Characterization of Aerobic Spore-Forming Bacteria Isolated From Raw Milk, Skim Milk Powder and UHT Milk

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ABSTRACT

Thermoduric and thermophilic aerobic spore-forming bacteria are the common causes of spoilage of pasteurized and Ultra-high-temperature (UHT) milk. The presence of high level of aerobic spore-forming bacteria in raw and recombined milk used in making UHT milk increased the spoilage and/or the reduced shelf life in the final product. In this study, 140 samples of (32) raw milk (RM), (8) skim milk powder (SMP) and (100) UHT milk were collected to isolate aerobic spore-forming bacteria. A total of 210 isolates of heat resistant bacteria were classified according to morphological, physiological and biochemical tests. Out of theses isolates, 144 strains from RM (102), SMP (25) and UHT milk (17) were expected to be *Bacillus spp*. The isolates were evaluated for proteolytic activity and lactose fermentation, 96.55% of the isolates were able to hydrolyze casein, while 42.76 % of the isolates were able to ferment lactose. Thermophilic *Bacillus* species were the predominant isolates from raw milk and skimmed milk powder. While, mesophilic *Bacillus* species were the predominant in UHT milk.

Key Words: Raw milk, Skimmed milk, UHT milk, spore-forming bacteria, Bacillus spp.

INTRODUCTION

Milk is one of the widely consumed products in the world, highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable temperatures. It is necessary to use great care in the collection and handling of milk samples to prevent any extraneous contamination and to control the growth of organisms during transportation and storage of the milk (Srilakshmi, 1999).There are several types of bacteria which changes the properties of milk. Psychrotrophic microorganisms are able to grow at temperature below 7^{o}C. There are seven types of bacteria which change the properties of milk. They are often produce proteolytic and lipolytic enzymes. They include bacteria such as *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Micrococcus luteus* and *Serratia marcescens.* Spores forming bacteria can withstand greater extremes of acidity, temperature and desiccation. Bacterial enzymes are most significant to milk spoilage (Megha & Annadurai, 2014; Zewail et al.,2015.).

The UHT treatment is usually carried out at temperatures between 135 – 150 °C for 1 – 10 seconds to achieve commercial sterility. The UHT processing of milk destroys all microorganisms that can grow under normal storage conditions (Lewis & Deeth, 2009 & Deeth, 2010). Almost all enzymes are also inactivated by UHT processing because the most enzymes in milk are inactivated at temperatures below 100 °C, but some bacterial proteinases and lipases needs temperatures above 150 °C for inactivation (Kessler, 2002).

Bacillus spp. are often present in raw milk and play an important role in the spoilage of UHT milk (Crielly *et al.,* 1994).The use of Ultra-high-temperature (UHT) processing should inactivate the spores of *Bacillus* species and result in fluid milk products with a long shelf-life without refrigeration. However, defects in UHT milk product stability have been reported (Klijn *et al.,* 1997). This nonstability appeared to be caused by the presence of highly heat-resistant bacterial endo-spores which were first detected in UHT milk from southern Europe in 1985 and were later identified in other countries both in and outside Europe (Hammer *et al.,* 1995). It thus became necessary to develop more efficient processes to inactivate this micro-organism completely and ensure milk commercial sterility.

Aerobic spore-forming members are of importance to the dairy industry as spores of these microorganisms, present in raw milk can survive pasteurization and other processing events and ultimately become incorporated into final products (Cook & Sandeman, 2000, Huck *et al.,* 2007, Coorevits *et al.,* 2008). Spores of *Bacillus spp.* appear regularly in stable environment and they usually represent a secondary contamination of milk during milking process. Besides predominant mesophilic species, e.g. *B. licheniformis, B. subtilis* and *B. pumilus,* dominant psychrotrophic isolates are represented by *B. cereus* strains. These aerobic spores are common in raw milk and linked to spoilage in pasteurized milk and UHT products (Stenfors & Granum, 2001, McGuiggan *et al.,* 2002). In liquid-based dairy products, bacterial spores are rarely a health issue but may cause product spoilage under inadequate pasteurization/storage conditions, leading to product downgrades and losses in

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revenue. For dairy powders, thermophilic spore-forming bacilli can survive in the final product (Burgess *et al.,* 2010). The presence of mesophilic sporeforming microorganisms in high levels in UHT products will be able to cause the deterioration and/ or the reduction of shelf life (Fernanda *et al.,* 2010). Also once present in a processing facility, spores from some thermophilic spore-former organisms can germinate and multiply in biofilms in dairy processing equipment (Burgess *et al.,* 2009, 2010).

Genera *Bacillus* are formed by Gram-positive rods able to produce endo-spores resistant to unfavourable external conditions (Logan & De Vos, 2009) that can be distinguished from other spore-formers (*Sporolactobacillus, Clostridium, Desulfotomaculum, Sporosarcina or Thermoactinomycetes*) due to their aerobic nature (strict or facultative), rod-shaped cells and catalase synthesis (Slepecky & Hemphill, 2006).

Most often, *Bacillus spp.* is detected and isolated by methods based on the resistance of spores to heating or ethanol. However, direct isolation of particular species requires a selective medium or other selective conditions that are available only for a few species (Logan & De Vos, 2009). Due to the large number of species and often incomplete descriptions of newly reported species, *Bacillus* species differentiation is difficult (Holt *et al.,* 1994).Traditional identification has been based on Gram stain, colony morphology, motility and biochemical testing, which are time-consuming, somewhat subjective, and labor intensive procedures. Both traditional and automated identification systems have difficulty identifying some *Bacillus* species and do not differentiate between strains (Webb *et al.,* 2003).

