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Seasonal and regional occurrence of heat-resistant spore-forming bacteria in the course of ultra-high temperature milk production in Tunisia

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ABSTRACT

Spore-forming bacteria, principally *Bacillus* species, are important contaminants of milk. Because of their high heat resistance, *Bacillus* species spores are capable of surviving the heat treatment process of milk and lead to spoilage of the final product. To determine the factors influencing the contamination of milk, spore-forming bacteria occurrence throughout the UHT milk production line during winter, spring, and summer was studied. The obtained results confirm that the total viable rate decreases rapidly throughout the production line of UHT milk showing the efficiency of thermal treatments used. However, the persistent high rate of spore-forming bacteria indicates their high heat resistance, especially in spring and summer. In addition, a significant variation of the quality of raw milk according to the location of the collecting centers was revealed. The molecular identification showed a high degree of diversity of heat-resistant *Bacillus* species, which are isolated from different milk samples. The distribution of *Bacillus* species in raw milk, stored milk, bactofuged milk, pasteurized milk, and UHT milk were 28, 10, 16, 13, and 33%, respectively. Six *Bacillus* spp. including *Bacillus licheniformis* (52.38%), *Bacillus pumilus* (9.52%), *Bacillus* sp. (4.76%), *Bacillus sporothermodurans* (4.76%), *Terribacillus aidingensis* (4.76%), and *Paenibacillus* sp. (4.76%) were identified in different milk samples.
Key words: spore-forming bacteria, milk, seasonal, geographical area, *Bacillus*

INTRODUCTION

Bacillus and *Paenibacillus* spp. are important spoilage bacteria in various sectors of the food industry, including dairy processing (Fromm and Boor, 2004; Scheldeman, 2004). These bacteria are of particular concern because they are capable of forming endospores and can thus survive pasteurization and other heat treatments commonly used to process raw food materials (Collins, 1981; Crielly et al., 1994). In addition, spores of *Bacillus* species are survival forms that are extremely resistant to most environmental stress factors (Andersson et al., 1995). The heat resistance of aerobic spore-formers isolated from dairy products was examined to give an overview of occurring highly heat-resistant spores (**HRS**). These spores have a special position among total microflora of milk with regard to their ability to survive thermal treatment of milk and subsequently to propagate in final products (Aboelnaga et al., 2002).

The *Bacillus* group has been identified as the prominent genera of gram-positive spore-formers in raw and pasteurized milk (Huck et al., 2007). The highly heterogeneous genus *Bacillus* comprises the largest species group of endospore-forming bacteria. Because of their ubiquitous nature, *Bacillus* spores can penetrate food production at several stages, resulting in significant economic losses and posing a potential risk to consumers due to the capacity of some *Bacillus* strains for toxin production (Ehling-Schulz and Messelhausser, 2013). The major spore-forming bacilli have contaminated and spoiled treated-UHT or sterilized milk, especially *Bacillus licheniformis*, *Bacillus cereus*, *Geobacillus stearothermophilus*, *Bacillus coagulans*, *Bacillus sporothermodurans*, *Brevisbacillus brevis*, *Paenibacillus lactis*, and *Bacillus sphaericus* (Pettersson et al., 1996; Cosentino and Palmas, 1997; Rombaut et al., 2002; Scheldeman, 2004; Aouadhi et al., 2014). Moreover, Huck et al. (2007) demonstrated that the principal contamination source of dairy products by spore-forming bacteria is the raw milk (Huck et al., 2007a). In addition, several entry points of theses microorganisms have been identified at the farm level including concentrate feeds, silage, bedding, manure, soil, wash water, clusters, teat cups, and filter cloths (Vaerewijck et al., 2001; te

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Table 1. Sampling plan of milk during 3 seasons and from different manufacturing steps of UHT Tunisian milk

