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A STUDY ON INCIDENCE AND GROWTH HAZARDS OF *BACILLUS CEREUS* IN EGYPTIAN PASTEURIZED MILK DURING REFRIGERATOR STORAGE

(With 2 Tables and One Figure)

By

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دراسة عن وجود ومخاطر نمو ميكروب الباسيلس سيرس فى الألبان المبسترة المصرية أثناء حفظها مبردة

عزة على التابعي ، زينب ابراهيم سليمان ، هدى أمين عايدية

يعتبر ميكروب الباسيلس سيرس من الميكروبات التى تتسبب فى فساد الألبان المبسترة وتقلل من فترة صلاحيتها حيث انه من الميكروبات المتجرثمة التى لا تتاثر بعملية البسترة. ويوجد منها بعض الأنواع التى لها القدرة على العيش فى درجات حرارة منخفضة. لذلك تم استبيان مدى وجود ذلك الميكروب فى الألبان المبسترة المصرية. تم تجميع عدد ٨٠ عينة من ألبان مبسترة بالحرارة العالية لفترة قصيرة (٧٢ درجة مئوية لمدة ١٥ ثانية)، ٤٠ عينة من كل من ألبان كاملة الدسم والمنزوعة الدسم ١. تم فحص الألبان عند بداية فتحها وكذلك يوميا أثناء حفظها عند درجة ٥ ° م حتى انتهاء صلاحيتها (١٥ يوم من الإنتاج) وقد أسفرت النتائج على وجود الميكروب فى ٣ (٧,٥%) من عينات اللبن الكامل الدسم من اجمالى عدد العينات التي تم فحصها في البداية. ولم يتم تسجيل اى زيادة فى العينات الايجابية خلال ٨ ايام من الفحص اثناء حفظها مبردة. ولكن بعد ذلك كان العدد الكلى للعينات الأيجابية فى الألبان المبسترة الكاملة الدسم ١٩ (٤٧,٥%) وفى عينات اللبن المنزوع الدسم ١٠ (٢٥%) وذلك حتى انتهاء تاريخ صلاحيتها. هذا ولم يتعدى العدد الكلى للميكروب فى العينات الايجابية الحد الذى يشكل خطرا على صحة المستهلك (٥١٠خلية/ مل). اثناء الحفظ عند درجة ٥ ° م. كذلك تم حفظ جزئين من عينة من اللبن التي اثبت التحاليل ايجابيتها لفحص الباسيلس سيرس وتحتوى على عدد قليل من الميكروب ، فى درجة ٢٥ و٣٢ درجة مئوية .وقد أظهرت النتائج أن الحفظ الغير جيد وخاصة فى فصل الصيف قد يؤدى إلى نمو الميكروب بصورة كبيرة تؤدى الى فساد المنتج وقد يؤدى إلى حدوث تسمم غذائي بالميكروب فى اقل من ٢٤ ساعة. كذلك تم الكشف عن قدرة المعزولات على إفراز إنزيمات تحلل الدهون والبروتين والتي تؤدى الى فساد المنتج وأظهرت النتائج أن ٩٦% و٦٠% من المعزولات لها القدرة على تحلل الدهون والبروتينات على التوالى. لذلك يمكن القول ان سلامة اللبن المبستر مسؤولية مشتركة بين المنتج والموزع والمستهلك عن طريق الحفظ الجيد. هذا وقد تم مناقشة التأثيرات الاقتصادية والصحية لوجود ذلك الميكروب فى الألبان المبسترة.