To prevent contamination of milk and dairy products with aerobic spore-forming bacteria, it is important to ensure that contamination of raw milk is minimised. To achieve this, the nature and origin of spores and in particular of spores in raw milk must be better understood. Although the incidence of *Bacillus* species in dairy products from different geographical areas has been widely investigated (Montanari *et al.*, 2004, Scheldeman *et al.*, 2005).

In Egypt, there is shortage in production of high quality raw milk. So, some dairy factories used low quality raw milk or milk powder (skimmed or full cream) or mix of raw milk with milk powder. Low quality of raw ingredients could increase the defects in UHT milk during production and/or during storage (shelf life). Therefore, the aims of study were to evaluate the microbiological quality of ingredients (raw milk and skimmed milk powder) that could be used in producing UHT milk and then isolate and characterize some of aerobic spore-forming bacteria (*Bacillus* species) from raw ingredients and ultra-high temperature treated milk (UHT Milk).

MATERIALS AND METHODS

Samples collection:

Thirty two raw cow's milk samples were collected during the period between May and September, 2015 from farms and milk collection centers in Alexandria and El-Beheira Governorates, Egypt. Samples were collected and were kept in ice box and transferred to lab for not more than 4hrs.

Eight samples of skimmed milk powder from five different brands and countries. Each sample (100g) was obtained in clean, dry and sterile polyethylene bags along with sterile spatula.

According to codex and Egyptian standards specifications (ESS), UHT milk product is not allowed to be released to marketing before incubation at 30°C for 7days. In a factory of dairying, UHT milk batch showed un-sterility defects after incubation period and before releasing, so one hundred samples of defected UHT milk were obtained from this batch. All samples were transferred to lab under sterile conditions.

Sample preparation:

Using aseptic technique, aliquots of 1 ml of the raw milk (RM) and UHT milk samples were transferred into test tube containing 9 ml sterile saline and mixed for 2 min using a vortex. On the other hand, skimmed milk powder (SMP) samples were rehydrated by dissolving 10 g samples with 90 ml of sterile saline in a sterile polyethylene bags and shaking them at 160 rpm for 5 min at room temperature using stomacher according to Standard Methods for the Examination of Dairy Products (1978).

Enumeration of total viable:

Total viable counts of all samples were enumerated using nutrient agar medium. The plates were incubated at 32°C for 48 h (Difco, 1984). The bacterial count was expressed as cfu/ ml or g milk.

Enumeration of aerobic spore-forming bacteria:

Aerobic spore-forming bacteria was enumerated on the same media used in total viable count but the samples of RM and SMP after preparation were heated in water bath at 80°C for 10 min then they cooled suddenly to the room temperature before transferring one ml aliquots in petri dishes. The plates were incubated at 32°C for 48 h (Standard Methods for the Examination of Dairy Products, 1978). The bacterial count was expressed as cfu/ ml or g milk.

Isolation of aerobic spore-forming bacteria:

Some of colonies, which suspected to be *Bacillus* spp. according to the colony morphology, spread with unusual characteristics (e.g., slimy, crusty, dry, embedded or forming skin-like pellicles), were sub cultured to purity on a non-selective medium Nutrient Agar (NA) plates. A total of 210 isolates "153, 33 and 24" from "RM, SMP and UHT milk respectively", were purified and characterized. The pure isolates were inoculated into nutrient broth (Merck, Germany) and stored in 15-20% glycerol at -20 °C for further analysis.

Classification of aerobic spore-forming isolates:

Pre-identification tests were carried out Gram type, cell morphology, catalase production, ability to growth at 7 ^{o}C and 50^{o}C, ability to hydrolyze casein and lactose fermentation.

Gram staining:

The biochemical structure of bacterial cell wall is considered to be the main responsible to constituent introduced in the Gram staining method according to (Difco, 1984).

Catalase test:

The presence or absence of catalase activity is an important taxonomical characteristic of bacteria. Aerobic and facultative anaerobic growing microorganisms exhibit catalase activity, while in obligate anaerobic bacteria this enzyme is absent, resulting in sensitivity to oxygen. For catalase test, 3% (v/v) of hydrogen peroxide solution was dropped on the colony under examination and observed for the production of effervescence. Yeast was used as a positive control for the catalase test (Wong *et al.,* 1988).

Growth at 7 $^{\circ}$C (Psychrotrophic aerobic spore-forming bacteria):

The ability of isolates to grow at 7°C was carried out as follow; each tested isolate was inoculated in nutrient agar medium at 7°C for 7-10 days. The growth was observed in the medium and an un-inoculated plate was incubated under the same condition as a control (Standard Methods for the Examination of Dairy Products, 1978).

Growth at 50 $^{\circ}$C (Thermophilic aerobic spore-forming bacteria):

The ability of isolates to grow at 50°C was carried out as following; each tested isolate was inoculated in nutrient agar medium at 50°C for 3 days. The growth was observed in the medium and an un-inoculated plate was incubated under the same condition as a control (Standard Methods for the Examination of Dairy Products, 1978).

Casein hydrolysis test:

The casein hydrolysis test is used to identify bacteria capable of hydrolyzing casein with the enzyme casease. The test was carried out as following inoculum from each tested isolate was streaked on petri dish of skim milk agar medium, Difco (1984).The inoculated petri dishes were incubated at 32 °C for 48 hrs. The growth was observed in the medium and an un-inoculated petri dish was incubated under the same condition as a control. The results are determined as clearing of the agar around the bacterial growth indicates casein hydrolysis.

Lactose fermentation test:

The purpose is to see if the isolates can ferment the lactose as a carbon source. The test was carried out as following; an inoculums from each tested pure isolate transferred aseptically to a sterile tube of phenol red lactose broth medium, Difco (1984).The inoculated tube was incubated at 35-37 $^{\circ}$C for 24 hrs. The growth was observed in the medium and an un-inoculated tube was incubated under the same condition as a control. The results are determined. A positive test consists of a color change from red to yellow.

