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Incidence and Diversity of Potentially Highly Heat-Resistant Spores Isolated at Dairy Farms

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The presence of highly heat-resistant spores of *Bacillus sporothermodurans* in ultrahigh-temperature or sterilized consumer milk has emerged as an important item in the dairy industry. Their presence is considered undesirable since they hamper the achievement of commercial sterility requirements. By using a selective 30-min heat treatment at 100°C, 17 Belgian dairy farms were screened to evaluate the presence, sources, and nature of potentially highly heat-resistant spores in raw milk. High numbers of these spores were detected in the filter cloth of the milking equipment and in green crop and fodder samples. About 700 strains were isolated after the selective heating, of which 635 could be screened by fatty acid methyl ester analysis. Representative strains were subjected to amplified ribosomal DNA restriction analysis, 16S rRNA gene sequencing, percent G+C content, and DNA-DNA reassociations for further identification. The strain collection showed a remarkable diversity, with representatives of seven aerobic spore-forming genera. *Bacillus licheniformis* and *Bacillus pallidus* were the most predominant species overall. Twenty-three percent of the 603 spore-forming isolates proved to belong to 18 separate novel species. These findings suggest that the selective heating revealed a pool of unknown organisms with a higher heat-resistant character. This study showed that high spore counts can occur at the dairy farm and that feed and milking equipment can act as reservoirs or entry points for potentially highly heat-resistant spores into raw milk. 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. Assessment and characterization of this particular flora are of great importance to allow the dairy or food industry to adequately deal with newly arising microbiological problems.

Raw milk represents a very suitable medium for the growth of bacteria, and the quality of milk is dependent on its microflora. *Bacillus* species and their spores, often present in raw milk (5, 49), play an important role in the bacterial deterioration of milk and milk products. To control the growth of *Bacillus* species, various kinds of heat treatments are used. The use of ultrahigh-temperature (UHT) processing or sterilization in conjunction with aseptic filling should result in fluid milk products with a long shelf life without refrigeration. Although these processes are designed to result in commercially sterile products, spoilage infrequently occurs because of recontamination during filling and is mostly caused by proteolytic activity of some *Bacillus* species (12, 50). Still, massive contaminations of sterilized or UHT-treated milk caused by heat-resistant mesophilic spore-forming bacteria have been reported (16). The causative organism producing highly heat-resistant spores (HRS) was first isolated from a bypass located directly after the heating section of an indirect heating device and was validly described later on as *Bacillus sporothermodurans* (30). Meanwhile, the problem of HRS spread to countries in and outside of Europe (15).

Despite the seasonal, regional, and methodological differences, general tendencies with regard to the compositions of the *Bacillus* flora in raw milk can be observed. *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus subtilis* generally constitute the predominant mesophilic spore-forming species (23, 31, 44, 45). *Bacillus cereus* is often the most common psychrotolerant species (44), whereas *Geobacillus stearothermophilus* and thermotolerant *B. licheniformis* isolates are the primary thermophilic or thermotolerant species (31). Remarkably, a large number of strains remained unidentified in these studies (e.g., up to 48% in the study reported by Sutherland and Murdoch [44]), possibly because of the limitations of the biochemical identification systems and the presence of as-yet-unknown *Bacillus* species.

The ubiquitous nature of aerobic spore-forming bacteria leads to numerous points of potential entry into raw milk (25). Soiling of the udder and teats is considered one of the most important factors in the contamination of raw milk by spores (49). High levels of aerobic spores, ranging from 10 to $>10^5$ CFU g^{-1}, were found in silage (41), and levels of 10^3 to 10^6 CFU g^{-1} were found in feed concentrate (47). When animals consume feed contaminated with spore-forming bacteria, large quantities of spores can be present in their feces which in turn can contaminate the udders and teats. te Giffel et al. (46) confirmed silage as an important source of the contamination of raw milk by comparing aerobic spore populations by means of random amplified polymorphic DNA analysis. In addition, inadequately cleaned milking equipment, pipelines, and farm bulk tanks may be important sources of contamination (14).

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Far less data on the presence of potentially highly heat-resistant spores (spores with an elevated resistance at higher temperatures) at the dairy farm level are available. Occasionally, *B. sporothermodurans* spores were reported in feed concentrate, silage, soy, and pulp (9, 37, 47). However, to prevent spoilage of milk and dairy products by aerobic spore-forming bacteria, it is important to minimize the initial contamination. To achieve this, the nature and origin of spores in raw milk, and in particular of those with a potentially high heat resistance, must be better understood.

In a study by Rombaut et al. (35), approximately one-third of all Belgian dairy farms were sampled, and total colony (TC) and total spore (TS) counts were determined. Their division of the dairy farms into four subsets, low TC and low TS, high TC and low TS, low TC and high TS, and high TC and high TS, with low counts situated between the 10th and 20th percentile and high counts situated between the 80th and 90th percentile, was used as a basis for a representative selection of farms for sampling in the present study.

This study was initiated to better understand the presence, sources, and nature of potentially highly heat-resistant spores in raw milk in general and the presence of *B. sporothermodurans* spores in particular. Seventeen dairy farms from the above-mentioned subsets at geographically diverse locations in Belgium were sampled in the winter of 1998 to 1999, since a higher incidence of spores (particularly mesophilic isolates) is usually observed in the winter period, when cows are housed indoors (5, 44). A heat treatment of 30 min at 100°C was used as a selection procedure for spore formers with a potential high heat resistance and compared to the more traditional 10-min 80°C heating isolation procedure.

MATERIALS AND METHODS

Sampling. Samples were collected over a 5-month period in the winter of 1998 to 1999. They were taken from raw milk, the milking equipment, green crop, and fodder at 17 dairy farms at geographically different locations in Belgium. Raw milk was kept on ice after sampling and was directly processed for determination of total colony and total spore counts. For the determination of potentially highly heat-resistant spores (total heat-resistant spore count [THRS]) (see below), 100 ml was subjected to chemical extraction, as described previously by Herman et al. (17). The pellet was resuspended afterwards in 10 ml of Ringer solution (Oxoid, Basingstoke, United Kingdom). Ten grams of fodder samples (feed concentrate and green crop) was homogenized with 100 ml of Ringer solution in a stomacher (Laboratory Blender 400; Seward Laboratory, London, United Kingdom) for 3 min and subsequently roughly filtered through porous sterile miracloth (Calbiochem Co., La Jolla, Calif.) to remove larger remnant pieces. Different parts of the milking installation were sampled after the cleaning procedure by means of swabs, which were then collected in 10 ml of Ringer solution.

Enumeration and isolation of strains. For TC and TS counts, 1 ml of the sample suspension and appropriate decimal dilutions were pour plated in duplicate directly or after heating for 10 min at 80°C, respectively. Milk plate count agar (Oxoid) was used for raw milk samples, and plate count agar (Oxoid) was used for all other samples. All plates were incubated at 30°C for 72 h prior to counting colonies. For enumeration and isolation of potentially highly heat-resistant spores (THRS), the initial spore suspension was heated for 30 min at 100°C, and after cooling on ice, 1 ml was immediately spread plated in duplicate onto large-diameter (14-cm) petri dishes containing brain heart infusion broth (Oxoid) supplemented with bacteriological agar no. 1 (15 g liter^{-1}; Oxoid) and filter-sterilized vitamin B_{12} (1 mg liter^{-1}; Sigma, St. Louis, Mo.), pH 6.8. Plates were incubated at 20°C for 72 h for psychrotolerant aerobic spore-forming bacteria (THRS at 20°C [THRS 20]) and for 48 h at 37°C for mesophilic (THRS 37) and at 55°C for thermotolerant or thermophilic (THRS 55) spore formers. Per sample category and per temperature, all visibly different colonies were picked off ($n = 701$). Pure cultures of these isolates were stored at −80°C.

