Incidence and public health significance of aerobic spore forming bacteria in Ultra Heat Treated (UHT) milk

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Abstract:
Ultra Heat Treatment (UHT) is the partial sterilization of milk by heating it for 1-2 seconds, at a temperature exceeding 135°C then kept inside the aseptic package.
A total of seventy five UHT milk samples were randomly collected from different retail outlets in Cairo and Giza governorates.
Collected samples were examined for enumeration and isolation of aerobic spore forming bacteria as well as *Bacillus cereus*. All prepared samples were inoculated on different bacteriological media and a number of biochemical tests were performed for the confirmation of the isolate.
The obtained results revealed that, all samples were positive for the presence of aerobic spore formers, while only 46 samples (61.33%) showed growth of *Bacillus cereus.*
The total count of aerobic spore forming bacteria and *Bacillus cereus* were within the span of 3.78 to 9.36 log CFU/ml and 2 to 5.69 log CFU/ml respectively.
A total of 191 pure isolates were obtained. They identified as *Bacillus subtilis, B. coagulans, B. circulans, B. brevis, B. cereus, B. licheniformis, B. pasteurii, B. pumilus, B. sphericus, B. badius, B. megaterium, B. marinus, B. insolitus, B. larvae* and *B. alvei*. Among the fifteen types of identified isolates, *Bacillus subtilis* and *Bacillus coagulans* were the most predominant species overall.

Key words: UHT milk, aerobic spore forming bacteria, *Bacillus cereus*, public health.

INTRODUCTION

Milk, the first food takes on birth, is a 'treasure-trove' of more than 200 ingredients, many with unique functional and nutritional properties which modern processing is capable of isolating and refining for a multiplicity of uses in the food and related industries (**Chatterjee and Acharya, 1992**).

Contaminated raw milk can be a source of harmful bacteria, such as those causing undulant fever, dysentery, salmonellosis, tuberculosis, etc so new scientific and efficient methods for preventing contamination of milk are required to overcome such problems.

Different methods of heat treatment are applied to raw milk in order to remove pathogenic organisms and to increase it's shelf life. The advent of Ultra High Temperature (UHT) treatment of milk, which was invented in the 1960s, and became generally available for consumption in the 1970s (**Elliott and Valerie, 2007)**, has added a new dimension to marketing of liquid milk in urban centers as well as remote areas.

UHT processing holds the milk at a temperature of 138°C for 1-2 seconds. Such powerful heat treatment kill all harmful microorganisms in milk and the products become have excellent keeping qualities and can be stored for a long period of time at ambient temperature.

Spore-forming microorganisms have a special position among total microflora of milk with regard to their greatest ability to survive thermal treatment of milk and subsequently to propagate in final products (**Mayr *et al.*, 1998; Abo-Elnaga *et al.*, 2002 and Vyletělová *et al.*, 2002**).

The microorganisms, causing spoilage in UHT milk, which is intended to be sterile, are either resistant types that have survived the heat treatment, or organisms that have contaminated the product after the heat treatment process. Contamination may either by heat labile organisms or heat resistant forms such as spores. Contaminating spores are, however, likely to be less heat resistant than those, which might survive the heat treatment **(Hassan et al., 2009).**

Post treatment contaminants in UHT milk may be either spores, which would not be expected to be heat resistant enough to survive the heat treatment or non heat resistant vegetative organisms. Organisms of first type will probably have entered from ineffectively sterilized plant downstream from the heat treatment stage of the process, which includes spores of *Bacillus cereus* and *Bacillus licheniformis* (**Wilson et al., 1960** and **Davies, 1975**). Organisms of second type will probably have entered through poorly sealed container after aseptic filling.
The most common spore-forming bacteria found in dairy products are *Bacillus licheniformis, B. cereus, B. subtilis, B. mycoides,*and *B. megaterium.*In one study, psychrotrophic *B. cereus* was isolated in more than 80% of raw milks sampled (**Meer et al., 1991**).

Aerobic and facultative anaerobic spore-forming bacteria of the genus *Bacillus* causing a serious problem in milk industry, due to the heat resistance of spores and ability of vegetative cells to produce extra-cellular enzymes causing deterioration of milk and milk products. *Bacillus* spp. are quite common in the agricultural environment and may contaminate milk from various sources either during the production, storage or processing.

Lowering this spore load by good hygienic measures could probably further reduce the contamination level of raw milk, in this way minimizing the aerobic spore forming bacteria that could lead to spoilage of milk and dairy products (**Westhoff and Dougerty, 1981**).This study was planned to enumerate and identify the aerobic spore forming bacteria and *Bacillus cereus* in UHT milk and their significance from the public health and economic points of view.