SUMMARY

A total of 80 random samples, forty of each of pasteurized full cream and skimmed milk were collected from local Egyptian markets. Samples were screened for the occurrence of *Bacillus cereus*. The incidence and total count of *B. cereus* in the different samples at the sterile opening were evaluated. 3 (7.5%) *B. cereus* positive samples were detected when the samples analyzed early. All milk samples were stored at 5 °C and evaluated for *B. cereus* up to their expiry date (at 72 °C for 15 s). No increase in incidence and count of *B. cereus* were observed during the first 8 days of storage at 50C but thereafter, an increase in the incidence and population of the microorganism with storage time was observed. *B. cereus* was detected in 19 (47.5%) and 10 (25%) of the investigated pasteurized full cream and skimmed milk samples, respectively at the end of refrigerator storage. Quantitive analysis of growth of *B. cereus* in refrigerated pasteurized milk confirms that no samples achieved hygienically undesirable limit 105 cfu/ml. Two portion of milk, proved to be positive for *B. cereus* were kept at ambient (25°C) and optimum (32°C) temperatures and evaluated for the growth of *B. cereus* during 24 hours. The data indicate that, if storage temperature is abused, less than 24 h are required for low initial levels of *B. cereus* cells to increase to levels that are sufficient to cause foodborne illness. Out of 50 *B. cereus* isolates tested, 48 (96 %) possessed lipolytic activity, while 30 (60%) showed proteolytic activity. *B. cereus* ability to elaborate protease and lipase may give an indication of their active role in the spoilage of pasteurized milk in abuse temperature. In spite of the fact that the producer is responsible for the safety of pasteurized milk, it is evident that this responsibility has to be shared with the retailer and consumer by applying proper cooling, especially in the summer period. *B. cereus* role in the keeping quality and hazards of pasteurized milk were discussed.

Key words: *Milk, pasteurized milk, Bacillus cereus.*

INTRODUCTION

The gradual transition of food retailing patterns from small stores to large supermarkets has been associated with an extension of the shelf life of the perishable foods. This applies also to pasteurized milk. Improving microbial safety and extending the shelf life of pasteurized milk have always been an important concern to the dairy industry. A major factor limiting realization of these goals is microorganisms

surviving the pasteurization process and/or contributing to post pasteurization contamination. *B. cereus* is a common bacterium that is often present in raw milk (Griffiths, 1992). Possible sources of contamination are the soil, faeces, bedding, feed, air, the milker, and the milking equipment (Van Heddeghem and Vlaemynck, 1992)

Under stress conditions, the *B. cereus* cells sporulate. The presence of spores in raw milk, their high resistance to the pasteurization temperature (Lin *et al.*, 1998 and Janstova and Lukasova, 2001), and also the adherence of spores and vegetative cells to metal surfaces (Faille *et al.*, 2001 and Peng *et al.*, 2001) make *B. cereus* an unwelcome, but very frequent contaminant of dairy products (Huang *et al.*, 1999).

Milk simply labeled "pasteurization" is usually treated with High Temperature/Short Time (HTST). In the HTST process, milk is forced between metal plates or through pipes heated on the outside by hot water, and is heated to 72 °C for 15-20 seconds. Pasteurized milk has a shelf life from only a couple of days in some countries to up to over 20 days in, e.g., USA (Rysstad and Kolstad, 2006). Pasteurization will actually induce spore germination, and in the absence of competing flora, *B. cereus* grows well (Granum and Lund, 1997). *B. cereus* species may be subdivided according to their thermotolerance: the psychrotrophes that can grow at temperatures as low as 4°C, but fail to grow at temperatures above 37°C, and the mesophiles, able to grow at temperatures ranging from 10°C to, sometimes even, 50°C (Kramer and Gilbert, 1989). Psychrotrophic strains of *B. cereus* are some of the most important organisms limiting the shelf life of pasteurized milk stored above 6°C (Griffiths, 1992). *B. cereus* is frequently found in pasteurized milk, causing spoilage because of the production of lipases and proteases. They can also exhibit a health risk to the consumer since they produce enterotoxins (Champagne *et al.*, 1994).

B. cereus play a very important role in the keeping quality of pasteurized milk. Therefore, the present study was undertaken in order to assess the existence and growth of *B. cereus* during refrigerator storage of pasteurized milk. The effect of abuse storage temperature on growth of *B. cereus* in milk and the proteolytic and lipolytic potentials of isolates were also investigated.