RESULTS AND DISCUSSION

Enumeration of total viable and aerobic spore-forming bacteria for raw milk, skim milk powder and UHT-milk:

The results in Table (1) show that the mean counts of total viable count ranged between logs 5.06 and 8.03 cfu/ml in raw milk samples. Results show that all RM samples were contain highly bacterial levels or counts according to the ESS (No, 0154-01/2005) the average of total number of micro-organisms should not exceed 100.000 per ml (log 5 cfu/ml) of raw cow's milk from primary production. Hence, 75% of raw milk samples may be considered of bad quality. As shown in Table (1) aerobic spore-forming bacteria count ranged between <10 and log 3.53cfu/ml in raw milk samples. This finding differs from those reported by (Mikolajcik & Simon, 1978), who found that raw milk is the usual source of spore-forming bacteria in finished dairy products. Their numbers before pasteurization seldom exceed 5,000 cfu/ ml.

Total viable bacteria of SMP ranged between log 2.4 and 3.2 cfu/g in samples of skim milk powder. Also, aerobic spore-forming bacteria count ranged between <10 and log 2.85 cfu/g in samples of skim milk powder.

Results in Table (1) show that the defected in un-sterility UHT milk batch was at level of 17%. The count of TVB was lower than 10 colonies per 0.1ml. EG-regulation 1623/2005 for long life sterilized milk requires that the number of colony counted from

incubated (30 -35°C for 7 days) unopened UHT-cartons, does not exceed 10 colony forming unit (cfu) per 1ml (Scheldeman *et al.,* 2006). *Bacillus spp.* spores may occur also in UHT milk, as reported by Bahout (2000) who found the spores in 18.3% samples investigated at a count of $2.6x10^2$ cfu/ ml.

Classification of aerobic spore-forming bacteria isolated from raw milk, skimmed milk powder and UHT milk:

Pre-identification results of obtained isolates from RM (153), SMP (33) and UHT-milk (24) are illustrated in Tables (3, 4 and 5).Two hundred and ten heat resistant (80°C /10 min) isolates were phenotypically identified. Results show that 66.67, 75.76 and 70.83% of total isolates for RM, SMP and UHT-milk were aerobic spore-forming bacteria. Gram-positive, rod-shaped bacteria that differentiate into heat-resistant endo-spores under aerobic conditions are placed in the genus *Bacillus* (Priest, 1993). After that the *Bacillus spp.* isolates were examined with pre-identification tests.

Bacillus spp. isolates were classified according to their growth ability at different temperatures into three divisions (7, 32and 50°C). Each division was classified to three sub-division according to their ability to hydrolyze casein and/or to ferment lactose. Depending on obtained data, the isolates classification is given at end in 9 groups as show in Table (2).

Table 1. Total viable and aerobic spore-forming bacteria counts for raw milk (RM), skim milk powder (SMP) and UHT milk

No. of sample	Sample					
	RM		SMP		Defected UHT-milk	
	TVB log cfu/ml	ASB log cfu/ml	TVB log cfu/g	ASB log cfu/g	TVB log cfu/0.1ml	ASB log cfu/0.1ml
1	6.82	2.56	3.08	1.95	<10	<10
2	6.83	3.51	2.40	<10	<10	<10
3	5.59	3.53	2.48	2.40	<10	<10
4	6.60	2.36	2.78	2.30	<10	<10
5	5.74	2.14	3.04	2.79	<10	<10
6	5.57	2.92	3.08	2.85	<10	<10
7	6.55	1.47	3.20	2.30	<10	<10
8	5.72	<10	3	2.08	<10	<10
9	5.98	<10	-	-	<10	<10
10	5.41	2.23	-	-	<10	<10
11	5.40	2.04	-	-	<10	<10
12	5.49	2.11	-	-	<10	<10
13	5.06	2.23	-	-	<10	<10
14	7.04	2.17	-	-	<10	<10
15	7.50	2.30	-	-	<10	<10
16	7.70	2.47	-	-	<10	<10
17	7.94	2.11	-	-	<10	<10
18	7.74	2.44	-	-	-	-
19	5.29	1.90	-	-	-	-
20	8.01	2.36	-	-	-	-
21	7.71	2.34	-	-	-	-
22	7.77	2.32	-	-	-	-
23	7.77	2.32	-	-	-	-
24	7.17	2.27	-	-	-	-
25	7.79	2.14	-	-	-	-
26	7.80	2.14	-	-	-	-
27	5.32	1.47	-	-	-	-
28	7.77	2.32	-	-	-	-
29	7.79	2.14	-	-	-	-
30	8.03	3.06	-	-	-	-
31	8.03	3.06	-	-	-	-
32	7.54	2.47	-	-	-	-

TVB: total viable bacteria ASB: aerobic sporeforming bacteria CFU: colony forming unit

Table 2. Classification of *Bacillus* species isolates.

Test	Group								
	A	B	C	D	E	F	G	H	I
Growth at 7°C	+	+	+	-	-	-	-	-	-
Growth at 32°C	+	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	+	+	+
Casein hydrolysis (CH)	+	+	-	+	+	-	+	+	-
Lactose fermentation (LF)	+	-	+	+	-	+	+	-	+

(+) *Bacillus* isolates positive for test **(-)** *Bacillus* isolates negative for test

Pre-identification of *Bacillus* species isolated from raw milk:

Data in Table(3) show the ability of 102 isolates from RM to grow at different temperatures. The results show that 18.62 and 55.88% of isolates were able to grow at 7 and 50 °C, respectively.