MATERIALS AND METHODS

Seventy-five Ultra Heat Treated (UHT) milk samples were randomly collected from different retail outlets in Cairo and Giza governorates. Collected samples were transferred directly to the laboratory with a minimum of delay to be examined microbiologically.

Preparation of samples (Longeveld et al., 1976):

Each sample of UHT milk was thoroughly mixed before being subjected to bacteriological examination. The surface of the retail packs was thoroughly swabbed with 70% alcohol. A sterile, single-service hypodermic needle of syringe inserted through the package wall for bacteriological examination.

Preparation of decimal dilution (APHA, 2004):

One ml of milk sample was aseptically transferred to 9 ml of sterile ¼ strength Ringer's solution and well mixed to obtain 1/10 dilution. One ml from the first dilution was added to 9 ml of sterilized diluents to obtain tenth fold decimal dilutions.

Enumeration of aerobic spore forming organisms (Collins & Lyne, 1984):

The previously prepared decimal dilutions were heated in a thermostatically controlled water bath at 80^{o}C for 10 minutes and suddenly cooled by ice. From each dilution 0.1 ml. was seeded evenly onto duplicate plates of Dextrose Tryptone Agar medium **(Oxoid manual, 2010).** Inoculated plates were incubated at 32^{o}C for 72 hours. The count of mesophilic aerobic spore formers was calculated and recorded.

Suspected colonies were picked up and inoculated into nutrient agar slopes and incubated at 37^{o}C for 24 hours to have a pure culture of isolates for further identification.

Enumeration of *Bacillus cereus* (Oxoid 2010):

Mannitol egg yolk phenol red polymyxin (MYP) agar medium was used for enumeration and isolation of *Bacillus cereus*. Plate count technique, (Spread plate inoculation). 0.1 ml amount from each prepared dilution of samples under

investigation was transferred and evenly spread onto the surface of MYP agar plates. All plates were incubated at 30°C for 24 hours.
According to the FDA method, typical *B. cereus* colonies on Mannitol Egg Yolk Agar (MYPDifco) supplemented with Polimixin B sulfate 0.1%, are surrounded by a precipitated zone which indicates lecithinase activity and a pink color is observed because mannitol is not fermented. The typical colonies were counted & recorded.

Identification of suspected aerobic spore forming colonies:
All typical and atypical colonies of aerobic spore formers and *B. cereus* were picked up and seeded onto nutrient agar slopes then incubated at 37°C for 24 hours. The culture was identified according to (***Krieg & Holt, 1986 and BAM on line, 2009***).

RESULTS

Table (1): Statistical analytical results of total aerobic spore formers and *Bacillus cereus* counts/ml of examined UHT milk samples.

Count/ml	No. of UHT samples	Positive samples		Min.	Max.	Mean	± S.E.M
		No	%				
Total aerobic spore formers	75	75	100	6×10^3	23×10^8	8.29×10^7	4.03×10^7
Bacillus cereus		46	61.3	100	10×10^5	2.91×10^4	1.12×10^4

Fig. (1): Statistical analytical results of total aerobic spore formers and *Bacillus cereus* counts/ml of examined UHT milk samples