Sampling	Collection season	Date	Production step	Sample origin
1	Winter	Jan. 6, 2014	Raw milk	Tankers from 2 farms
			Raw milk	Storage tank
			Bactofuged milk	Bactofuge unit
			Standardized milk	The cream separator
			Pasteurized milk	Pasteurizer output
			UHT milk	Package (production Jan. 7, 2014)
2	Winter	Jan. 22, 2014	Raw milk	Tankers from 7 farms
			Raw milk	Storage tank
			Bactofuged milk	Bactofuge unit
			Standardized milk	The cream separator
			Pasteurized milk	Pasteurizer output
			UHT milk	Package (production Jan. 23, 2014)
3	Spring	Mar. 19, 2014	Raw milk	Tankers from 6 farms
			Raw milk	Storage tank
			Bactofuged milk	Bactofuge unit
			Standardized milk	The cream separator
			Pasteurized milk	Pasteurizer output
			UHT milk	Package (production Mar. 20, 2014)
4	Summer	Jun. 13, 2014	Raw milk	Tankers from 6 farms
			Raw milk	Storage tank
			Bactofuged milk	Bactofuge unit
			Standardized milk	The cream separator
			Pasteurized milk	Pasteurizer output
			UHT milk	Package (production Jun. 14, 2014)

Giffel et al., 2002; Scheldeman et al., 2005; Huck et al., 2008). The processing plant has also been identified as a source of spore-forming bacteria, and there might be potential for milk contamination due to the presence and persistence of *Bacillus* and *Paenibacillus* spp. in processing environments (Lin et al., 1998).

An improved understanding of the sources of potentially HRS throughout the production line of UHT milk is necessary to prevent or reduce their presence in final product and to increase product shelf life (Meer et al., 1991). To achieve this, the nature and origin of spores and in particular of spores in raw milk must be better understood.

According to the literature reports, variations in the spore-forming bacterial community within regions (Ranieri and Boor, 2009), seasons (Phillips et Griffiths, 1986; Sutherland and Murdoch, 1994), production runs (Scott et al., 2007), pasteurization conditions (Ranieri and Boor, 2009; Monsallier et al., 2012), and processing facilities (Huck et al., 2007) were evaluated. Although the incidence of *B. sporothermodurans* and other heat-resistant bacteria in Tunisian milk (raw milk, pasteurized milk, and UHT milk) and the characterization of their phenotype and genotype properties have been evaluated by Aouadhi et al. (2014), the factors influencing their incidence and their origin have not previously been studied. The present study aims to evaluate the quality of different types of milk. In addition, the factors influencing the presence of *Bacillus* and related genera in a milk chain in Tunisia and the source of these bacteria in packaged fluid milk were determined.

MATERIALS AND METHODS

Milk Sampling

Forty-one samples were taken at different stages during the UHT milk manufacturing. Twenty-one samples of raw milk have been provided from different collected centers situated in northern and northwest of Tunisia during winter, spring, and summer periods.

Milk collection was achieved in a dairy plant in Tunisia and the different sampling points are tankers of raw milk originated from different collecting centers, raw milk storage tank, bactofuged milk, standardized milk, pasteurized milk, and UHT milk (Table 1).

Determination of Milk Quality and Isolation of Bacteria. The quality of different types of milk samples was evaluated using 2 parameters: total and spore-forming bacteria counts. In fact, the total flora presented in different samples of milk was determined by plating on plate count agar after serial dilutions prepared in peptone water (0.01%). The determination of spore-forming bacteria from UHT milk was obtained by spreading 0.1 mL of product on brain-heart infusion agar supplemented with 1 mg/L of vitamin B_{12}. After incubation for 48 or 72 h at 37°C, the plates were examined and the counts of individual microbial groups were

Table 2. Heat treatments for the isolation of high resistant bacteria from different types of Tunisian milk

Treatment	Temperature (°C)	Duration of treatment (min)	Number of isolated bacteria
1	100	10	112
2	100	30	96
3	100	40	52
4	100	50	40

expressed as colony-forming units per milliliter of milk. Typically, colonies representing each visually distinct morphology were selected and transplanted several times to obtain pure culture. Only strains representing gram-positive characters were used in this study. Furthermore, isolation of these bacteria from raw, bactofuged, standardized, and pasteurized milk was realized after 4 heat treatments at 100°C with varying the duration of treatment (Table 2) to destroy vegetative bacteria and make isolation of heat-resistant bacteria that survive the heat pre-treatment more easily. The isolated bacteria from the third and fourth heat treatment (100°C for 40 min and 100°C for 50 min) were heated another time at 100°C for 40 min to select the highly heat-resistant bacteria, so 21 isolated bacteria survived treatment at 100°C for 40 min.