MATERIALS and METHODS

1. Milk samples and sampling:

A total of eighty, freshly produced (day 2 of production) pasteurized milk samples (at 72. °C for 15 s), forty of each of

pasteurized full fat milk and skimmed milk, packaged in plastic bottles (15 days shelf life) were collected from local Egyptian markets over a period of 6 months. The samples were transported in cooler boxes with ice to the laboratory and analyzed within 1h after collection.

Milk samples were aseptically opened under laminar flow. The incidence of *B. cereus* in the milk samples was investigated. All milk samples were tested for *B. cereus* at the sterile opening and daily during keeping the samples at 5°C, the recommended temperature for cold storage of pasteurized milk until their expiry date.

In addition, 2 portion of milk, proved to be positive were stored in incubators at ambient (25°C) and optimum (32°C) temperatures and then tested for growth of *B. cereus* at intervals of 0, 3, 7, 10, 15, and 24 h. The average number of colonies on the duplicate plates was recorded for each sample at each time to study the effect of abuse storage temperature on growth of *B. cereus* in milk.

2. Isolation and enumeration of *B. cereus*:

For each milk sample, 50 ml were added to 450 ml Butterfield's phosphate-buffered and blended for 2 min at 18,000-20,000 rpm. Serial dilutions from 10-1 to 10-6 were prepared. Isolation procedures followed the methods described by Health Protection Agency (2004), using B. *cereus* selective agar (PEMBA) supplemented with polymyxin B (Oxoid) and egg yolk emulsion (Oxoid) as selective plating medium. Plates were inoculated with each dilution of sample (including 1:10) by spreading 0.1 ml evenly onto surface of each plate with sterile glass spreading rod. The inoculated Petri dishes were incubated at 30°C for 24 h. Typical 2 to 5 mm in diameter, turquoise to peacock blue in color with flat ground glass surface and surrounded by a grey to turquoise halo of dense precipitate (egg yolk reaction) were counted.

3. Confirmation of *B. cereus*

Turquoise to peacock blue colonies were isolated and confirmed as *B. cereus*. 5 or more presumptive positive colonies plates were Picked and transferred to nutrient agar (NA) slants and incubated at 30°C or for 24h for biochemical confirmation as *B. cereus*. All isolates from NA were examined by Gram stain for morphologic characterization. The identities of all suspected colonies were confirmed by testing for glucose fermentation, Voges-Proskauer reaction, nitrate reduction, and β-hemolytic activity. *B. cereus* strains should be positive for all four reaction. Motility test, rhizoid growth on NA plates were applied to differentiate typical strains of *B. cereus* from other members of the cereus group (FDA, 1998).

4. Determination of proteolytic and lipolytic activities of isolates

The proteolytic activity was tested by inoculating the strains on 50 percent skim milk agar. Then plates were incubated at 5°C for 10 days. The presence of clear zone of hydrolysis around the colonies confirmed as positive (APHA, 1978).

The lipolytic activity was tested by culturing the strains on tributyrin agar. The plates were incubated at 25°C for 3 days and screened for the presence of clear zone of hydrolysis (Harrigan and Mc Cance, 1976).

RESULTS

Table 1: Statistical analytical results of *B. cereus* incidence and counts in examined full cream and skimmed pasteurized milk samples during storage at 50C.

Items	0-8 days		9 -11days		12-13 days		14-15 days	
	Full cream	Skimmed	Full cream	Skimmed	Full cream	Skimmed	Full cream	Skimmed
No. of total samples	40	40	40	40	40	40	40	40
ND	37	40	30	34	26	32	21	30
Positive	3 (7.5%)	0	10 (25%)	6 (15%)	14 (35%)	8 (20%)	19 (47.5%)	10 (25%)
Minimum	2.5 x102	-	4.5 x102	3.5 x102	2.0 x102	3.5 x102	2.0 x102	2.0 x102
Maximum	5.0 x102	-	2.5 x103	9.0 x102	4.0 x103	2.0 x103	8.2 x103	7.2 x103
Mean	3.5 x102	-	1.1 x103	6.1 x102	1.6 x103	8.6 x102	2.8 x103	3.0 x103
S.D	1.3 x102	-	5.9 x102	2.1 x102	1.3 x103	5.5 x102	2.4 x103	2.6 x103
±S.E	7.6 x101	-	1.9 x102	8.7 x101	3.4 x102	1.9 x102	5.5 x102	8.1 x102

0 Time: Second day of production.
ND: Non detectable (<102).
NB: % was calculated according to the total number of each sample (40).