Fatty acid analysis. The culture conditions for fatty acid methyl ester (FAME) analysis were previously described (37). The methods used for fatty acid extraction, methyl ester preparation, and separation by gas chromatography were done according to the method described by Vancanneyt et al. (48). The whole-cell FAME profiles were identified and clustered using the Microbial Identification System software and database (TSBA, version 4.0; MIDI, Newark, Del.). A similarity index of ≥0.550 (on a scale of 0 to 1.0) was considered as an indicative FAME identification.

Preparation of DNA. Whole-cell DNA template for PCR amplification was extracted from pure cultures according to the method described by Pitcher et al. (32). For DNA-DNA reassociation experiments, high-purity total genomic DNA was prepared on a larger scale as extensively described previously by Logan et al. (22).

ARDRA. Amplified ribosomal DNA restriction analysis (ARDRA) was performed as described previously by Heyndrickx et al. (20). Numerical analysis and data interpretation for identification purposes were done as previously delineated by Vaerewijck et al. (47).

16S rRNA gene sequencing and comparison. The 16S rRNA genes were amplified by PCR using conserved primers pA and pH (36). The PCR products were purified and subsequently sequenced using an ABI 310 sequencer (Applied Biosystems, Foster City, Calif.) as previously described (37). A combination of the sequencing primers described by Coenye et al. (2) was used to obtain a continuous stretch of the 16S rRNA gene. Sequence assembly was implemented using GeneBase software (Applied Maths, Sint-Martens-Latem, Belgium). The BioNumerics software package, version 3.0 (Applied Maths), was used for construction of a phylogenetic tree based on the neighbor-joining method. The FASTA program (29) was applied to find the most closely related sequences from the EMBL database.

DNA-DNA reassociations. DNA-DNA reassociations were performed at 37°C with photobiotin-labeled probes in microplate wells, according to the method described by Ezaki et al. (10), using an HTS7000 Bio Assay reader (Perkin-Elmer, Norwalk, Conn.) for the fluorescence measurements as extensively described previously by Willems et al. (51).

DNA base composition. The DNA base composition was determined by high-performance liquid chromatography using further specifications given previously by Logan et al. (22).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers AJ535639, AY257870, AY373318 to AY373323, AY382189 to AY382192, AY397764 to AY397774, AY442983 to AY442988, AY443034 to AY443039.

RESULTS

Presence of spores and potentially highly heat-resistant spores at the dairy farm level. (i) Total spore (TS) content. The percentages of the samples belonging to a given spore (TS) content category after heating at 80°C for 10 min are shown in Table 1. TS counts ranged from undetected to approximately 10^7 CFU g^{-1} in green crop and fodder samples.

At the milking equipment level, the majority of the samples in the different spore categories (teat cups, clusters, connection points with the pipeline, and collection tanks) had spore contents in the range of 10 to 10^3 CFU swab^{-1}. The spore content of the filter cloth ranged from 10^3 to 10^5 CFU g^{-1}. Unfortunately, we were able to sample this item on only two occasions.

Relatively high total spore counts were observed within the different green crop samples. The total spore counts of the majority of the samples were in the range of 10^3 to 10^5 CFU g^{-1}. The total spore content of green maize appeared to be highly variable.

Over 75% of the feed concentrate samples had TS counts of $>10^4$ CFU g^{-1}. The highest determined value for feed concentrate was 7.2×10^6 CFU g^{-1}. Mixtures of soy, linseed, cereals, wheat, and barley had somewhat lower spore levels.

(ii) Presence of potentially highly heat-resistant spores (THRS). In comparison to the TS counts, clearly lower THRS 20, THRS 37, and THRS 55 counts were observed.

TABLE 1. Overview of the spore load at the dairy farms[a]

Sample (no. of isolates)	% TS content							Avg concn
	≤10	>10–≤10^2	>10^2–≤10^3	>10^3–≤10^4	>10^4–≤10^5	>10^5–≤10^6	>10^6–≤10^7	
Raw milk (CFU ml^{-1})								
Total (18)	5.6	33.3	38.9	16.7	5.6			5.46×10^3
Milking machine (CFU $swab^{-1}$)								
Teat cups (33)	21.2	54.5	21.2	3.0				3.84×10^2
Cluster (12)	16.7	50.0	16.7	16.7				1.05×10^3
Connection point pipeline (6)		33.3	50.0		16.7			8.89×10^3
Filter cloth (2)[b]				50.0	50.0			1.19×10^4
Collection tank (11)	18.2	18.2	45.5	18.2				5.18×10^2
Total (64)	17.2	43.8	26.6	9.4	3.1			
Green crop (CFU g^{-1})								
Ensilage (12)			8.3	25.0	50.0	16.7		1.44×10^5
Green maize (15)	6.7		26.7	33.3	20.0	6.7	6.7	2.86×10^5
Hay/straw (5)				40.0	40.0	20.0		1.01×10^5
Other (4)[c]				50.0	50.0			1.31×10^4
Total (36)	2.8		13.9	33.3	36.1	11.1	2.8	
Fodder (CFU g^{-1})								
Feed concentrate (25)		4.0		20.0	40.0	16.0	20.0	8.3×10^5
Pulp (2)				50.0	50.0			2.1×10^4
Other (5)[d]			40.0	40.0	20.0			1.0×10^4
Total (32)		3.1	6.3	25.0	37.5	12.5	15.6	

[a] Total spore counts are expressed as percentages of the samples belonging to a given spore content category after heating at 80°C for 10 min. The number of samples analyzed per category of sample is mentioned in parentheses.

[b] The whole filter cloth was recovered for sampling; therefore, concentration is expressed as CFU per gram for this sample category.

[c] Other samples included luceme, chopped corn, grass, and ensiled beetroot and leaves.

[d] Other samples included soy (2), soy mixed with linseed, cereals, and a mixture of wheat, barley, and linseed.

Table 2 represents a more detailed view of the distribution of the THRS counts across the different sample categories. In at least half of all samples per category, no spores were detected after heating for 30 min at 100°C and subsequent incubation at 20°C (THRS 20). However, substantial levels of these spores of psychrotolerant species were detected in feed concentrate and several self-made mixtures.

The THRS 37 counts showed a different picture. In all raw milk samples, potentially highly heat-resistant spores of mesophilic flora were detected, even though they were detected in rather small numbers. For milking equipment and green fodder samples, half or more of the samples had undetectable levels of THRS 37, with the exception of the filter cloth and hay and straw samples. One of the latter samples contained as much as 7.9×10^3 THRS 37 g^{-1}. In general, fodder samples showed higher THRS 37 counts than samples from other categories.

Thermotolerant or thermophilic counts of potentially highly heat-resistant spores (THRS 55) were below 10 CFU ml^{-1} in most raw milk samples. For the milking apparatus, high levels were again observed in the filter cloth. The majority of the other samples in this category had mainly low counts. In contrast, high THRS 55 counts were revealed in some fodder samples.

Identification approach of the potentially highly heat resistant spore formers. (i) Isolation. Following the 30-min heating at 100°C and the incubation at three different temperatures, a total of 701 isolates were obtained. As shown in Table 3, most of the potentially very heat-resistant spore formers originated from fodder. In general, most isolates were recovered following incubation at 37 or 55°C.

(ii) Identification approach. A polyphasic identification strategy similar to the one described by Vaerewijck et al. (47) was applied. In the hierarchical approach, all isolates were initially screened by numerical analysis of their FAME profiles, resulting in clusters of closely related strains. FAME analysis provides a useful tool for grouping *Bacillus* isolates but is not always as useful for exact species identification (21). Therefore, for each of the obtained FAME clusters, a representative strain was selected which was subsequently subjected to ARDRA (n = 106). Representative strains of the ARDRA clusters were further subjected to 16S rRNA gene sequence analysis (n = 37), percent G+C measurements (n = 24), and DNA-DNA reassociations (n = 10) to obtain a consensus identification (see Table 4, last column). This consensus identification was then recombined with the composition of the FAME and ARDRA clusters to obtain the results shown in Tables 5 to 7.