Microbial cultures were stored at (−20°C) on brain-heart infusion agar supplemented with 20% glycerol.

Phenotypic Characterization of Isolates. One hundred twelve isolates were examined for colony and cell morphology, and for motility. Colony morphology was described using standard microbiological criteria, followed by Gram staining and microscopic observation (Logan et al., 2009).

Catalase and oxidase tests were also carried out, and the casein hydrolysis test was performed as described by Claus and Berkeley (1986).

Identification by PCR Methods. Forty 5 isolates showing different phenotypic characteristics were selected. Bacterial DNA was extracted from pure bacteria culture according to the methods provided by GF-1 Bacterial DNA extraction Kit (Vivantis Technologies, Subang Jaya, Selangor, Malaysia) user's guide protocol. Therefore, the molecular identification of selected isolates was conducted by different PCR primers. The first and second were species-specific PCR BSPO and gapC (CarthaGenomics Advanced Technologies, Iman Abou Hanifa city, Tunisia), which allow the characterization, respectively, of *B. sporothermodurans* and *Paenibacillus* sp. (Scheldeman et al., 2002; Zadoks et al., 2005).

The third PCR primer is for the amplification of housekeeping gene *rpoB* (encoding the β subunit of RNA polymerase) based on available gene sequences for different *Bacillus* spp. (Huck et al., 2007). This gene present in all gram-positive bacteria had previously been shown to be an appropriate target for DNA sequencing-based characterization of broad groups of gram-positive bacteria (La Duc et al., 2004). Because *rpoB* is generally less well conserved among closely related organisms than those for 16S rDNA, many authors hypothesize that sequences from these genes would provide increased discrimination among the isolated *Bacillus* species over 16S rDNA sequence data. The *rpoB* fragment was amplified using primers described by Drancourt et al. (2004).

The PCR reaction mixture of each PCR contained 5 μL of 10× PCR buffer (with $MgCl_2$), 0.2 μL of 25 m*M* deoxynucleotide triphosphates, 0.5 μL of each primer stock solution (25 m*M*), 0.2 μL of Taq polymerase (5 U/μL), 5 μL of chromosomal DNA, and 13.6 μL of sterilized distilled water in a total volume of 25 μL. The PCR conditions released in this study for each gene are given in Table 3.

Sequencing of 16S RNA Genes

The identification of selected isolates of *Bacillus* species was confirmed by sequencing the 16S rRNA genes after PCR amplification, using the universal primers forward Bact16F27N (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse 16R1525 (5′-CTACGGCTACCTTGTTACGA-3′; Invitrogen), as described by Weisburg et al. (1991). The PCR reaction mixture contained 5 μL of 10× PCR Buffer (with $MgCl_2$), 0.2 μL of 25 m*M* deoxynucleotide triphosphates, 0.5 μL of each primer stock solution (25 m*M*), 0.2 μL of Taq polymerase (5 U/μL), 5 μL of chromosomal DNA, and 13.6 μL of sterilized distilled water in a total volume of 25 μL.

The PCR was performed in a thermal cycler (Bio-Rad T100, Bio-Rad, Hercules, CA). Thirty thermal cycles were carried out as following: denaturation at 94°C for 45 s, hybridation at 55°C for 1 min, and extension at 72°C for 2 min. The first cycle was preceded by initial denaturation at 94°C for 3 min, and the last one was followed by a final extension step at 72°C for 7 min.

Five microliters of each PCR product were electrophoresed on 1.5% (wt/vol) agarose gel using Tris

Table 3. Primers and polymerase chain reaction (PCR) cycling conditions

Gene	Primers (5′-3′)	Reference	Size (bp)	PCR cycling conditions
rpoB	CCT CTT CTT ATC AGT GGT TTC TTG CGG TTT GGA ATK ACA GTM GC	Durak et al., 2006	397	94°C, 2:00 min 20 × (94°C, 0:45 min; 50°C, 1:00 min; 72°C, 1:00 min) 72°C, 5:00 min
gapC	TTG GTA TTA ACG GTT TCG GTC CAA GTT GAG CAG TGT AAG ACA TTT C	Zadoks et al., 2005	906	94°C, 4:00 min 35 × (94°C, 1:00 min; 50°C, 1:00 min; 72°C, 1:00 min) 72°C, 7:00 min
BSPO	ACG GCT CAA CCG TGG AG GTA ACC TCG CGG TCT A	Scheldeman et al., 2002	660	95°C, 1:30 min 30 × (95°C, 0:15 min; 60°C, 0:15 min; 72°C, 3:00 min) 72°C, 8:00 min

borate-EDTA. Gel was stained with Run-Safe DNA Gel Strain (7 μL/100 mL; Thermo Fisher Scientific, Waltham, MA) and visualized under UV light.