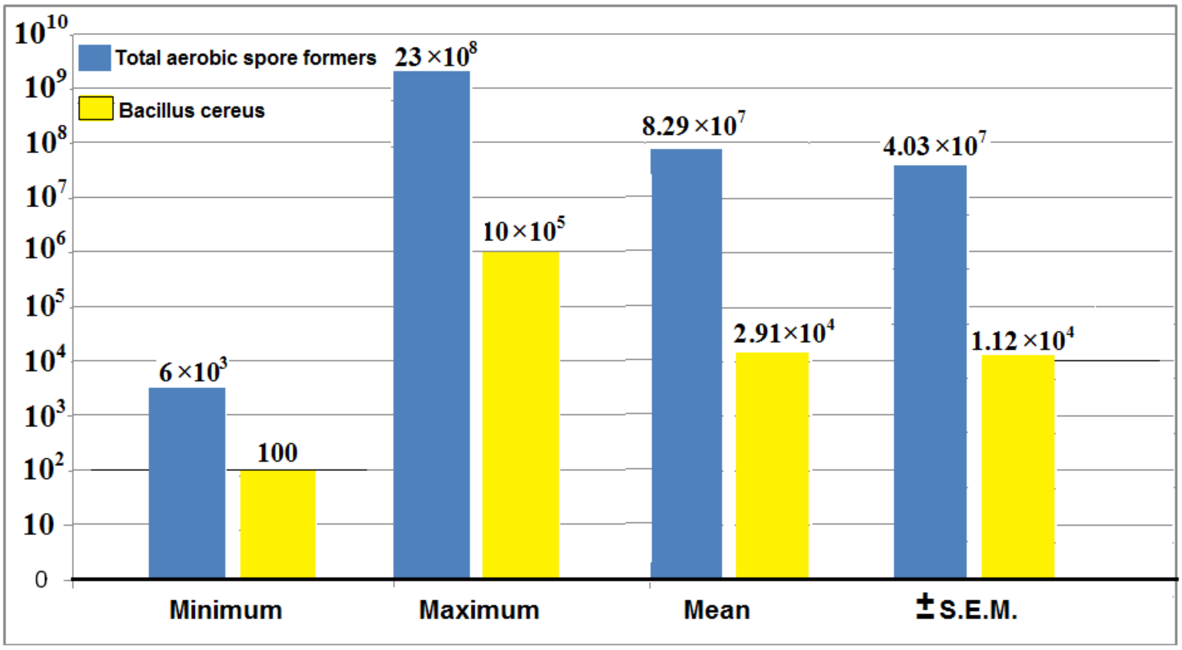


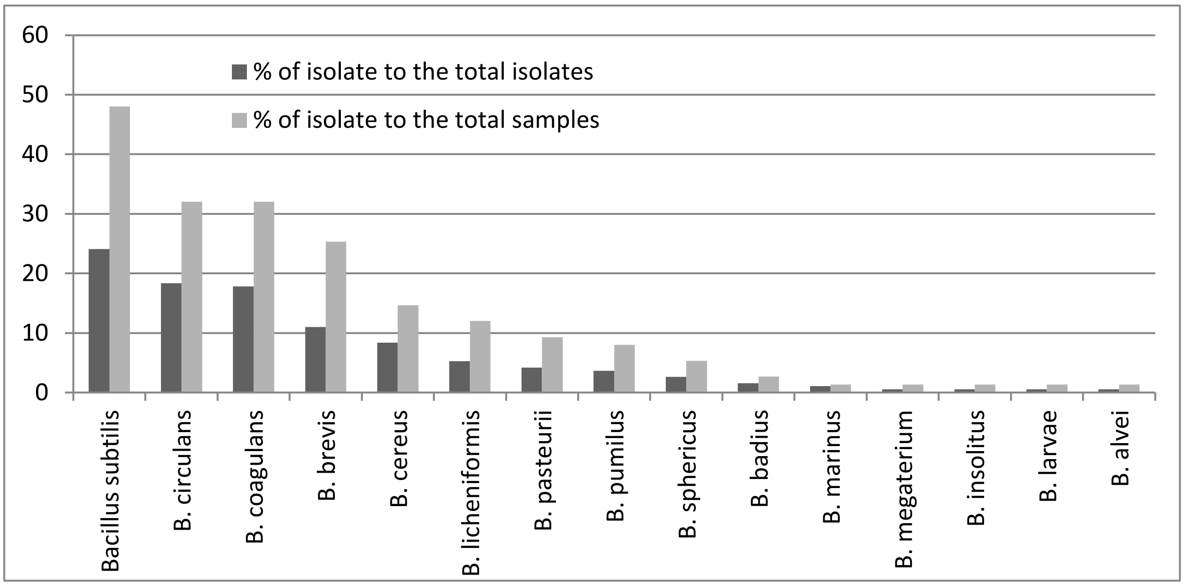
Table (2): Frequency distribution of the examined samples based on their aerobic spore formers and Bacillus cereus count/ml

Intervals	Aerobic spore formers		*Bacillus cereus*	
	No.	%	No.	%
10^2 ----10^4	4	5.34	27	58.69
10^4----10^6	29	38.66	19	41.31
10^6----10^8	33	44	0	0
10^8----10^{10}	9	12	0	0
Total	**75**	**100.00**	**46**	**100**

Table (3): Incidence (%) of aerobic spore formers isolated from examined UHT milk samples

Types of isolates	% of isolate to the total isolates		% of isolate to the total samples	
	No. of isolates	%	No. of samples	%
Bacillus subtilis	46	24.08	36	48
B. circulans	35	18.33	24	32
B. coagulans	34	17.80	24	32
B. brevis	21	11	19	25.3
B. cereus	16	8.38	11	14.66
B. licheniformis	10	5.24	9	12
B. pasteurii	8	4.18	7	9.3
B. pumilus	7	3.67	6	8
B. sphericus	5	2.62	4	5.33
B. badius	3	1.57	2	2.66
B. marinus	2	1.05	1	1.33
B. megaterium	1	0.52	1	1.33
B. insolitus	1	0.52	1	1.33
B. larvae	1	0.52	1	1.33
B. alvei	1	0.52	1	1.33
Total	**191**	**100**	----	----

Fig. (2): Incidence of aerobic spore formers isolated from examined UHT milk samples



Out of 195 Bacillus cereus isolates from MYP agar medium, 181(92.8%) were positive.