All of *Bacillus spp.* isolates were able to hydrolyze casein. The most important spoilage organism in the dairy industry is undoubtedly *B. cereus*, causing 'bitty cream' (floating clumps of fat) due to lecithinase activity and 'sweet curdling' (curdling of milk without acidification) due to protease activity (Heyndrickx & Scheldeman, 2002).While, 35.29% of isolates were able to hydrolyze casein and ferment lactose.

The results revealed to 47.37, 30.77 and 50% of psychrotrophic, mesophilic and thermophilic *Bacillus spp.* isolates, respectively were able to ferment lactose. As show in Fig. (1), *Bacillus spp.* isolates from RM samples were classified to 6 groups according to data given in Table (2). The group H (37.25%) was the predominant group, group G (18.63%), group E (17.65%), group B (9.8%), group A (8.83%) and group D (7.84%) were also present. While, noticed that the groups C, F and I were not present in *Bacillus spp.* isolates from raw milk. According to bibliographic data, it was reported by Burton (1988) that *Bacillus licheniformis* is the most common spore isolated from raw milk.

Pre-identification of *Bacillus* species isolated from skimmed milk powder:

Pre-identification results of obtained 25*Bacillus spp.* isolates from 8 samples of SMP were showed in Table (4). The results show that 100 % of isolates were able to grow at 32°C, only 64% grow at 50°C and no grow at 7°C. Thermophilic bacilli are the dominant contaminants of spray-dried milk powders. While, the isolates from SMP were not exhibited any growth at 7°C. hydrolyze casein and lactose ferment

According to the results illustrated in Table (4), it was revealed that the proportion of 88% for total *Bacillus spp.* isolates was able to hydrolyze the casein. Also, 72.72% of hydrolyzed casein isolates were able to ferment lactose. On the other hand, 12% of isolates were able to ferment lactose.

The data in Table (4) show that 81.25% of thermophilic *Bacillus spp.* isolates were able to hydrolyze casein, the same ratio was able to ferment lactose. At same time, 62.5% of these isolates were able to hydrolyze casein and ferment lactose. On the other hand, 100% mesophilic *Bacillus spp.* isolates were able to hydrolyze casein, while, 66.67 % of these isolates were able to hydrolyze casein and ferment lactose.

The *Bacillus spp.* isolates form SMP samples were classified to 5 groups according to data given in Table (2) and Fig. (2). The group G (40%) was the predominant group, followed by group D (24%) then group E, group H and group I (12%). While, noticed that the groups A, B, C and F were not present in *Bacillus spp.* isolates from SMP samples. The presence of thermophilic strains of *G. stearothermophilus, B. licheniformis* and *B. subtilis* has been confirmed in both raw milk and milk powders in numerous instances (Phillips & Griffiths, 1990, Crielly *et al.* 1994, Murphy *et al.* 1999).

Pre-identification of *Bacillus* species isolated from UHT milk:

The results in Table (5) of *Bacillus spp.* isolates from defected UHT milk show that 29.41% of total isolates were able to grow at 7 °C. While, the isolates were not exhibited any growth at 50°C. On the other hand, results showed that the ability of 94.12% of isolates to hydrolyze the casein. *Bacillus* species can cause spoilage in sterilized milk due to their production of proteolytic and lipolytic enzymes or recontamination during the filling of sterilized milk (Chen *et al.,* 2004).

While, results revealed to 43.75 % of isolates were able to hydrolyze the casein and ferment the lactose. Also, 41.18 % of total isolates were able to ferment the lactose. Spoilage of UHT and sterilized milk only occurs occasionally and can mostly attribute to recontamination with proteolytic *Bacillus* species during the filling step (Schroder, 1984, Foschino *et al.,* 1990).

Table 3. Pre-identification of *Bacillus* species isolated from raw milk.

Samples	Isolates	Pre-identification test						Pre-identification
		Gram stain	Catalase	Growth at 7°C	Growth at 50°C	Casein hydrolysis	Lactose fermentation	
	RM1	+	+	-	-	+	-	*Bacillus spp.*GE
	RM3	+	+	+	-	+	+	*Bacillus spp.*GA
1	RM4	+	+	-	-	+	-	*Bacillus spp.*GE
	RM5	+	+	+	-	+	-	*Bacillus spp.*GB
	RM2	+	+	+	-	+	+	*Bacillus spp.*GA
	RM3	+	+	-	-	+	+	*Bacillus spp.*GD
2	RM4	+	+	+	-	+	+	*Bacillus spp.*GA
	RM5	+	+	-	+	+	+	*Bacillus spp.*GG
	RM6	+	+	+	-	+	+	*Bacillus spp.*GA
	RM1	+	+	-	+	+	+	*Bacillus spp.*GG
	RM3	+	+	-	-	+	-	*Bacillus spp.*GE
	RM5	+	+	-	-	+	-	*Bacillus spp.*GE
	RM6	+	+	-	-	+	-	*Bacillus spp.*GE
	RM7	+	+	-	+	+	-	*Bacillus spp.*GH
3	RM8	+	+	-	+	+	-	*Bacillus spp.*GH
	RM9	+	+	-	+	+	-	*Bacillus spp.*GH
	RM10	+	+	+	-	+	-	*Bacillus spp.*GB
4	RM1	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	+	+	-	*Bacillus spp.*GH
5	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	-	+	-	*Bacillus spp.* GE
	RM2	+	+	-	-	+	-	*Bacillus spp.* GE
6	RM3	+	+	-	-	+	-	*Bacillus spp.* GE
	RM4	+	+	+	-	+	-	*Bacillus spp.*GB
	RM5	+	+	+	-	+	+	*Bacillus spp.*GA
7	RM3	+	+	-	-	+	+	*Bacillus spp.*GD
8	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
9	RM3	+	+	-	+	+	+	*Bacillus spp.*GG
	RM2	+	+	+	-	+	+	*Bacillus spp.*GA
10	RM3	+	+	-	+	+	-	*Bacillus spp.*GH
	RM6	+	+	-	+	+	-	*Bacillus spp.*GH
11	RM3	+	+	+	-	+	+	*Bacillus spp.*GA
11	RM5							*Bacillus spp.* GH
	RM1						-	*Bacillus spp.*GB
12	RM2	+	-	+	+	-	+	*Bacillus spp.*GG
	RM3	+	+	-	+	+	+	*Bacillus spp.*GG
13	RM2	+	+	+	-	+	+	*Bacillus spp.*GA
14	RM1	+	+	-	+	+	+	*Bacillus spp.*GG
	RM4	+	+	+	-	+	-	*Bacillus spp.*GB
	RM3	+	+	-	+	+	-	*Bacillus spp.*GH
	RM4	+	+	+	-	+	+	*Bacillus spp.*GA
15	RM5	+	+	-	+	+	-	*Bacillus spp.*GH
	RM6	+	+	-	+	+	-	*Bacillus spp.*GH
	RM7	+	+	-	+	+	-	*Bacillus spp.*GH