The amplicons were sequenced in both orientations on the MegaBACE 1000 capillary sequencer (Amersham BioSciences, Little Chalfont, UK), in the Genomic and Biomedical Ontogenetic Laboratory at the Pasteur Institute of Tunisia. The sequence data were edited and analyzed using into the BioEdit version 5.0.9 and the RDP Sequence Aligner programs (Gordon et al., 1973). The consensus sequence was adjusted to be conform to the 16S rRNA gene secondary structure model (Ewing and Green, 1998). Obtained sequences were compared with available databases using the GenBank BLASTN combined with Ez-Taxon (http://eztaxon-e.ezbiocloud.net/) search tools.

Statistical Analysis

Data were analyzed using the SAS v. 9.1.3 program (SAS, 1990, SAS Institute Inc., Cary, NC). Analysis of variance and Duncan's multiple range method were used to compare any significant differences between solvents and samples. Values were expressed as means ± standard deviations. Differences were considered significant at $P < 0.05$. All the analyses were carried out in triplicate, and the values were the average of 3 replicates.

RESULTS AND DISCUSSION

Study of the Microbiological Quality of Milk

The microbiological quality of different types of collected milk from spring, winter, and summer was evaluated by the determination of total flora and aerobic-spore-forming bacteria counts. Figure 1 shows the mean log (cfu/mL) of the total flora and heat-resistant bacteria in all raw milk samples collected from different origins. The microbial quality of raw milk, particularly the incidence of HRS, depends on its origin. The rate of total flora is almost the same in all samples of raw milk (10^6 cfu/mL), but the level of aerobic-spore-forming bacteria differs between samples. The HRS is present only in 3 samples. Our findings are in agreement with preview reports described by Scheldeman et al. (2005) and Coorevits et al. (2008), who suggested that raw milk is the typical source of contamination for spore-forming bacteria in dairy products. Accordingly, Ozrenk and Incy (2008) who worked on raw milk samples collected from different local points of the Van Province in Turkey, found that climatic conditions, seasonal variation, and regional differences are the most important sources of variation in microbial composition