(iii) FAME screening. A total of 16, 6, 8, and 36 isolates of raw milk, the milking equipment, green crop, and fodder, respectively, could not be analyzed since they failed to grow according to the prescribed growth conditions for FAME analysis. These isolates were not further considered. The FAME profiles of the remaining isolates were subjected to a clustering analysis per sample category (data not shown). The 32 isolates for which the FAME analysis clearly indicated that they did not

TABLE 2. Overview of the THRS counts in the different samples from dairy farms and at three different incubation temperatures (psychrotolerant, mesophilic, and thermotolerant aerobic spore-forming bacteria)[a]

Sample (no. of isolates)	% THRS[a]													
	20°C				37°C					55°C				
	ND[e]	≤ 10	>10–$\leq 10^2$	$>10^2$	ND	≤ 10	>10–$\leq 10^2$	$>10^2$–$\leq 10^3$	$>10^3$	ND	≤ 10	>10–$\leq 10^2$	$>10^2$–$\leq 10^3$	$>10^3$
Raw milk (CFU ml^{-1})														
Total (18)	55.6	44.4				88.9	11.1			16.7	77.8	5.6		
Milking machine (CFU $swab^{-1}$)														
Teat cups (33)	87.9	12.1			78.8	18.2	3.0			78.8	18.2	3.0		
Cluster (12)	100.0				50.0	41.7		8.3		75.0	8.3	16.7		
Connection point pipeline (6)	100.0				50.0	50.0				83.3	16.7			
Filter cloth (2)[b]	50.0		50.0				50.0	50.0				50.0	50.0	
Collection tank (11)	100.0				72.7	27.3				81.8	18.2			
Total (64)	92.2	6.3	1.6		67.2	26.6	3.1	3.1		76.6	15.6	6.3	1.6	
Green crop (CFU g^{-1})														
Ensilage (12)	75.0	25.0			66.7	16.7	8.3	8.3		58.3	33.3		8.3	
Green maize (15)	100.0				86.7	6.7			6.7	80.0	6.7	6.7	6.7	
Hay/straw (5)	100.0				20.0	20.0	20.0	20.0	20.0		40.0	60.0		
Other (4)[c]	75.0		25.0		100.0					25.0	50.0	25.0		
Total (36)	88.9	8.3	2.8		72.2	11.1	5.6	5.6	5.6	55.6	25.0	13.9	5.6	
Fodder (CFU g^{-1})														
Feed concentrate (25)	56.0	16.0	16.0	12.0	24.0	12.0	32.0	28.0	4.0	20.0	12.0	20.0	32.0	16.0
Pulp (2)	100.0				50.0		50.0			50.0		50.0		
Other (5)[d]	60.0	20.0		20.0	20.0	40.0	20.0	20.0			40.0	40.0	20.0	
Total (32)	59.4	15.6	12.5	12.5	25.0	15.6	31.3	25.0	3.1	18.8	15.6	25.0	28.1	12.5

[a] THRS counts, selecting for potentially highly heat-resistant spores, are expressed as percentages of the samples belonging to a given spore content category after heating at 100°C for 30 min. The numbers of samples analyzed per category of sample are shown in parentheses.

[b] The whole filter cloth was recovered for sampling; therefore, concentration is expressed as CFU g^{-1} for this sample category.

[c] Other samples included lucerne, chopped corn, grass, and ensiled beetroot and leaves.

[d] Other samples included soy (2), soy mixed with linseed, cereals, and a mixture of wheat, barley, and linseed.

[e] ND, none detected.

belong to aerobic spore-forming species were regarded as contaminants and were not further considered. For the remaining 603 isolates (166 raw milk, 138 milking equipment, 43 green crop, and 256 fodder isolates), clusters of similar strains were delineated at a Euclidian distance maximum of 15 in each of the four individual clustering analyses. In each clustering analysis, FAME profiles of several culture collection strains of *B. sporothermodurans* were included. For some groups, an indicative similarity index of ≥0.550 was obtained, and these isolates could therefore be allocated to *B. licheniformis*, *Bacillus sphaericus*, *B. subtilis* group, *B. cereus* group, and *Brevibacillus agri*. Other strains could not unambiguously be identified.

(iv) ARDRA. A total of 106 representative isolates were subjected to ARDRA, together with the type strains of *B. sporothermodurans*, *B. subtilis* subsp. *subtilis*, *B. licheniformis*, *Brevibacillus brevis*, *Geobacillus kaustophilus*, and *Paenibacillus polymyxa*. A cluster analysis revealed 30 groups of highly similar patterns and eight strains occupying a single position. The identification obtained for the dairy farm isolates according to the ARDRA database is given per cluster or single strain in Table 4.

TABLE 3. Number of potentially highly heat-resistant isolates picked off per sample category and per incubation temperature

Sample category	No. of isolates at the given incubation temp (°C)			Total no.
	20	37	55	
Raw milk	9	90	87	186
Milking machine	7	71	80	158
Green crop	7	22	27	56
Fodder	30	104	167	301
Total				701

For ARDRA cluster 12, the FAME identification as *B. licheniformis* was further confirmed here and by an extensive genotypic study (7), including five of the dairy farms isolates (R-6452, R-6646, R-6979, R-7199, and R-7478). Similarly, the ARDRA results further confirmed the FAME identification as *B. subtilis* group (ARDRA cluster 13). In addition to confirming FAME identifications, ARDRA enabled nine additional identifications at the species level. ARDRA clusters 4, 6, 7, 8, 24, 28, 29, and 30 and strain R-6443 were identified as *Bacillus smithii*, *B. circulans*, *Bacillus oleronius*, *B. sporothermodurans*, *Brevibacillus brevis*, *Aneurinibacillus aneurinilyticus*, *Aneurinibacillus thermoaerophilus*, *Virgibacillus proomii* and *Paenibacillus thiaminolyticus*, respectively (Table 4). The strains in ARDRA clusters 6, 7, 8, 12, 13, and 24 grouped together with the type strain of the respective species analyzed simultaneously in this study. For ARDRA cluster 8, the identification as *B. sporothermodurans* strains was further confirmed by a positive reaction with the primers described previously by Scheldeman et al. (37). The remaining clusters could not be allocated at the species level, and only an indicative relatedness at the genus level was obtained.

TABLE 4. Strain composition of the ARDRA clusters standing for selected representatives of the FAME analysis[a]