G: Group

Continuous

Cont. Table 3.

Samples	Isolates	Pre-identification test						Pre-identification
		Gram stain	Catalase	Growth at 7°C	Growth at 50°C	Casein hydrolysis	Lactose fermentation	
	RM1	+	+	-	+	+	-	*Bacillus spp.*GH
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
16	RM3	+	+	-	+	+	-	*Bacillus spp.*GH
	RM4	+	+	-	+	+	+	*Bacillus spp.*GG
	RM5	+	+	-	+	+	+	*Bacillus spp.*GG
	RM1	+	+	-	-	+	+	*Bacillus spp.*GD
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
17	RM3	+	+	-	-	+	+	*Bacillus spp.*GD
	RM4	+	+	-	-	+	-	*Bacillus spp.*GE
	RM5	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	+	+	-	*Bacillus spp.*GH
	RM2	+	+	-	+	+	+	*Bacillus spp.*GG
18	RM3	+	+	-	+	+	+	*Bacillus spp.*GG
	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM7	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	+	+	+	*Bacillus spp.*GG
19	RM2	+	+	-	-	+	-	*Bacillus spp.* GE
	RM3	+	+	-	-	+	-	*Bacillus spp.* GE
	RM1	+	+	-	-	+	+	*Bacillus spp.*GD
	RM2	+	+	-	-	+	-	*Bacillus spp.*GE
20	RM4	+	+	+	-	+	-	*Bacillus spp.*GB
	RM5	+	+	-	+	+	-	*Bacillus spp.*GH
21	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	+	-	+	-	*Bacillus spp.*GB
22	RM2	+	+	-	-	+	-	*Bacillus spp.*GE
	RM4	+	+	+	-	+	-	*Bacillus spp.*GB
23	RM1	+	+	-	+	+	+	*Bacillus spp.*GG
	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
24	RM5	+	+	-	+	+	-	*Bacillus spp.*GH
	RM6	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	-	+	-	*Bacillus spp.*GE
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
25	RM3	+	+	+	-	+	-	*Bacillus spp.*GB
	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM1	+	+	-	+	+	-	*Bacillus spp.*GH
26	RM3	+	+	-	-	+	+	*Bacillus spp.*GD
	RM4	+	+	+	-	+	-	*Bacillus spp.*GB
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
27	RM3	+	+	-	+	+	-	*Bacillus spp.*GH
	RM2	+	+	-	+	+	+	*Bacillus spp.*GG
28	RM3	+	+	-	+	+	-	*Bacillus spp.*GH
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
29	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM5	+	+	-	+	+	+	*Bacillus spp.*GG
	RM2	+	+	-	+	+	+	*Bacillus spp.*GG
30	RM4	+	+	-	+	+	+	*Bacillus spp.*GG

Cont. Table 3.

Samples	Isolates	Pre-identification test						Pre-identification
		Gram stain	Catalase	Growth at 7°C	Growth at 50°C	Casein hydrolysis	Lactose fermentation	
31	RM1	+	+	-	-	+	-	*Bacillus spp.* GE
	RM2	+	+	-	-	+	-	*Bacillus spp.* GE
	RM3	+	+	-	-	+	+	*Bacillus spp.*GD
	RM4	+	+	-	+	+	-	*Bacillus spp.*GH
	RM5	+	+	-	+	+	+	*Bacillus spp.*GG
	RM6	+	+	-	+	+	-	*Bacillus spp.*GH
	RM7	+	+	-	+	+	+	*Bacillus spp.*GG
	RM8	+	+	-	-	+	-	*Bacillus spp.*GE
32	RM1	+	+	-	-	+	+	*Bacillus spp.*GD
	RM2	+	+	-	+	+	-	*Bacillus spp.*GH
	RM3	+	+	-	+	+	+	*Bacillus spp.*GG
	RM4	+	+	-	-	+	-	*Bacillus spp.*GE

A: *Bacillus* isolates grow at 7, 32°C, hydrolyze casein & lactose ferment. B: *Bacillus* isolates grow at 7, 32°C & hydrolyze casein.
C: *Bacillus* isolates grow at 7, 32°C & lactose ferment.
D: *Bacillus* isolates grow at 32°C, hydrolyze casein & lactose ferment. E: *Bacillus* isolates grow at 32°C & hydrolyze casein.
F: *Bacillus* isolates grow at 32°C & lactose ferment.
G: *Bacillus* isolates grow at 32, 50°C, hydrolyze casein & lactose ferment.
H: *Bacillus* isolates grow at 32, 50°C & hydrolyze casein.
I: *Bacillus* isolates grow at 32, 50°C & lactose ferment