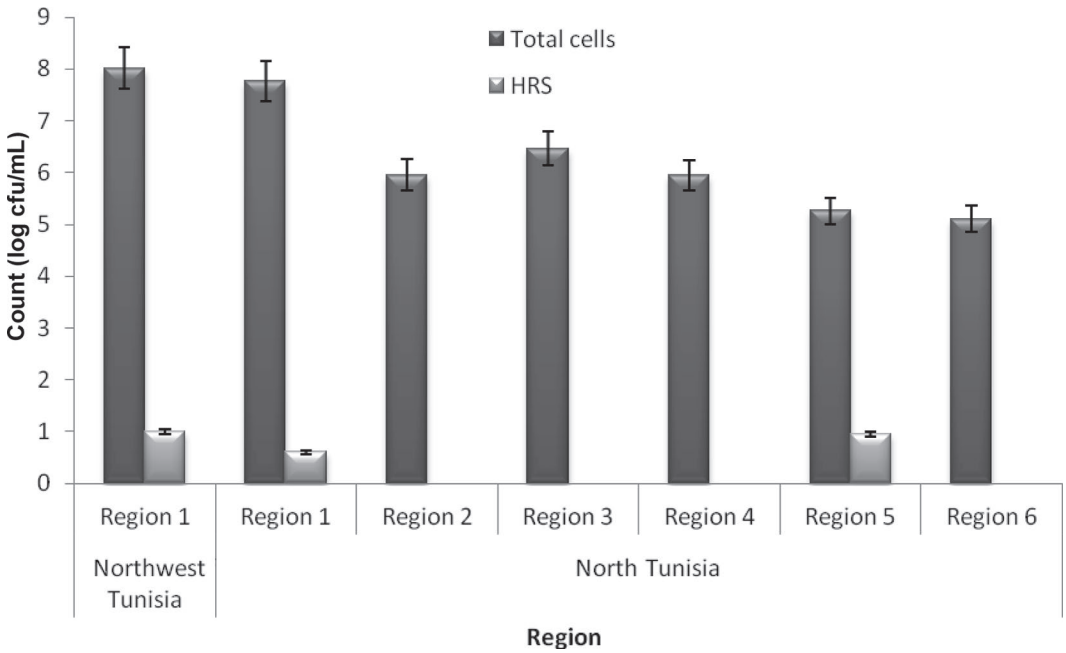

Figure 1. Total and heat-resistant spore (HRS) counts in different samples of raw milk. Values given are means (error bars represent SD) of 3 independent experiments.

of milk (Ozrenk and Incy, 2008). Moreover, many researchers found that regional incidence of spores in raw milk can be linked to the pasturing cattle, the milking equipment and the farm bulk tanks (Sutherland and Murdoch, 1994; Slaghuis et al., 1997; Lukasova et al., 2001). In addition, Verdier-Metz et al. (2009) showed that each farmer's milking practices may contribute to the diversity of bacteria found in the milk, which they produce after analysis of different samples of bulk milk collected from 67 dairy farms in the Savoie regions of France.

The farm environment (silage, feed, animal faces, bedding, soil, and so on) is associated with poor hygienic practices and affects the quality of raw milk (Meer et al., 1991; te Giffel et al., 1997; Magnusson et al., 2007).

The second factor influencing the microbiological quality of milk is seasonal variation. The mean log (cfu/mL) of total flora and heat-resistant bacteria during the manufacturing process of UHT milk at different seasons is summarized in Figure 2. The results demonstrated a decreasing number of total flora during the UHT milk process (10^6 cfu/mL in raw milk to 10^2 cfu/mL in bactofuged milk). This result proved the efficiency of heat treatment in reducing the total flora. The incidence of the HRS throughout the lines of production of UHT milk was lower in winter and higher in spring and summer mostly in pasteurized and bactofuged milk (10–25 cfu/mL of HRS, respectively). Our findings are in agreement with Lukasova et al. (2001) who found a high incidence of *Bacillus* sp. in raw milk in August. Also, McKinnon and Pettipher (1983) suggested that one possible reason responsible for the seasonal distribution of psychrotrophic bacilli in raw milk is that they are derived from summer pasture and enter milk due to increased contamination of the cow's udders. In general, the occurrence of *Bacillus* sp. in raw milk is usually attributed to seasonal effects (Sutherland and Murdoch, 1994). Hay and dust are considered to be sources of these bacteria during winter months, whereas dirty teats by soil are the sources during the humid summer months.

On the other hand, microorganisms can enter into the milk chain through dairy processing. Results shown in Figure 3 indicate that the total viable count rate decreases rapidly throughout the production line of UHT milk (from 10^6 to 1 cfu/mL in UHT milk), showing the efficiency of thermal treatments used. However, the existence of aerobic spore-forming bacteria in different segments of UHT milk production lines is considerable. Also, the increasing number of spore-forming bacteria between raw and UHT milk (1–8 cfu/mL of HRS, respectively) proves the possible contamination of milk by these spores in dairy industries. So, additional sources in the factory, such as equipment, packaging materials (Pirttijarvi et al., 2000), milk storage tanks (Boudjemaa et al., 2004; Svensson et al., 2004), pasteurizers (te Giffel et al., 1997), conditioner (Eneroth et al., 2001), and various postpasteurization sections (Salustiano et