ARDRA cluster or single strain	Dairy farm isolates in cluster	Farm/sample/Temp (°C)[b]	ARDRA identification[c]	Strain selected for 16S rRNA gene sequencing (accession no.)	FASTA interpretation (accession no.)[d]	G + C content (mol%)[b]	Proposed consensus identification
Clusters							
1	R-6640 R-7412 R-7750 R-7751	4/MI17/55 13/RM14/55 16/RM17/55 16/RM17/55	Related to *Ureibacillus thermosphaericus*	R-7750 (AY373318)	99.7% *Ureibacillus thermosphaericus* DSM10633^T (AB101594) 99.7% *Ureibacillus thermosphaericus* P-11^T (X90640)	ND	*Ureibacillus thermosphaericus*
2	R-6785 R-7428	7/KV13/37 7/KV21/37	No identification	R-7428 (AY422987)	95.9% *Virgibacillus carmonensis* LMG 20946^T (AJ316302) 95.7% *Virgibacillus necropolis* LMG 19488^T (AJ315056)	37.4	*Virgibacillus* sp. nov. A
3	R-6678 R-6962 R-7413	4/KV5/55 8/KV15/55 13/RM14/55	Related to *Bacillus smithii*	R-7413 (AY422985)	95.7% *Bacillus methanolicus* C1 (X64465) 95.6% Bacterium LMG 18435 (AJ250318) 95.2% *Bacillus sporothermodurans* M215^T (U49079)	33.8	*Bacillus* sp. nov. B
4	R-6511 R-6515 R-7159 R-7170 R-7484 R-7820	1/MI3/55 1/MI3/55 11/KV18/55 11/RM12/55 14/KV23/55 16/SI34/55	*Bacillus smithii*	R-7170 (AY373319)	99.7% *Bacillus smithii* DSM 4216^T (Z26935) 97.0% *Bacillus smithii* DSM 4216^T (X60643)	ND	*Bacillus smithii*
5	R-7409 R-7440 R-7499 R-7764	14/KV25/55 13/MI53/55 14/SI28/55 16/MI59/55	Related to unknown *Bacillus* sp.	R-7499 (AY373320)	99.8% uncultured bacterium pPD10 (AF252322) 99.6% uncultured compost bacterium 4–50 (AB034714) 99.0% *Bacillus thermoamylovorans* CNCM I-1873^T (L27478)	36.9	*Bacillus thermoamylovorans*[e]
6	R-7349 R-8031	12/SI21/37 14/MI63/37	*Bacillus circulans*			ND	*Bacillus circulans*
7	R-6450 R-6691 R-7770	1/MI3/37 4/KV8/37 16/MI61/37	*Bacillus oleronius*			ND	*Bacillus oleronius*
8	R-6710	3/KV4/55	*Bacillus sporothermodurans*			ND	*Bacillus sporothermodurans*
9	R-6484 R-6486 R-6488 R-6650 R-6739 R-7165	1/MI6/55 1/RM1/55 1/RM1/55 4/RM4/55 4/MI20/55 11/RM12/55	Related to *Bacillus smithii*	R-6488 (AY397764)	99.6% *Bacillus* sp. TAT112 (AB066341; E63292) 99.5% *Bacillus* sp. TAT105 (AB066342; E63291) 99.5% *Bacillus* sp. 115898 (AF071858) 93.7% *Bacillus thermoamylovorans* CNCM I-1378^T (L27478)	34.3	*Bacillus* sp. nov. C
10	R-6476 R-6490 R-6503	1/MI6/55 1/RM1/55 1/MI3/55	Unidentified thermophilic *Bacillus* sp.	R-7748 (AY373321)	99.9% uncultured *Geobacillus* sp. Td (AJ564615) 99.7% *Bacillus pallidus* DSM 3670^T (Z26930) 98.6% "*Bacillus thermoalkalophilus*" DSM 6866 (Z26931)	38.8	*Bacillus pallidus*[f]

	R-6984 R-6987 R-7176 R-7339 R-7411 R-7748	9/SI15/55 9/KV16/55 11/RM12/55 12/KV19/55 13/RM14/55 16/RM17/55					
11	R-6707 R-7433	3/KV4/55 13-KV22/55	Related to *Geobacillus thermoglucosidasius* and *Geobacillus kaustophilus*	R-6707 (AY397770)	99.0% *Geobacillus toebii* SK-1^{T} (AF326278) 98.0% *Geobacillus thermoglucosidasius*ATCC 43742^{T} (AB021197) 98.0% *Geobacillus* sp. R-7653 (AY397769) 97.9% *Geobacillus caldoxylosilyticus* S1812^{T} (AF067651)	42.6	*Geobacillus* sp.
	R-7653	15/KV26/55		R-7653 (AY397769)	99.9% *Geobacillus caldoxylosilyticus* Tj (AJ564618) 99.5% *Geobacillus caldoxylosilyticus* S1812^{T} (AF067651) 98.0% *Geobacillus toebii* SK-1^{T} (AF326278) 98.0% *Geobacillus thermoglucosidasius* ATCC 43742^{T} (AB021197) 98.0% *Geobacillus* sp. R-6707 (AY397770)	43.5	*Geobacillus* sp.
12	R-6451 R-6708 R-6936	1/MI3/37 3/KV4/55 7/KV13/37	*Bacillus subtilis*			ND	*Bacillus subtilis* group[g]
13	R-6719	4/KV6/55	*Bacillus licheniformis*			ND	*Bacillus licheniformis*
14	R-6760 R-7794	5/RM5/37 16/KV30/37	Related to *Bacillus galactosidilyticus*	R-6760 (AJ535639)	99.9% low-G+C-content gram-positive bacterium M51 (AB116129) 98.1% *Bacillus galactosidilyticus* LMG 17892^{T} (AJ535638)	39.2	*Bacillus* sp. nov. D[h]
15	R-6521	1/MI3/55	Related to "*Bacillus carotarum*"	R-6521 (AY382192)[i]	97.7% *Bacillus* sp. 112442 JS2 (AF071857) 97.7% *Bacillus farraginis* R-6540^{T} (AY443036) 97.3% *Bacillus fordii* R-7190^{T} (AY443039) 97.2% *Bacillus* sp. R-6930 (AY382191) 97.1% *Bacillus fortis* R-6514^{T} (AY443038)	40.8	*Bacillus* sp. nov. E[j]
	R-6538	1/MI3/55		R-6538 (AY443035)	99.2% *Bacillus farraginis* R-8039 (AY443034) 98.9% *Bacillus farraginis* R-6540^{T} (AY443036)	ND	*Bacillus farraginis*[k]
16	R-6558 R-7157	4/KV5/20 11/KV18/37	Related to "*Bacillus carotarum*"	R-6558 (AY422986)	99.7% *Bacillus flexus* IFO 15715^{T} (AB021185) 98.3% *Bacillus megaterium* IAM 13418^{T} (D16273)	37.9	*Bacillus flexus*
17	R-6539 R-6540 R-6735 R-7346 R-7789	1/MI3/55 1/MI3/55 3/SI5/55 12/KV19/37 16/KV30/55	Related to "*Bacillus carotarum*"	R-6540 (AY443036)	99.4% *Bacillus farraginis* R-6915 (AY443037) 98.9% *Bacillus farraginis* R-6538 (AY443035) 96.3% *Bacillus fortis* R-6514^{T} (AY443038)	43.7	*Bacillus farraginis*[k]
	R-6915 R-7148 R-7343	7/KV13/37 11/SI18/55 12/KV19/55		R-6915 (AY443037)	99.2% *Bacillus farraginis* R-6540^{T} (AY443036) 95.8% *Bacillus fortis* R-6514^{T} (AY443038)	ND	*Bacillus farraginis*[k]
18	R-6438 R-6674	1/MI3/37 14/MI20/37	No identification	R-7190 (AY443039)	99.3% *Bacillus* sp. 112442 JS2 (AF071857) 97.7% *Bacillus fortis* R-6514^{T} (AY443038)	41.9	*Bacillus fordii*[k]