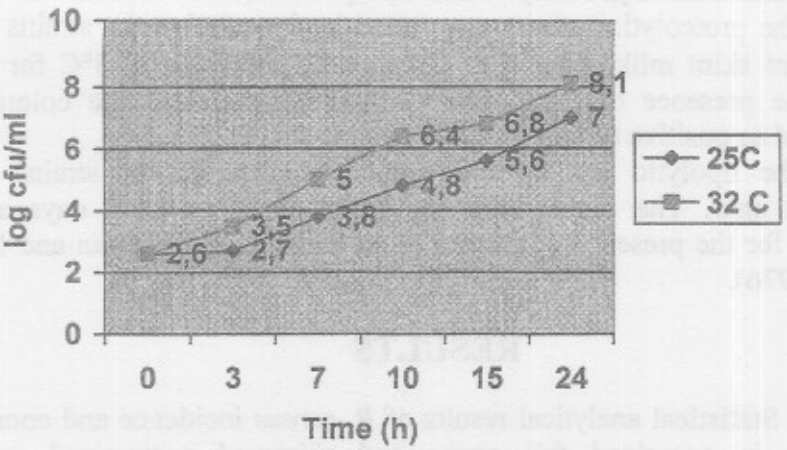

Fig. 1: Growth of *Bacillus cereus* in pasteurized milk during storage at ambient (25°C) and optimum (32°C).

Table 2: Lipolytic and proteolytic activities of *B. cereus* isolated from the examined milk samples.

N0. of tested isolates	Lipolytic		Proteolytic	
50	No.	%	No.	%
	48	96	30	60

DISCUSSION

The prevalence and total count of *B. cereus* in examined samples during refrigerator storage up to their expiry date were presented in Table (1).

On examination on the day of opening (day 2 of production) only 3 (7.5%) of examined full cream milk samples were found to be positive for *B. cereus* cr. However, no positive samples could be detected in examined skimmed milk samples on the day of opening. The initial count of *B. cereus* during the first 8 days of storage at 50C was low, numbers ranged from 2.5 x102 to 5.0 x102 with a mean count of 3.5 x102 ± 7.6 x101 cfu/ml were observed in the positive samples recorded. Improved temperature control of stored milk during sale may be reflected in the lower reported prevalence.

For the first 8 days of storage at 50C, there was no increase in number of positive samples and count of *B. cereus* but thereafter, an increase in the incidence and population of the microorganism with

storage time was observed. The achieved results in Table (1) showed that higher incidence of *B. cereus* positive samples was recorded from pasteurized full eam milk samples during observation period. *B .cereus* positive samples were detected in 19 (47.5%) and 10 (25%) of the investigated pasteurized full cream and skimmed milk samples, respectively at the end of refrigerator storage. In this concern, Larsen and Jorgensen (1996) reported that vegetative cells and spores of *B. cereus* tend to adhere to fat droplets of the milk. The presented results indirectly corroborate with that findings by Schiegelova *et al.* (2003) they reported that products made from skimmed milk (curd cheese) were contaminated in one case only (3.2% of products), while products with higher fat content (even if made with different production technologies) in 10–16% of cases.

The obtained results regarding the presence of *B. cereus* in pasteurized milk were supported by other reports. Isolation of *B. cereus* from samples of pasteurized milk (35%) have been reported by Ahmed *et al.* (1983). *B. cereus* was detected in 2% of Chinese pasteurized milk samples at average counts of 280/ml (Wong *et al.,* 1988).