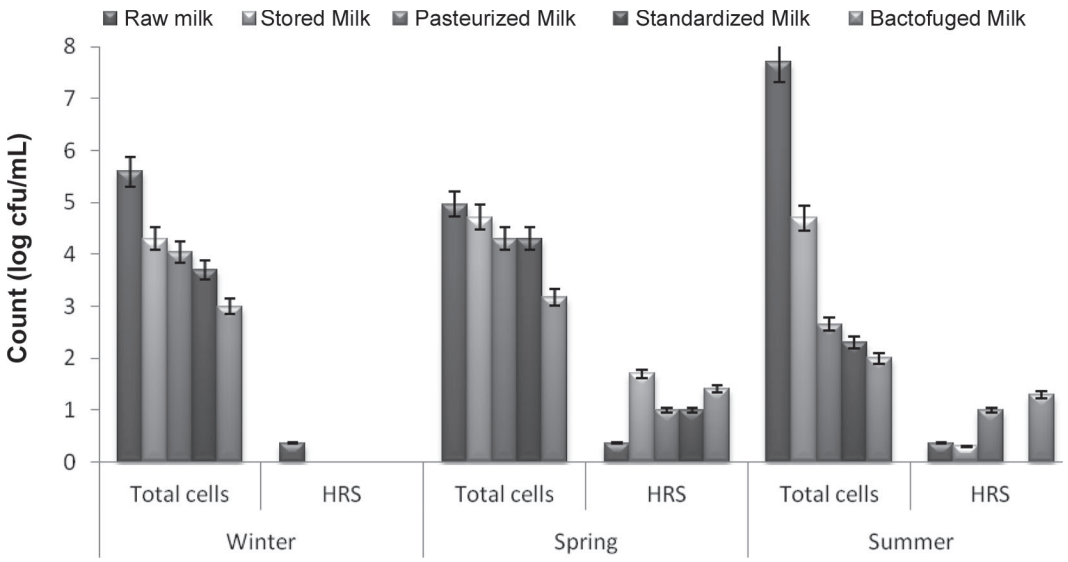

Figure 2. Effect of seasons and dairy processing in the total and heat-resistant spore (HRS) counts of milk. Values given are means (error bars represent SD) of 3 independent experiments.

al., 2009) are a significant sources of contamination of milk and dairy products with spore-forming bacteria.

Identification of Isolated Strains

The majority of isolates, obtained after a heat treatment of 40 min at 100°C, present biochemical and microbiological characteristics that match completely with *Bacillus* species. They were positive to the Gram reaction and catalase tests. Variable results were obtained for oxidase reaction and all isolates hydrolyzed casein. This phenotypic identification was followed by a molecular study to identify the isolated species. Twenty-one isolates were selected for molecular identification. The amplification of BSPO gene demonstrate that one isolate from raw milk harbored this gene. Only one isolate from UHT milk reacted positively in gap C-PCR. However, all strains reacted positively in rpoB-PCR, which proves that all isolates belonged to the genus *Bacillus* in concordance with phenotypic identification

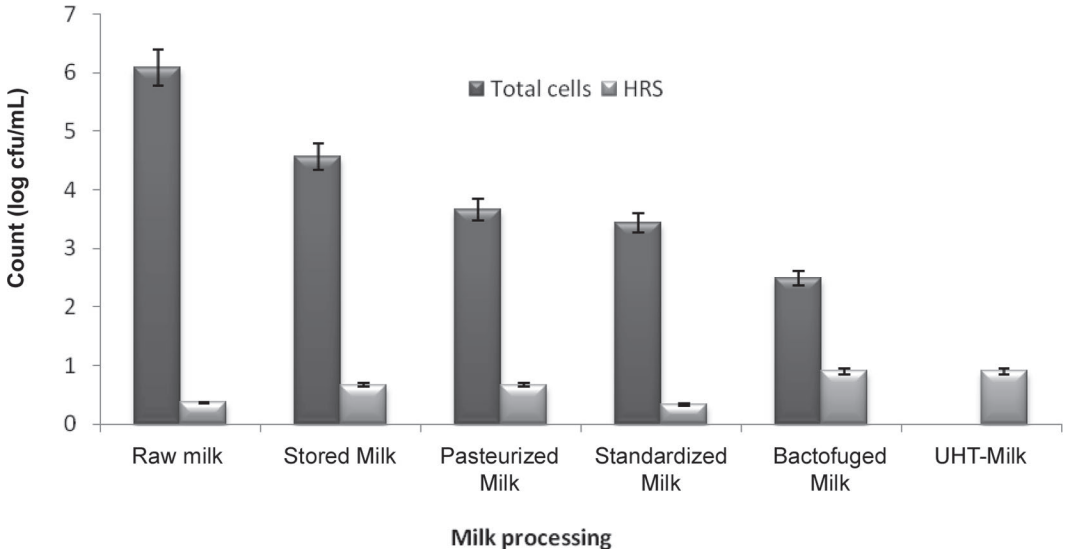

Figure 3. Total and heat-resistant spore (HRS) counts throughout the UHT milk production line. Values given are means (error bars represent SD) of 3 independent experiments.