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TABLE 4—*Continued*

ARDRA cluster or single strain	Dairy farm isolates in cluster	Farm/sample/Temp (°C)[b]	ARDRA identification[c]	Strain selected for 16S rRNA gene sequencing (accession no.)	FASTA interpretation (accession no.)[d]	G + C content (mol%)[b]	Proposed consensus identification
	R-7190 R-7399 R-7795	11/RM12/37 13/KV22/37 16/KV30/37					
19	R-6470 R-6761 R-6767	1/RM1/37 5/RM5/37 5/RM5/20	No identification	R-6767 (AY422988)	99.4% Low G + C Gram-positive bacterium M54 (AB116132) 99.3% *Bacillus sp.* LMG 19636 (AF329473) 95.3% *Virgibacillus picturae* LMG 19492^{T} (AJ3I5060)	35.4	*Virgibacillus* sp. nov. F
20	R-6466 R-8030	1/SI1/37 17/KV31/37	Related to *Paenibacillus glucanolyticus*	R-6466 (AY397766)	98.8% *Paenibacillus* sp. LMG 20245 (AJ316315) 94.6% *Paenibacillus* sp. R-7204 (AY382190) 94.4% *Paenibacillus favisporus* (GMP01^{T} (AY208751) 94.3% *Paenibacillus cineris* LMG 18439^{T} (AJ575658)	ND	*Paenibacillus* sp. nov. G
21	R-6435 R-6472 R-7193 R-7191	1/MI3/37 1/RM1/37 11/RM12/37 11/RM12/37	Related to *Paenibacillus lautus*	R-6472 (AY257870)	98.8% *Paenibacillus lactis* MB 1871^{T} (AY257868) 97.1% *Paenibacillus* sp. SB45-2B (AF395029) 97.1% *Paenibacillus lautus* JCM 9073^{T} (AB073188)	51.7	*Paenibacillus lactis*[l]
22	R-7160 R-7818	11/KV18/55 16/SI34/55	Related to *Geobacillus thermoglucosidasius* and *Geobacillus kaustophilus*	R-7160 (AY397768)	99.5% *Bacillus* sp. E2 (AB089215) 99.3% *Geobacillus stearothermophilus* BGSC 9A21 (AY297092) 99.1% *Bacillus vulcani* 3s-1^{T} (AJ293805) 99.0% *Geobacillus thermocatenulatus* DSM 730^{T} (Z26926) 99.0% *Geobacillus theroleovorans* DSM 5366^{T} (Z26923) 98.7% *Geobacillus stearothermophilus* DSM 22^{T} (AJ294817) 97.4% *Geobacillus thermodenitnficans* DSM 465^{T} (Z26928)	51.0	*Geobacillus* sp.
23	R-6469 R-6547 R-8033	1/RM1/37 2/SI4/55 17/MI62/55	Related to *Brevibacillus thermoruber*	R-6469 (AY397767)	98.7% *Brevibacillus* sp. 96452 (AF227853) 98.5% *Brevibacillas brevis* DSM 30^{T} (AB101593) 98.0% *Brevibacillus formosus* NRRL NRS-863^{T} (D78460)	52.7	*Brevibacillus* sp.
24	R-6738	4/MI20/55	*Brevibacillus brevis*			ND	*Brevibacillus brevis*
25	R-6982 R-7201 R-7815	9/RM10/55 11/RM12/37 16/KV29/37	Related to *Brevibacillus borstelensis*	R-7201 (AY373322)	99.7% *Brevibacillus borstelensis* LMG 15536 (AF378230) 99.6% *Brevibacilus brostelensin* NRRL NRS-818^{T} (D78456) 97.5% *Brevibacillus* sp. R-7745 (AY397774) 97.4% *Brevibacillus* sp. R-6774 (AY422983) 96.9% *Brevibacillus invocatus* LMG 18962^{T} (AF378232)	51.8	*Brevibacillus borstelensis*

26	R-6956 R-7155 R-7418 R-7745	8/MI35/37 11/KV18/37 13/RM14/37 16/RM17/37	Related to *Brevibacillus agri*	R-7745 (AY397774)	99.1% Brevibacillus sp. R-6774 (AY422983) 98.8% *Brevibacillus invocatus* LMG 18962^{T} (AF378232) 97.4% *Brevibacillus brevis* DSM 30^{T} (AB101593) 97.4% *Brevibacillus borstelensis* NRRL NRS-818^{T} (D78456) 97.0% *Brevibacillus centrosporus* NRRL NRS-664^{T} (D78458) 97.0% *Brevibacillus formosus* NRRL NRS-863^{T} (D78460)	50.5	*Brevibacillus* sp.
	R-6774 R-7045	5/MI23/37 10/M140/37		R-6774 (AY422983)	99.6% *Brevibacillus invocatus* LMG 18962^{T} (AF378232) 99.1% *Brevibacillus* sp. R-7745 (AY397774) 97.4% *Brevibacillus borstelensis* LMG 15536 (AF378230) 97.1% *Brevibacillus brevis* DSM 30^{T} (AB101593)	49.1	*Brevibacillus* sp.
27	R-7032 R-7152	9/KV16/55 11/KV18/37	Related to *Brevibacillus choshinensis*	R-7152 (AY397773)	96.4% *Brevibacillus borstelensis* LMG 15536 (AF378230) 96.2% *Brevibacillus* sp. R-7745 (AY397774) 96.2% *Brevibacillus thermoruber* DSM 7064^{T} (Z26921)	52.0	*Brevibacillus* sp. nov. H
28	R-6958 R-7401	8/MI35/37 13/KV22/37	*Aneurinibacillus aneurinilyticus*			ND	*Aneurinibacillus aneurinilyticus*
29	R-7443 R-7637	13/KV21/55 15/RM16/55	*Aneurinibacillus thermoaerophilus*			ND	*Aneurinibacillus thermoaerophilus*
30	R-6638 R-6762	3/RM3/37 5/RM5/37	*Virgibacillus proomii*	R-6762 (AY373323)	98.8% *Virgibacillus proomii* LMG 12370^{T} (AJ012667) 96.9% *Virgibacillus pantothenticus* IAM 11061^{T} (D16275)	36.4	*Virgibacillus proomii*
Single strains							
R-6443		1/MI3/37	*Paenibacillus thiaminolyticus*			ND	*Paenibacillus thiaminolyticus*
R-6507		1/MI3/55	No identification	R-6507 (AY397765)	99.4% bacterium strain 31293 (AF227859) 99.3% bacterium strain 31295 (AF227835) 92.1% *Paenibacillus azoreducens* CM1^{T} (AJ272249)	57.9	*Paenibacillus* sp. nov. I
R-6782		7/RM8/37	No identification	R-6782 (AY422984)	99.4% low G + C gram-positive bacterium HTA437 (AB002642) 99.3% low G + C gram-positive bacterium HTA506 (AB002643) 97.3% *Bacillus barbaricus* VII-B3-A2^{T} (AJ422145)	43.9	*Bacillus barbaricus*
R-6928		7/KV13/37	No identification	R-6928 (AY397772)	99.0% *Paenibacillus* sp. MB 2039 (AY257871) 92.8% *Paenibacillus thiaminolyticus* IFO 15656^{T} (AB073197) 92.7% *Paenibacillus larvae* subsp. *pulvifaciens* DSM 3615^{T} (AB073204) 92.7% *Paenibacillus larvae* subsp. *larvae* DSM 7030^{T} (AB073205) 92.7% *Paenibacillus popilliae* ATCC 14706^{T} (AB073198)	ND	*Paenibacillus* sp. nov. J

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TABLE 4—*Continued*

ARDRA cluster or single strain	Dairy farm isolates in cluster	Farm/sample/Temp (°C)[b]	ARDRA identification[c]	Strain selected for 16S rRNA gene sequencing (accession no.)	FASTA interpretation (accession no.)[d]	G + C content (mol%)[b]	Proposed consensus identification
R-6930		7/SI12/37	Related to *Bacillus coagulans*	R-6930 (AY382191)	97.5% *Bacillus fortis* R-6514^T (AY443038) 97.2% low G + C gram-positive bacterium M52 (AB116130)	ND	*Bacillus* sp. nov. K
R-7204		1/RM12/37	No identification	R-7204 (AY382190)	95.7% *Paenibacillus* sp. LMG 20245 (AJ316315) 94.5% *Paenibacillus daejeonensis* AM141^T (AF391124) 94.4% *Paenibacillus illinoisensis* JCM 9907^T (AB073192)	ND	*Paenibacillus* sp. nov. L
R-7487		15/RM16/37	Related to *Paenibacillus macerans*	R-7487[i] (AY397771)	96.4% *Paenibacillus macerans* IAM 12467^T (AB073196) 93.7% *Paenibacillus lautus* JCM 9073^T (AB073188)	ND	*Paenibacillus* sp. nov. M
R-7652		15/KV26/55	No identification	R-7652 (AY382189)	99.3% uncultured synthetic wastewater bacterium tmbr11-6 (AF309811) 98.6% uncultured synthetic wastewater bacterium tmbr11-29 (AF309809) 94.4% *Paenibacillus kobensis* DSM 10249^T (AB073363) 93.9% *Bacillus chitinolyticus* IFO 15660^T (AB021183)	55.0	*Paenibacillus* sp. nov. N

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[a] The dairy farm isolates in clusters 8, 12, 13, and 24 grouped together with the type strains *B. sporothermodurans* LMG 17894^T, *B. subtilis* subsp. *subtilis* LMG 7135^T, *B. licheniformis* LMG 12363^T, and *Brevibacillus brevis* LMG 16703^T, respectively, simultaneously analyzed in this study. When appropriate, the FASTA search interpretation of the determined 16S rRNA gene sequence is given. The proposed identification is given in the last column and is further discussed in the text.