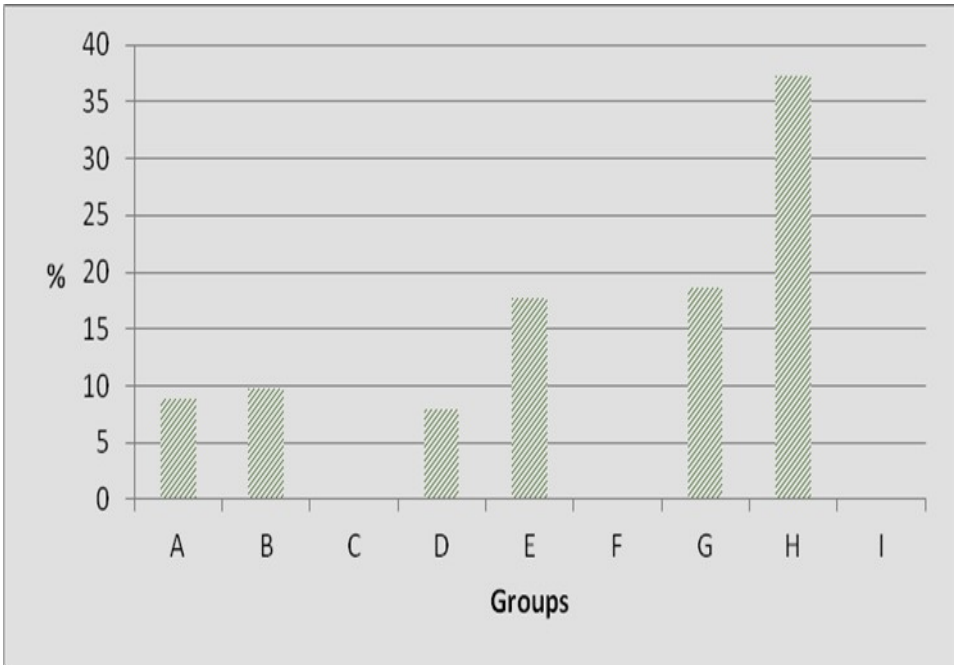

Figure 1. Classification of *Bacillus* species isolated from raw milk.

A: *Bacillus* isolates grow at 7, 32°C, hydrolyze casein & lactose ferment. B: *Bacillus* isolates grow at 7, 32°C & hydrolyze casein.
C: *Bacillus* isolates grow at 7, 32°C & lactose ferment.
D: *Bacillus* isolates grow at 32°C, hydrolyze casein & lactose ferment. E: *Bacillus* isolates grow at 32°C & hydrolyze casein.
F: *Bacillus* isolates grow at 32°C & lactose ferment.
G: *Bacillus* isolates grow at 32, 50°C, hydrolyze casein & lactose ferment.
H: *Bacillus* isolates grow at 32, 50°C & hydrolyze casein. I: *Bacillus* isolates grow at 32, 50°C & lactose ferment.

Table 4. Pre-identification of *Bacillus* species isolated from skim milk powder

Samples	Isolates	Pre-identification test						Pre-identification
		Grams' stain	Catalase	Growth at 7°C	Growth at 50°C	Casein hydrolysis	Lactose fermentation	
1	SMP1	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP2	+	+	-	-	+	-	*Bacillus spp.*GE
	SMP3	+	+	-	+	+	+	*Bacillus spp.*GG
2	SMP2	+	+	-	-	+	-	*Bacillus spp.*GE
	SMP3	+	+	-	-	+	+	*Bacillus spp.*GD
	SMP4	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP5	+	+	-	+	+	+	*Bacillus spp.*GG
3	SMP1	+	+	-	+	-	+	*Bacillus spp.*GI
	SMP3	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP4	+	+	-	+	+	+	*Bacillus spp.*GG
4	SMP1	+	+	-	+	-	+	*Bacillus spp.*GI
	SMP2	+	+	-	-	+	+	*Bacillus spp.*GD
	SMP3	+	+	-	-	+	+	*Bacillus spp.*GD
5	SMP2	+	+	-	-	+	-	*Bacillus spp.*GE
	SMP3	+	+	-	+	+	-	*Bacillus spp.*GH
6	SMP1	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP2	+	+	-	-	+	+	*Bacillus spp.*GD
	SMP3	+	+	-	-	+	+	*Bacillus spp.*GD
	SMP5	+	+	-	+	-	+	*Bacillus spp.*GI
7	SMP1	+	+	-	+	+	-	*Bacillus spp.*GH
	SMP2	+	+	-	-	+	+	*Bacillus spp.*GD
	SMP3	+	+	-	+	+	+	*Bacillus spp.*GG
8	SMP2	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP3	+	+	-	+	+	+	*Bacillus spp.*GG
	SMP4	+	+	-	+	+	-	*Bacillus spp.*GH

A: *Bacillus* isolates grow at 7, 32°C, hydrolyze casein & lactose ferment.
B: *Bacillus* isolates grow at 7, 32°C & hydrolyze casein. C: *Bacillus* isolates grow at 7, 32°C & lactose ferment.
D: *Bacillus* isolates grow at 32°C, hydrolyze casein & lactose ferment.
E: *Bacillus* isolates grow at 32°C & hydrolyze casein. F: *Bacillus* isolates grow at 32°C & lactose ferment.
G: *Bacillus* isolates grow at 32, 50°C, hydrolyze casein & lactose ferment.
H: *Bacillus* isolates grow at 32, 50°C & hydrolyze casein. I: *Bacillus* isolates grow at 32, 50°C & lactose ferment.