Counts of *B. cereus* in 38 samples of pasteurized milk in the Netherlands showed that all contained spores with a mean of around 100/100 ml (Notermans *et al.,* 1997), with counts highest in the summer. *B. cereus* could be isolated from 133 (40%) from pasteurized milk in household refrigerators in The Netherlands, they found low *B. cereus* counts, less than five per ml in the samples that were stored below 7°C. (Te Giffel *et al.,* 1997). Eneroth *et al.* (2001) conducted a study on pasteurized milk samples from Sweden they reported that counts of *B. cereus* ranging from 10 to 1200 CFU/ml in the samples taken from consumer packages. however, counts of up to 104 cfu/ml have also been recovered from bottled pasteurized milk (86.7%) by Ionescu *et al.* (1966). Moreover, Larsen and Jorgensen (1996) analyzed 458 samples of pasteurized milk and cream from 3 Danish dairies and found the total viable count of *B. cereus* ranging from 103 to 3 X 105 cfu / ml. The high prevalence of *B. cereus* in milk reported by these researcher may be due to survival of spores during pasteurization and subsequent re-growth.

Many dairy scientists believe that contamination of pasteurized milk with *Bacillus spp.* results from spores that were present in the raw milk and survived the pasteurization process (Griffiths and Phillips, 1990 and Sutherland and Murdoch, 1994). However, other data in the literature suggest that post pasteurization contamination might significantly contribute to *Bacillus* counts in pasteurized milk (Crielly *et al.,* 1994).

The results recorded in Table (1) pinpoint that *B. cereus* was found to be present in two-day pasteurized milk samples in relatively small numbers and the count did not increase during first 8 days of storage, but after this time an increase in the incidence and population of the microorganism with storage time were observed. At 9- 11 days of storage, the milk samples had *B. cereus* count ranged from 4.5x102 to 2.5x103 in examined pasteurized full cream milk and 3.5x102 to 9.0 x102 in skimmed milk samples. The count increased up to 8.2 x103 and to 7.2 x103 cfu/ml at the end of observation period in examined pasteurized full cream and skimmed milk samples, respectively. The present findings suggest that the growth of *B. cereus* naturally present in pasteurized milk as regarded as a thermoduric psychrotrophic species, can survive pasteurization and grow at refrigeration temperatures .Several strains of *B. cereus* are psychrotrophic and are capable of growth in milk at temperatures as low as 4-6°C (Andersson *et al.*, 1995). Possibly mesophilic *B. cereus* may be facultative in the sense that they develop psychrophilic activity after adaptation to prolonged low temperature storage.

Since psychrotrophs have a significant impact on the keeping quality of pasteurized milk the findings in this study suggest that the ability of *B. cereus* to grow at low temperature occurs during storage. In this concern, Kevin *et al.* (1998) reported that the detection of psychrotrophic strains of the *B. cereus* group in dairy products is at present extremely slow (5 to 10 days), due to the inability to discriminate psychrotrophic and mesophilic strains by means other than growth at low temperature. Psychrotrophic bacteria have been recognized as a recurring problem in the refrigerated storage and distribution of perishable food products, which is particularly pertinent to the dairy industry (Sorhaug and Stepaniak, 1997). It has been estimated that 25% of all shelf life problems associated with conventionally pasteurized milk and cream products in the United States may be linked to this class of thermoduric bacteria, with a large number of the contaminants being psychrotrophic *B. cereus* and *B. mycoides* (Meer *et al.*, 1991).

In evaluating these data in term of the practical keeping problem in home refrigerators, good quality commercially pasteurized milk should maintain desirable quality for at least 8 days when stored at 50C. It has been considered that a number of 105 *B. cereus* present in pasteurized milk should be regarded as hazardous to the consumer (Notermans *et al.*, 1997). Quantitive analysis of growth of *B. cereus* in

refrigerated pasteurized milk confirms that no samples achieved hygienically undesirable limit 105 cfu/ml.