[b] Abbreviations: KV, cattle feed (fodder); MI, milking installation; RM, raw milk; SI, silage (green crop); ND, not determined; Temp, isolation temperature.

[c] Similarity coefficients of at least 90% were considered an acceptable ARDRA identification (47). Values between 80 and 90% or below 80% are represented as "related to" or "no identification," respectively.

[d] The highest similarity is mentioned, as are relevant scores higher or equal to 97%. Values lower than 97% are given only when they are either the highest score itself or the highest scores with type strains of recognized species.

[e] A DNA-DNA reassociation value of 90% was found between strains R-7499 and *B. thermoamylovorans* LMG 18084^T.

[f] A DNA-DNA reassociation value of 85% was found between strains R-7748 and *B. pallidus* LMG 19006^T.

[g] The phylogenetic *B. subtilis* group contains the following species: *B. atrophaeus, B. amyloliquefaciens, B. mojavensis, B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, and *B. vallismortis.*

[h] A DNA-DNA reassociation value of 33% was found between strains R-6760 and *B. galactosidilyticus* LMG 17892^T (19).

[i] For strains R-6521 and R-7487, only a smaller stretch of 950 and 813 bp, respectively, of 16S rRNA gene sequence was obtained.

[j] An extended polyphasic study excluded R-6521 from the species *B. farraginis*, *B. fortis*, and *B. fordii* (38).

[k] The new species *B. farraginis*, *B. fortis*, and *B. fordii* were described in the course of this study (38).

[l] *P. lactis* was described as a new species following a polyphasic study including these strains (36).

(v) Further identification using 16S rRNA gene sequencing, DNA-DNA reassociations, and percent G+C content. Both the nucleotide sequence accession numbers for the sequences determined in this study and the summarized results of the FASTA search are presented in Table 4. All available data were taken into consideration to obtain the consensus identification (last column of Table 4).

For representative strains of six ARDRA clusters (1, 4, 5, 14, 25, and 30) and two single strains (R-6782 and R-6930), the FASTA search revealed a similarity of at least 97% with a 16S rRNA gene sequence representing the type strain of one single recognized species. Therefore, ARDRA clusters 1, 4, 5, 25, and 30 and strain R-6782 were identified as *Ureibacillus thermosphaericus*, *B. smithii*, *Bacillus thermoamylovorans*, *Brevibacillus borstelensis*, *V. proomii*, and *Bacillus barbaricus*, respectively. The 16S rRNA gene identification for ARDRA cluster 5 was further confirmed by a high DNA-DNA reassociation value (90%) between R-7499 and *B. thermoamylovorans* LMG 18084^T. However, for cluster 14, the high 16S rRNA gene similarity score obtained by FASTA was not confirmed, since only a low DNA-DNA reassociation value (33%) was found between R-6760 and *Bacillus galactosidilyticus* LMG 17892^T (19). Hence, ARDRA cluster 14 most probably represents a novel *Bacillus* species related to the latter species. Even though R-6930 (single strain in ARDRA clustering) showed 97.5% 16S rRNA gene sequence similarity to the type strain of *Bacillus fortis* (R-6514^T), it occupied a more distinct phylogenetic position and showed restriction patterns somewhat different from that of R-6514^T with three of the five restriction enzymes used for ARDRA. For these reasons, R-6930 should probably not be attributed to *B. fortis* and most likely represents a novel *Bacillus* species.

Although the FASTA search revealed scores over 97%, no exact species identification could be assigned for the ARDRA clusters 10, 11, 16, 22, 23, and 26 with these data, because type strains of more than one recognized species matched the determined 16S rRNA gene sequence in this similarity range. The strains in ARDRA clusters 11 and 22 are therefore designated *Geobacillus* sp., and the ones in clusters 23 and 26 are designated *Brevibacillus* sp., indicating that they probably belong to still-to-be-determined recognized species of these genera. A high DNA-DNA reassociation value (85%) between R-7748 and *Bacillus pallidus* LMG 19006^T confirmed relatedness at the species level between these strains. Consequently, ARDRA cluster 10 is identified as *B. pallidus*. Strain R-6558 (ARDRA cluster 16) showed high 16S rRNA gene sequence similarities with both *Bacillus flexus* and *Bacillus megaterium* but can be assigned to *B. flexus* since its whole-cell protein profile was highly similar to that of *B. flexus* LMG 11158^T (P. Scheldeman, unpublished data).

For another six ARDRA clusters (2, 3, 9, 19, 20, and 27) and the individual strains R-6507, R-6928, R-7204, R-7487, and R-7652, no 16S rRNA gene sequence similarity of at least 97% with type strains of valid species was observed. Since only low mutual similarities (<97%) were found among these sequences, the corresponding strains represent 11 separate new species in the genera *Bacillus*, *Brevibacillus*, *Paenibacillus*, and *Virgibacillus* (42).

Strains from ARDRA clusters 15, 17, and 18 were recently described as three new species (*Bacillus farraginis*, *B. fortis*, and *B. fordii*, respectively) following an extensive polyphasic taxonomic study (38). The latter study excluded R-6521 from any of these three species, despite having a FASTA score over 97% with each of these species; consequently, the latter strain represents another new *Bacillus* species. In addition, R-6685 was not assigned to any of these three new species (38), and since no supplementary data are available, this strain is further designated as *Bacillus* sp. The four strains in ARDRA cluster 21 were included together with a number of isolates from UHT milk in a polyphasic taxonomic study leading to the description of the new species *Paenibacillus lactis* (36).

Table 4 also contains the results of the percent G+C analysis. Although the DNA base composition for the genus *Geobacillus* was reported to range between 48.2 and 58 mol% G+C content (27), one species in this genus, *G. toebii*, was described with a significantly lower value of 43.9 mol% (43). The latter value corroborates well with the values obtained for R-6707 and R-7653 (cluster 11). The percent G+C values obtained for strains R-7499, R-7748, R-6558, R-7201, and R-6762 (Table 4) are in line with the reported values for their proposed identification as *B. thermoamylovorans* (3), *B. pallidus* (39), *B. flexus* (33), *Brevibacillus borstelensis* (40), and *V. proomii* (18). For the strains designated as *Brevibacillus* sp., the obtained percent G+C values did not allow the ability to unequivocally distinguish between several *Brevibacillus* species with DNA base compositions in the 49 to 53 mol% range (13).

Presence and diversity of potentially highly heat-resistant spores at the dairy farms. A phylogenetic analysis revealed more than 10% 16S rRNA gene sequence divergence among the potentially highly heat-resistant spore-forming isolates (data not shown). This wide diversity was reflected in the assignment of the farm isolates to seven spore-forming genera including *Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, *Paenibacillus*, *Ureibacillus*, and *Virgibacillus*.

When all consensus identifications are recombined with the initial FAME groupings of 603 spore-forming isolates, an overview of the potentially highly heat-resistant spore formers present at dairy farms is obtained. Table 5 represents a summary of the identifications per sample category. *B. pallidus* and *B. licheniformis* were overall the most frequently isolated species. The predominant species isolated from raw milk, fodder, green crop, and the milking equipment were, respectively, *B. licheniformis*, *B. subtilis* group, *B. farraginis*, and *B. pallidus*. Seventy-five isolates represented 14 different and as-yet-undescribed species in the genera *Bacillus* (five species), *Brevibacillus* (one species), *Paenibacillus* (six species), and *Virgibacillus* (two species) (which are designated sp. nov. A to N in Tables 4 to 7). An additional 65 isolates were assigned to the four species *B. farraginis*, *B. fortis*, *B. fordii*, and *P. lactis*, which were newly described on the basis of isolates from this study (36, 38). Out of 603 isolates, 140 (23.2%) belonged to 18 previously unknown (including the four above-mentioned species) aerobic spore-forming species. For another 5.5% of these isolates, no identification was obtained. These strains either held a separate position in the FAME clustering of a given sample category and were not further analyzed or clustered together with *B. sporothermodurans* strains in FAME but did not react in a species-specific PCR test (37).