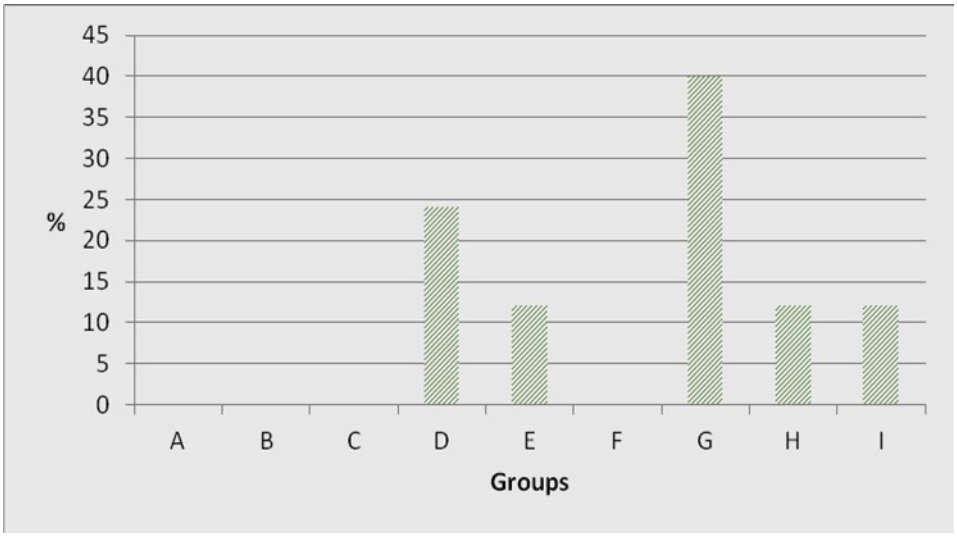

Figure 2. Classification of *Bacillus* species isolated from skim milk powder

Table 5. Pre-identification of *Bacillus* species isolated from UHT milk

Samples	Isolates	Pre-identification test						Pre-identification
		Grams' stain	Catalase	Growth at 7°C	Growth at 50°C	Hydrolyze the casein	Lactose fermentation	
2	UHT1	+	+	+	-	+	-	*Bacillus spp.*GB
	UHT2	+	+	+	-	+	-	*Bacillus spp.*GB
4	UHT1	+	+	+	-	+	+	*Bacillus spp.*GA
5	UHT1	+	+	-	-	+	-	*Bacillus spp.*GE
	UHT2	+	+	-	-	+	-	*Bacillus spp.*GE
7	UHT1	+	+	-	-	+	-	*Bacillus spp.*GE
8	UHT1	+	+	-	-	+	+	*Bacillus spp.*GD
9	UHT1	+	+	-	-	+	-	*Bacillus spp.*GE
	UHT2	+	+	-	-	+	-	*Bacillus spp.*GE
11	UHT1	+	+	+	-	+	+	*Bacillus spp.*GA
	UHT2	+	+	-	-	-	+	*Bacillus spp.*GF
12	UHT1	+	+	-	-	+	-	*Bacillus spp.*GE
14	UHT2	+	+	-	-	+	+	*Bacillus spp.*GD
15	UHT1	+	+	-	-	+	+	*Bacillus spp.*GD
	UHT2	+	+	-	-	+	-	*Bacillus spp.*GE
16	UHT1	+	+	-	-	+	+	*Bacillus spp.*GD
17	UHT1	+	+	+	-	+	-	*Bacillus spp.*GB

A: *Bacillus* isolates grow at 7, 32°C, hydrolyze casein & lactose ferment. B: *Bacillus* isolates grow at 7, 32°C & hydrolyze casein.
C: *Bacillus* isolates grow at 7, 32°C & lactose ferment.
D: *Bacillus* isolates grow at 32°C, hydrolyze casein & lactose ferment. E: *Bacillus* isolates grow at 32°C & hydrolyze casein.
F: *Bacillus* isolates grow at 32°C & lactose ferment.
G: *Bacillus* isolates grow at 32, 50°C, hydrolyze casein & lactose ferment.
H: *Bacillus* isolates grow at 32, 50°C & hydrolyze casein. I: *Bacillus* isolates grow at 32, 50°C & lactose ferment.