The gross abuse trials were presented in Fig (1). Based on the worst case situation, we considered the possibility that consumers might left portions of milk not use it immediately, or that unused might be left un refrigerated, and outgrowth of any *B. cereus* present in milk could occur. An increase in the population of *B. cereus* with storage time was observed in both samples and its level had reached 7 and 8.1 log cfu/ml after 24 hours in samples stored at 25 and 32 °C respectively. Approximately 3 to 7 hours were required to reach 5 log cfu/ml during storage at 32°C, and 10 to almost 15 h during storage at 25°C. Indicating a possibility of larger mesophilic bacterial activity at 25 and 32°C than psychrotrophic activity at 5°C. Chandler and McMeein (1985) consider the storage temperature as the basic factor influencing the growth of the microorganisms in the spoilage of milk. Several reports shown that the organism can proliferate in pasteurized milk depending on the storage temperature (Crielly *et al.*, 1994). According to Adams and Moss (2000), these microorganisms can grow and spoil a product quite rapidly at ambient temperature, so refrigerated storage is often a requirement for an acceptable shelf-life. In this respect, Feijoo *et al.* (1997) studied the outgrowth of *B. cereus* in whipping cream stored at 23, and 32°C, from an initial inoculum of 1 x102 cfu /ml, within 9 h at 32°C and 11 h at 23°C, numbers reached 105 to 106 cfu/ml. In this concern, Bean and Griffin (1990) reported that 94% of outbreaks involving *B. cereus* were attributed to improper holding temperature; therefore, the importance of maintaining adequate refrigerated storage of dairy products is obvious. There are two forms of *B. cereus* food poisoning. The rapid-onset emetic illness occurs one to five hours after the consumption of food contaminated with a heat stable enterotoxin produced by *B. cereus* during growth in the food. The second form is a diarrhoeal illness, which becomes evident eight to sixteen hours after ingestion of contaminated food (Helgason *et al.*, 2000).

During storage under low temperature the milk undergoes spoilage due to proteinases and lipases released by psychrotrophic bacteria (Sorhaug and Stepaniak, 1997).

The number of *Bacillus* isolates positive for proteolysis and lipolysis is given in Table (2). Out of a total 50 isolates, 48 (96%) and 30 (60%) of total *B. cereus* isolated were found to be lipolytic and proteolytic respectively. Meer *et al.* (1993) concluded that 57% of the *Bacillus* isolates obtained from 59 Grade A milk samples in Oregon, US were lipolytic. Almeida *et al.* (2000) detected that 92% of the *Bacillus*

isolates were found to have proteolytic and or lipolytic activity. Matta and Punj (1999) obtained 59 lipolytic *Bacillus* isolates and showed that *B. cereus* (32.2%) was the predominant lipolytic organism. It has been estimated that 25% of all shelf life problems associated with conventionally pasteurized milk and cream products may be linked to a large number of the contaminants being psychrotrophic *B. cereus*, the organism is associated with defects such as off flavours, sweet curdling and bitty cream caused by proteinase, lipase and phospholipase enzymes (Meer *et al.*, 1991). Brown (2000) described *Bacillus* spp. as microorganisms that cause significant economic losses. Low concentrations of *B. cereus* can cause defects such as coagulation and off-odors due to proteolysis and lipolysis (Andersson *et al.*, 1995). This defects are detected at the moment of reaching the concentration of microorganisms of 5x105 - 107 cfu/ml (Marth and Steele, 1998).

Based on the results obtained, *B. cereus* levels on examined Egyptian pasteurized milk samples were less than that considered harmful. The data indicate that, if storage temperature is abused, less than 24 h are required for low initial levels of *B. cereus* cells to increase to levels that are sufficient to cause foodborne illness. Pasteurized milk stored under proper conditions, at 5 °C, and consumed within the expiration date in general cause no problems. The producer is responsible for the safety of pasteurized milk, it is evident that this responsibility has to be shared with the retailer and consumer by applying proper cooling, especially in the summer period. It is evident from these studies that there might be a possibility of alteration of milk by the proteolytic and lipolytic activities of the *B. cereus* isolates in the present study, their activity may result in the degradation of milk during transport and storage, hence care is to be taken while handling and storage of pasteurized milk to avoid spoilage.

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