To evaluate the spread of the different potentially highly heat-resistant spore-forming species at the dairy farm level, the

TABLE 5. Summary of the presence of a given species in the different sample categories[a]

Identification	Presence (%)				
	Raw milk	Fodder	Green crop	Milking equipment	Overall
Bacillus barbaricus	1.2				0.3
Bacillus cereus group			2.3		0.6
Bacillus circulans			2.3	1.4	0.9
Bacillus farraginis[b]		8.6	16.3	2.9	6.9
Bacillus flexus		1.2			0.3
Bacillus fordii[b]	1.2	2.0		5.8	2.2
Bacillus fortis[b]		2.0		0.7	0.7
Bacillus licheniformis	22.3	5.5	9.3	11.6	12.2
Bacillus oleronius		1.2		4.3	1.4
Bacillus pallidus	15.1	15.2	11.6	22.5	16.1
Bacillus smithii	1.2	2.7	4.7	7.2	4.0
Bacillus sphaericus				0.7	0.2
Bacillus sporothermodurans		5.9	2.3		2.0
Bacillus subtilis group	1.2	25.4	4.7	2.9	8.5
Bacillus thermoamylovorans		0.4	4.7	2.9	2.0
Bacillus sp.[c]		0.4			0.1
Bacillus sp. nov. B	3.0	3.9			1.7
Bacillus sp. nov. C	6.0			1.4	1.9
Bacillus sp. nov. D	0.6	1.2			0.4
Bacillus sp. nov. E				0.7	0.2
Bacillus sp. nov. K			4.7		1.2
Aneurinibacillus aneurinilyticus		0.4		1.4	0.5
Aneurinibacillus thermoaerophilus	1.2	2.0			0.8
Brevibacillus agri	4.8	2.0	11.6	7.2	6.4
Brevibacillus borstelensis	7.2	2.3			2.4
Brevibacillus brevis				0.7	0.2
Brevibacillus spp.	4.8	1.2	4.7	5.8	4.1
Brevibacillus sp. nov. H		2.7			0.7
Geobacillus spp.		5.9	11.6		4.4
Paenibacillus lactis[b]	4.2			2.9	1.8
Paenibacillus thiaminolyticus				2.2	0.5
Paenibacillus sp.[d]				2.9	0.7
Paenibacillus sp. nov. G		1.2	4.7		1.5
Paenibacillus sp. nov. I				1.4	0.4
Paenibacillus sp. nov. J	3.0	2.0			1.2
Paenibacillus sp. nov. L	0.6				0.2
Paenibacillus sp. nov. M	2.4				0.6
Paenibacillus sp. nov. N		0.4			0.1
Virgibacillus proomii	3.6				0.9
Virgibacillus sp. nov. A		0.8			0.2
Virgibacillus sp. nov. F	5.4				1.4
Ureibacillus thermosphaericus	6.6			0.7	1.8
No identification[e]	4.2	3.9	4.7	9.4	5.5

[a] All FAME, ARDRA, and 16S rRNA gene sequence data were considered.

[b] *P. lactis*, *B. farraginis*, *B. fortis*, and *B. fordii* were described as new species following their isolation in this study (36, 38).

[c] Strain R-6685 could not be attributed to the three new species *B. farraginis*, *B. fortis*, and *B. fordii* (38) and is therefore designated as *Bacillus* sp.

[d] Four strains (R-6440, R-6441, R-6449, and R-6461) clustered together with *P. lactis* isolates in FAME but are reported as *Paenibacillus* sp. because a taxonomic study excluded them from *P. lactis* (36).

[e] Strains which held a single position in the FAME clustering of a given sample category and were not further analyzed or strains which clustered together with *B. sporothermodurans* strains in FAME but reacted negatively in a PCR with the primers described by Scheldeman et al. (37).

identification data were classified per farm (Table 6). *B. licheniformis*, *B. pallidus*, *B. farraginis*, and *B. subtilis* group were not only the predominant species in different sample categories but were also the most widely spread among dairy farms. Other species such as *B. sporothermodurans* and several *Paenibacillus* spp. were less frequently isolated.

Table 7 compares the presence of potentially highly heat-resistant species present in raw milk to their possible sources in the other dairy farm samples. *A. thermoaerophilus*, *Brevibacillus borstelensis*, *Bacillus* sp. nov. B and D, and *Paenibacillus* sp. nov. J were found only in fodder samples and raw milk. Especially, feed concentrate, and to a lesser extent soy, seemed to be a major source for these species. Among the species occurring only in raw milk and the milking equipment, *U. thermosphaericus* was isolated exclusively from the teat cups, and *Bacillus* sp. nov. C was isolated solely from the filter cloth, while *P. lactis* was recovered from several parts of the milking system. However, both the milking equipment and the fodder tended to be the most important sources for the potentially highly heat-resistant spores occurring in raw milk.

Three incubation temperatures were used following the selective heat treatment at 100°C. When the identification data are classified under the heading 20, 37, or 55°C, a broad temperature growth range for some species is observed. Representatives of both *B. licheniformis* and the *B. subtilis* group were isolated following incubation at all three temperatures. Although primarily mesophilic, psychrotolerant and thermotolerant isolates of *B. farraginis*, *Brevibacillus borstelensis*, and even *B. sporothermodurans* were occasionally recovered. Other species for which isolates were obtained following incubation at 20°C were *B. flexus*, *B. cereus* group, *Brevibacillus* sp., and *Virgibacillus* sp. nov. F. The isolates identified as *B. pallidus*, *B. smithii*, *B. thermoamylovorans*, *A. thermoaerophilus*, *Geobacillus* sp., and *U. thermosphaericus* were exclusively recovered after incubation at 55°C, which is in line with their identification as thermophilic species. From the previously undescribed species found in this study, *Bacillus* spp. nov. B, C, and E and *Paenibacillus* spp. nov. I and N can be regarded as thermophilic species since they were found only following incubation at 55°C. From *Bacillus* sp. nov. D, *Paenibacillus* spp. nov. G, L, and M, and *Virgibacillus* sp. nov. A, only mesophilic isolates were observed. Finally, *Brevibacillus* sp. nov. H was mainly recovered after incubation at 55°C, whereas *Virgibacillus* sp. nov. F was mainly found after incubation at 37°C.

DISCUSSION

Incidence of spores and potentially highly heat-resistant spores at dairy farms. (i) Incidence of spores. A higher incidence of spores in raw milk is usually observed in the winter period, when cows are housed indoors (5, 44). Average spore levels ranging from 10^0 to 10^3 CFU ml^{-1} are usually reported (5, 23, 35, 46, 49). The relatively high average spore count of raw milk in this study (Table 1) is possibly attributed to one extreme value of 9×10^4 CFU ml^{-1} and to the fact that 10 out of the 17 farms selected for sampling from the study of Rombaut et al. (35) were reported to have high TS counts.