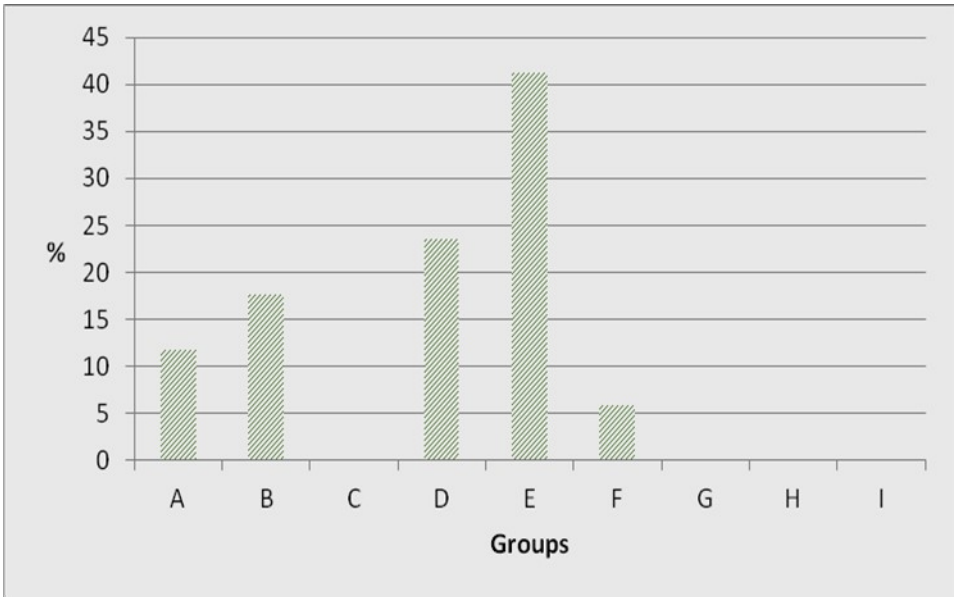

Figure 3.Classification of *Bacillus* species isolated from UHT milk

A: *Bacillus* isolates grow at 7, 32°C, hydrolyze casein & lactose ferment. B: *Bacillus* isolates grow at 7, 32°C & hydrolyze casein.
C: *Bacillus* isolates grow at 7, 32°C & lactose ferment.
D: *Bacillus* isolates grow at 32°C, hydrolyze casein & lactose ferment. E: *Bacillus* isolates grow at 32°C & hydrolyze casein.
F: *Bacillus* isolates grow at 32°C & lactose ferment.
G: *Bacillus* isolates grow at 32, 50°C, hydrolyze casein & lactose ferment.
H: *Bacillus* isolates grow at 32, 50°C & hydrolyze casein. I: *Bacillus* isolates grow at 32, 50°C & lactose ferment.

All psychrotrophic *Bacillus ssp*. Isolates were able to hydrolyze the casein. At the same time 40% of these isolate were able to ferment the lactose. Bahout (2000) mentioned that the presence of *B. cereus* in UHT milk. On the other hand, 91.67% of mesophilic *Bacillus ssp.* isolates were able to hydrolyze the casein and 36.36% of isolates able to hydrolyze the casein were able to ferment the lactose.

As shown in Fig. (3) *Bacillus* spp. isolates from UHT milk samples were classified to 5 groups according to data given in Table (2). The group E (41.18%) was the predominant group, group D (23.53%), group B (17.65%), group A (11.76%) and group F (5.88%) were also present. While, noticed that the groups C, G, H and I were not present in *Bacillus spp.* isolates from UHT milk samples. Klijn *et al.* (1997), Montanari *et al.* (2004) demonstrated that *B. sporothermodurans* was the predominant sporigenous micro-organisms in UHT milk. Cosentino *et al.* (1997) reported the presence of aerobic spore formers, including *B. sphaericus, B. licheniformis* and *B. brevis*, in Sardinian UHT milk samples.

Burton (1988) reported that *Bacillus stearothermophilus* and *Bacillus licheniformis* may be of the most thermophilic spore formers commonly found in UHT milk and dairy products. While, as stated by Datta & Deeth (2007) *Bacillus stearothermophilus* and *Bacillus flavothermus* spores can be so great in milk powder that UHT processing is ineffective as a commercial sterilization method.

CONCLUSION

Raw milk, skimmed milk powder and some of UHT milk samples were contaminated with aerobic spore-forming bacteria. Isolates from raw milk were psychrotrophic, mesophilic and thermophilic *Bacillus* species. Isolates from Skimmed milk powder were mesophilic and thermophilic *Bacillus* species. While, isolates from UHT milk were psychrotrophic and mesophilic *Bacillus* species. There were differences among isolates in ability to hydrolyze the casein and ferment lactose. There for future works is concluded to identify the isolates by molecular methods and prevent or reduce the spore forming bacterial counts in raw milk to enhance the quality of UHT milk in Egypt.

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الملخص العربي

صفات البكتيريا المتجرثمة الهوائية المعزولة من اللبن الخام، اللبن الفرز المجفف واللبن المعقم

سامح يعقوب، شريف شمسية، سامح عوض، حامد زينه، نبيل صفوت

البكتيريا المتجرثمة المقاومة للحرارة والمحبة للحرارة المرتفعة من أهم مسببات فساد الألبان المبسترة والمعقمة، حيث أن وجود هذه البكتيريا بأعداد كبيرة فى اللبن الخام واللبن المجفف المستخدم فى صناعة اللبن المعقم تؤدى الى حدوث فساد أو تقليل فترة الصلاحية للمنتج النهائى. لذلك أجريت هذه الدراسة بهدف عزل والتعرف على البكتيريا المتجرثمة الهوائية من اللبن الخام،اللبن الفرز المجفف، واللبن المعقم.

تم تجميع ١٤٠ عينة من اللبن الخام (٣٢)، اللبن الفرز المجفف (٨)، واللبن المعقم (١٠٠) تم الحصول من خلالها على ٢١٠ من عزلات البكتيريا المقاومة للحرارة العالية، ثم تم تصنيفها اعتمادا على الاختبارات المورفولوجية، الفسيولوجية والكيموحيوية، وأظهرت النتائج أن ١٤٤ عزلة (لبن خام ١٠٢، لبن فرز مجفف ٢٥ ولبن معقم ١٧) كانت أنواع بكتيرية تابعة لجنس الباسيلس (*Bacillus*)، وتم تقييم النشاط التحللى والقدرة على تخمير اللاكتوز لهذه العزلات ،ووجد أن ٩٦,٥٥% لها القدرة على تحليل الكازين، ٤٢,٧٦% لها القدرة على تخمير اللاكتوز وكانت الأنواع البكتيرية من جنس الباسيلس (*Bacillus*) المحبة لدرجة الحرارة المرتفعة هى السائدة فى عينات اللبن الخام واللبن الفرز المجفف، بينما الأنواع المحبة لدرجة الحرارة المتوسطة كانت هى السائدة فى عينات اللبن المعقم.