DISCUSSION

Raw milk is the usual source of spore-forming bacteria in finished dairy products. Their numbers before heat treatment seldom exceed 5,000/ml (**Mikolajcik & Simon, 1978**); however, they can also contaminate milk after processing (**Griffiths & Phillips, 1990**).

The total count of aerobic spore forming bacteria in the examined UHT milk samples ranged from 6×10^3 to 23×10^8 CFU/ml, with mean value of 8.29×10^7 CFU/ml (table 1 and figure 1).

Results presented in (table 2) showed that 62 (82.66) out of 75 examined samples were contained aerobic spore formers with the range of 10^4 - $<10^8$/ml. This is normally unacceptable because sterilization or ultra-heat treatments of milk are essential to ensure microbial safety and enzymatic stability of milk (**Korhouen et al., 1988**) allowing prolongation of its shelf life.

The relatively high average aerobic spore count of UHT milk samples (table 1) was probably due to either low microbiological quality of the raw milk under process and/or contamination after heating process, as was suggested by several reports (**Uraz *et al.* 1981; Burton, 1983; Yetismeyen, 1997; Metin, 1998 and Tekinsen & Tekinsen, 2005**).

Aerobic and facultative anaerobic spore-forming bacteria of the genus *Bacillus* present a serious problem in milk industry, due to the heat resistance of spores and ability of vegetative cells to produce extra cellular enzymes causing milk and milk product deterioration. *Bacillus* species are quite common in the agricultural environment and may contaminate milk from various sources both during the production, storage and processing (**Janstova and Lukasova, 2001**).

Examination of 75 samples of retail UHT milk samples revealed that, 46 samples (61.3%) were contain Bacillus cereus in a count ranged from 100 to 10×10^5/ml with an average value of 2.91×10^4 /ml (table 1 and figure 1). The count of the total Bacillus cereus was more than 100 in 27 samples (58.69%), and more than 10000 CFU/ml in 19 of examined samples (41.31%) (table 2).

Out of the 191 isolated strains, only 16 etrains (8.38%) were identified as *B. cereus* (table 3). **Nagarajan et al., (1990)** found that, *B. cereus* spores survived 100°C for 10 min in sterile milk. The destruction of spores independence on the temperature and time was much quicker as compared to *B. subtilis* and *B. licheniformis* (**Janstova and Lukasova, 2001**).

B. cereus strains are able to produce six types of toxins, five of which are enterotoxins and an emetic one, which can be heat-stable or heat-labile depending on the strain **(From et al., 2007 and Logan & Rodrigez-Diaz, 2006).**

B. cereus causes self-limiting (24-48 hours) food-poisoning syndromes (diarrheal form and an emetic form). The diarrheal form is characterized by abdominal cramps, profuse watery diarrhea, and occasionally, fever and vomiting. The emetic form is characterized by nausea, vomiting, and malaise, occasionally with diarrhea **(Logan and Rodrigez-Diaz, 2006)**.

Food illness caused by *B. cereus* are commonly found in places where there are improper food handling. Between 1973–1985, *B. cereus* caused 17.8% of the total bacterial food poisonings in Finland, 11.5% in the Netherlands, 0.8% in Scotland, 0.7% in England and Wales, 2.2% in Canada, 0.7% in Japan, and 15.0% between 1960–1968) in Hungary **(Kotiranta et al., 2000).**

As in 2008; 103 confirmed outbreaks have been reported in the USA **(Venkitanarayanan and Doyle, 2008).** In Norway, *B. cereus* was the most common microbe isolated from foodborne illnesses in 1990 **(Kotiranta et al., 2000)**.

