and *Lb. rhamnosus*) as a culture adjunct to suppress the availability of *B. cereus* and its toxin production during cheese manufacture and storage.

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