Table 4. Molecular identification of aerobic spore-forming bacteria isolated from Tunisian milk

Isolates	Origin	PCR results			Phylogenetic identification	
		rpoB	*gapC*	*BSPO*	Species	Similarity (%)
EC1	Raw milk	+	−	−	*Bacillus pumilus*	100
EC2	Raw milk	+	−	−	*Bacillus* sp.	97
EC3	Bactofuged milk	+	−	−	*Bacillus licheniformis*	99
EC4	UHT milk	+	−	−	*Terribacillus aidingensis*	98
EC5	Stored milk	+	−	−	*B. licheniformis*	99
EC6	Raw milk	+	−	+	*Bacillus sporothermodurans*	97
EC7	UHT milk	+	−	−	*B. licheniformis*	99
EC8	UHT milk	+	−	−	*B. licheniformis*	99
EC9	UHT milk	+	−	−	*B. licheniformis*	98
EC10	UHT milk	+	+	−	*Paenibacillus* sp.	98
EC11	UHT milk	+	−	−	*B. pumilus*	97
EC12	UHT milk	+	−	−	*B. licheniformis*	99
EC13	Raw milk	+	−	−	*B. pumilus*	98
EC14	UHT milk	+	−	−	*B. licheniformis*	99
EC15	Raw milk	+	−	−	*B. licheniformis*	98
EC16	UHT milk	+	−	−	*B. pumilus*	98
EC17	Pasteurized milk	+	−	−	*B. licheniformis*	98
EC18	Pasteurized milk	+	−	−	*B. licheniformis*	99
EC19	UHT milk	+	−	−	*B. licheniformis*	98
EC20	UHT milk	+	−	−	*B. pumilus*	99
EC21	UHT milk	+	−	−	*B. pumilus*	98

results. Table 4 shows the results of the sequence alignment and the comparison of partial 16S rDNA genes of the strains analyzed in this study with sequences of the National Center for Biotechnology Information databases. Phylogenetic analysis indicated that all isolated strains are related to 6 species of the *Bacillus* genus. Despite the small number of samples and identified species, our data present a prospective picture on spore-forming bacteria biodiversity in Tunisian milk.

According to Table 5, *B. licheniformis* is the most commonly isolated species of *Bacillus*, present in milk at various processing stages, and this genus represents 52.38% of all isolates. Our findings are in agreement with Crielly et al. (1994) and Vyletelova et al. (2002), who found that *B. licheniformis* strains were present in milk samples collected from all sampling sites. In addition, Anderton et al. (2008) found that *B. licheniformis* was the most commonly isolated species of *Bacillus* found in milk at all stages of processing and was ubiquitous in the farm environment. However, previous studies have shown that some *Bacillus* species, such as *B. licheniformis*, have been linked to potential food poisoning (Rodríguez-Lozano et al., 2010) and spoilage of several dairy products (Meer et al., 1991). Of the 4 *Bacillus* species isolated from UHT milk, we detected the presence of *Paenibacillus* sp. (4.76%), which was isolated for the first time in UHT milk produced in Tunisia. These findings are in agreement with the results of many authors such as Scheldeman (2004) who signaled the presence of *Paenibacillus* sp. in both raw and heat-treated milk. Moreover, *Paenibacillus* spores have been isolated from silage and feed concentrates, which may be the origin of this contamination.