In addition to the public health importance of *B. cereus* it is influencing the maintenance and quality of milk, it changes the milk odour and taste due to the production of the enzymes proteinase, lipase and phospholipase which may remain active in the milk after the enzyme-producing microbes have been destroyed **(Meer et al., 1991).**

Out of 191 aerobic spore formers strains isolated from 75 examined UHT milk samples, 46 (24.08%) Bacillus *subtilis,* 35(18.33%) B. *circulans,* 34(17.80%) *B. coagulans*, 21(11%) B. *brevis*, 16(8.38%) *B. cereus*, 10(5.24%) *B. licheniformis*, 8(4.18%) *B. pasteurii*, 7(3.67%) *B. pumilus*, 5(2.62%) *B. sphericus*, 3(1.57%) *B. badius*, 2(1.05%) *B. marinus* and one (0.52%) each of *B. megaterium, B. insolitus, B. larvae* and *B. alvei* (table 3 & fig. 2)

Bacillus species are widely distributed in nature, and commonly associated with a variety of foods. Their presence in foods are important because the formation of their spores allows them to be resistant to heat, freezing, chemicals, and other adverse environments that the food undergoes during processing and preparation. Although the vegetative cells are killed by these conditions, the spores can survive and need harsher conditions to be inactivated. Traditionally these microorganisms have been associated with the spoilage of food products; however, recently they have been linked to potential food poisoning issues **(Rodríguez-Lozano et al., 2010)**.

Many of the *Bacillus* species can produce enzymes, including proteases, lipases, and amylases among others. These enzymes are synthesized prior to heat treatment. The process is sufficient to eliminate viable organisms but does not inactivate the preformed heat stable enzymes. These remain active after the process causing spoilage **(Barefoot and Adams, 1998).**

Thermally resistant protease enzyme can affect the quality of ultra-high-temperature (UHT) milk in various ways, but largely by producing bitter peptides (**Sørhaug & Stepaniak, 1991 and Shah, 1994**). Phospholipases are heat stable and can result in the development of bitter off-flavors due to the release of fatty acids by milk's natural lipase (**Chrisope & Marshall, 1976 and Fox et al., 1976**). Heat-stable bacterial lipases have been associated with the development of rancid flavors in UHT milk (**Adams & Brawley, 1981**).
Coagulation of the casein of milk by chymosin-like proteases produced by many of these bacilli occurs at a relatively high pH (**Choudhery & Mikolajcik, 1971**).

Ledenbach and Marshall (2009) reported that, lactose-fermenting *B.* ***circulans*** was the dominant spoilage microbe in aseptically packaged pasteurized milk.

From the food safety point of view, *B. cereus* is the most important species, known for its ability to form toxins causing food borne illnesses. The United States Environmental Protection Agency does not consider *B. subtilis* and *B. licheniformis* to be human pathogenic organisms (**USEA, 2009 and 2010**). In contrast, the Health Protection Agency (HPA) in the United Kingdom has reported a total of 17 outbreaks of gastroenteritis attributed to *B. subtilis* and 5 other outbreaks attributed to *Bacillus* spp. in England and Wales along the period 1992-2006 (**HPA, 2010**). Previous reports by the HPA also include several cases of foodborne illnesses associated with *B. subtilis* and *B. licheniformis* **(Martimer, 1975 and Turbull, 2009)**.

From et al., 2007 showed that, the production of pumilacidins by a *B. pumilus* strain implicated in a small foodborne outbreak related to the consumption of rice in a Chinese restaurant. The rice was shown to have a level of *B. pumilus* of 10^5/g. High levels of *Bacillus* spp. may be related to food borne cases (**Duc et al., 2009**), possibly by infection or by the presence of the organism causing a reaction in the consumer.
B. licheniformis together with *B. subtilis*, *B. pumilus* and *B. cereus* are able to grow in milk and milk products at cold-storage temperatures and even cause alimentary diseases (**Christiansson, 1992**).

This study revealed isolation a diversity of aerobic spore-forming species which are either able to survive heating of milk and /or due to post-heat treatment contamination. These organisms or their heat resistant enzymes play important role in spoilage potential, or health risks.

CONCLUSIONS

It is concluded from this study that, there is an increase in the number of aerobic spore forming bacteria in the examined samples of UHT milk sold in Egypt and a large diversity of *Bacillus species* were isolated and identified. These organisms and their heat resistant enzymes synthesized prior to heat treatment play an important role in limiting the shelf life of UHT milk and potential health risks. This high incidence could be attributed to poor handling conditions, storage procedures and Post-heat treatment contamination.
It is of importance to conclude that, raw milk used for the UHT milk should be chosen of extreme care, physco-chemical and particularly microbiological qualities, because of that, raw milk under process affects directly the quality characteristics of the final UHT milk.

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