Interestingly, we demonstrate the presence of *Terribacillus aidingensis* (4.76%), a spore-forming, moderately halophilic bacterium. This species was isolated from

Table 5. Distribution of *Bacillus* species isolated from different milk samples

Taxonomic characteristics	Number of strains/milk					Percentage
	Raw milk	Stored milk	Bactofuged milk	Pasteurized milk	UHT milk	
B. sporothermodurans	1	0	0	0	0	4.76
Terribacillus aidingensis	0	0	0	0	1	4.76
B.licheniformis	1	1	1	2	6	52.39
B. pumilus	2	0	0	0	4	28.57
Paenibacillus sp.	0	0	0	0	1	4.76
Bacillus sp.	1	0	0	0	0	4.76
Percentage	23.81	4.76	4.76	9.53	57.14	100

field soil in Japan (An et al., 2007), from a mountain in China (Peng et al., 2015), and from salt mines in Pakistan (Roohi et al., 2012), and this is the first time it was isolated from the dairy product (UHT milk). The main phenotypic characteristic of the genus *Terribacillus* is the formation of ellipsoidal endospores (Liu et al., 2010). We can hypothesize that soil is the source of raw milk contamination by these bacteria (Christiansson et al., 1999). *Bacillus sporothermodurans*, the highly heat-resistant bacteria, was isolated from raw milk (4.76%), and we found that 9.52% of the isolated strains were identified as *B. pumilus* and 4.76% as *Bacillus* sp. These results were partially in agreement with the findings of Aouadhi et al. (2014) who studied the incidence of heat-resistant spore formers from 3 types of Tunisian milk and reported the presence of 7 *Bacillus* species in both raw and pasteurized milk (*B. sporothermodurans*, *B. cereus*, *B. subtilis*, *B. licheniformis*, *Brevibacillus brevis*, *B. sphaericus*, and *B. pumilus*) and the persistence of 4 *Bacillus* species in UHT milk (*B. sporothermodurans*, *B. cereus*, *B. sphaericus*, and *B. licheniformis*).

Distribution of Different Species Isolated from Different Types of Milk

The distribution of *Bacillus* species in the different types of analyzed milk is summarized in Table 5. In the raw milk, 4 *Bacillus* species were present, and for the UHT milk the predominant isolated strains belonged to *B. licheniformis. Bacillus* species can be present in milk deriving from a variety of sources, but psychrotrophic strains of *Bacillus* sp. are introduced into milk as spores from pasture or as a result of improper cleaning of bulk tanks (Phillips and Griffiths, 1986). In addition, we noticed the presence of spore-forming bacteria in bactofuged and pasteurized milk, and these results are in correlation with the findings of Faille et al. (2001) who suggested that pasteurized milk may be spoiled by spore formers (e.g., *Bacillus* sp.) that have survived heat treatment or entered the milk process after heat treatment by recontamination of the milk. So, the presence of these spore-forming bacteria in pasteurized milk, which should have been inactivated by pasteurization, could be due to postpasteurization contamination or to their resistance to thermal treatments. Therefore, we note that significant numbers of the field strains are resistant to thermal treatments during the pasteurization operations and even after the sterilization process UHT milk (12 strains). Of these 12 heat-resistant strains, the species found are *Paenibacillus* and *Terribacillus aidingensis* in UHT milk. However, heat-resistant strains are also present belonging to pathogenic *Bacillus* sp. implicated in some cases of food poisoning, namely *B. licheniformis* (6 strains). Our findings indicate that a considerable diversity of *Bacillus* sp. exists in raw milk and some of these spore formers appear to persist over time in UHT processing. These findings are consistent with a variety of other reports that have shown that various bacteria can persist in some cases for extended periods of time in food processing plants. In addition, the contamination of milk after the high temperature sterilization may result from several sources, but 2 important ones are the seals in the homogenizer and the air supply to the aseptic packaging unit. Kessler (1994) showed that spores trapped under seals had enhanced heat stability, largely attributable to a very low water activity in their microenvironment, and could act as a reservoir of spore contamination.

CONCLUSIONS

This study revealed a large diversity of spore-forming species, which are able to survive heating for 40 min at 100°C. These results show clearly the persistence of a potential risk of foodborne illness due to UHT milk consumption despite the application of heat treatment at UHT. Therefore, it seems to be evident to talk about a direct link of contamination with highly HRS from the raw milk on the dairy farm to the final product in the dairy. It is important to conclude that raw milk used for the UHT milk should be chosen with extreme care and high microbiological qualities. Also, a chain management approach taking into account the entire chain from raw materials via processing to final products will be the most effective way to control and to reduce spores in various food production processes and to prevent spoilage of foods. Moreover, it is necessary to investigate new strategies to eliminate bacterial spores from UHT milk and other dairy products. Interesting research still needs to be conducted in this area.

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