In general, lower spore levels were observed in the milking equipment, with the exception of the filter cloth (Table 1). It should be taken into account, however, that the different parts of the milking system were sampled after the heat-cleaning procedure. High levels of spores in feed concentrate were also previously reported (41, 47). The high incidence of spores in various green fodder samples (Table 1) corroborated with the findings of Slaghuis et al. (41), Lukášová et al. (23), and te Giffel et al. (46). On the whole, green crop and fodder can account for a substantial attribution to the spore load at the dairy farm. te Giffel et al. (46) concluded from their random

TABLE 6. Dissemination of the isolated spore-forming species across the sampled dairy farms

% of dairy farms with isolation of a given spore-forming species[a]			
≥75	≥50	≥25	<25
B. licheniformis	*Brevibacillus agri*	*Brevibacillus borstelensis*	*B. circulans*
B. pallidus	*Brevibacillus* spp.	*Brevibacillus* sp. nov. H	*B. flexus*
B. farraginis[b]	*B. fordii*[b]	*P. lactis*[b]	*P. thiaminolyticus*
B. subtilis group	*B. smithii*	*B. oleronius*	*Paenibacillus* sp. nov. G
	Bacillus sp. nov. B	*B. sporothermodurans*	*Paenibacillus* sp. nov. M
	Geobacillus spp.	*Bacillus* sp. nov. C	*A. aneurinilyticus*
	No identification[c]	*Bacillus* sp. nov. D	*B. barbaricus*
		U. thermosphaericus	*Bacillus* sp. nov. K
		B. fortis[b]	*Paenibacillus* sp. nov. I
		B. thermoamylovorans	*Virgibacillus* sp. nov. A
		Paenibacillus sp. nov. J	*B. sphaericus*
		A. thermoaerophilus	*B. thuringiensis*
		V. proomii	*Bacillus* sp.
		Virgibacillus sp. nov. F	*Bacillus* sp. nov. E
			Brevibacillus brevis
			Paenibacillus sp.
			Paenibacillus sp. nov. L
			Paenibacillus sp. nov. N

[a] Arranged in mathematical order of predominance.

[b] *P. lactis*, *B. farraginis*, *B. fortis*, and *B. fordii* were described as new species following their isolation in this study (36, 38).

[c] Strains which held a single position in the FAME clustering of a given sample category and not further analyzed or which clustered together with *B. sporothermodurans* strains in FAME but reacted negatively in a PCR with the primers described by Scheldeman et al. (37).

amplified polymorphic DNA study that silage is an important source of spores in raw milk. Likewise, one can expect feed concentrate to be an important contamination source of spores in raw milk.

(ii) Incidence of potentially highly heat-resistant spores. All data discussed above deal only with the presence of spores in general and do not indicate possible sources of potentially highly heat-resistant spores at the dairy farm level. Although the isolation of *B. sporothermodurans* spores from various feeds after a heat treatment at 100°C has occasionally been reported (9, 37, 47), far less was known on sources and/or numbers of potentially highly heat-resistant spores. The present study shows that significant numbers of spores can indeed be recovered after a 30-min 100°C heat treatment. Since spores of thermotolerant or thermophilic spore-forming bacteria are typically more heat resistant (28), the higher THRS 55 and THRS 37 counts compared to the THRS 20 counts are not surprising (Table 2).

A high variability in THRS counts among the samples of a given category is observed, ranging from no spores to over 10^3

TABLE 7. Comparison of the number of isolates of the potentially highly heat-resistant spore-forming species found in raw milk with their numbers in the other samples at the dairy farms

Identification	No. of isolates of the given species per sample category[b]:														Total no. of isolates
	Raw milk (18)	Milking equipment					Fodder				Green crop				
		Teat cups (33)	Clusters (12)	Connection point (6)	Filter cloth (2)	Tank (11)	Concentrate (25)	Pulp (2)	Soy (2)	Other (3)	Silage (12)	Green maize (15)	Hay/straw (5)	Other (4)	
B. licheniformis	37	1	3	1	7	4	12		2			1	3		71
B. pallidus	25	2	21	1	7		37		2		1		3	1	100
Brevibacillus borstelensis	12						6								18
U. thermosphaericus	11	1													12
Bacillus sp. nov. C	10				2										12
Virgibacillus sp. nov. F	9														9
Brevibacillus agri	8	1		2	6	1	4			1		1	4		28
Brevibacillus spp.	8	1	2	3	2		3						1	1	21
P. lactis[a]	7		1	1	2										11
V. proomii	6														6
Bacillus sp. nov. B	5						8		2						15
Paenibacillus sp. nov. J	5						5								10
Paenibacillus sp. nov. M	4														4
B. barbaricus	2														2
B. fordii[a]	2		5	1	2		5								15
B. smithii	2	2	6	1		1	7					2			21
B. subtilis group	2	1	2		1		54		11					2	73
A. thermoaerophilus	2						4		1						7
Bacillus sp. nov. D	1						3								4
Paenibacillus sp. nov. L	1														1

[a] *B. fordii* and *P. lactis* were described as new species following their isolation in this study (36, 38).

[b] Number of samples per category is given in parentheses.

spores per g or ml. Per individual sample, these THRS counts did not necessarily reflect the corresponding TS count (data not shown). This result may be explained by the fact that the counts of the classical (TS) and 100°C (THRS) heating are not entirely comparable because of the different experimental setup (media and incubation temperatures). However, it cannot be excluded that some samples might indeed constitute some kind of reservoir of spores with a potentially high heat resistance.

Remarkable diversity of potentially highly heat-resistant spores. In addition to the search for sources of potentially heat-resistant spores at the dairy farm level, another goal of this study was to obtain an overall assessment of the diversity of these spores. After all, any strategic effort to control the strains found within a given setting (whether it be raw milk production or an industrial setting) must be based on the knowledge of the numbers and identity of strains present.

A selective heat treatment followed by incubation at three different temperatures on a rich medium resulted in a large collection of isolates. Since classical identification methods are usually time consuming and not always unambiguously interpretable because of their dependence on phenotypic gene expression, the hierarchical identification procedure applied in this study was based on a combination of chemotaxonomic and genomic methods, for which well-documented identification databases were available. Despite this elaborate identification approach, 23% of the isolates could not be identified to the species level; they belong to 18 separate previously unknown aerobic spore-forming taxa, of which four have recently been described elsewhere (36, 38). Combined with the remarkable diversity of spore formers belonging to seven different genera, these findings suggest that the selective heating reveals a pool of unknown organisms with a more heat-resistant character. Although *B. thermoamylovorans* was taxonomically described as a non-spore-forming organism (3), its isolation here after a 30-min 100°C heat treatment implicates the presence of spores.

Previous studies have focused either specifically on the presence of *B. cereus* at dairy farms (see, e.g., references 1 and 41) or on the presence of spore formers in a particular sample (see, e.g., references 5 and 49). Few studies, however, used a more stringent heat treatment than 10 min at 80°C. Vaerewijck et al. (47) isolated 11 strains from six feed concentrate samples after heating for 30 min at 100°C. The species found by the latter authors were also recovered in the present study. However, from the species found after a classical 10-min heating at 80°C (47), only *B. flexus*, *B. licheniformis*, and *Brevibacillus borstelensis* were found in the present study. This result suggests that a different flora is revealed from a similar sample, depending on the heat treatment used for isolation, and that the isolates from the present study may therefore indeed have a more heat-resistant character.

de Silva et al. (9) isolated *B. licheniformis*, *B. sporothermodurans*, and *Brevibacillus borstelensis* from silage after heating at 100°C for 60 min alongside two unassigned isolates and three isolates regarded as new species. One of these three isolates (112442 JS2) showed 99.3% 16S rRNA gene sequence similarity with the type strain of *B. fordii* (Table 4). After a heat treatment of grass and maize silage for 30 s at 125 and 130°C, te Giffel et al. (46) isolated members of the *B. subtilis* group, *B. licheniformis*, *B. oleronius* or *B. sporothermodurans*, *Aneurinibacillus*, and *Paenibacillus* spp. Several of these species were also found in green crop samples from the present study.

In most studies, spore-forming bacteria occurring in raw milk were isolated after the classical heating procedure at 80°C for 10 min, and their identification was based mainly on classical biochemical tests. In one study, however, raw milk was subjected to a heat treatment of 30 s at temperatures between 90 and 130°C (46). At temperatures above 120°C, only isolates of *B. licheniformis*, *B. subtilis* group, *Aneurinibacillus*, *Brevibacillus*, and *Paenibacillus* were found, whereas *B. cereus* was solely recovered after more moderate heat treatments (90 and 105°C for 30 s). That finding is in line with the recovery of one single isolate (out of 603) assigned to the *B. cereus* group in this study and the more numerous isolates of the heat-resistant species mentioned.

Incidence of potentially highly heat-resistant spores at the dairy farm. Overall, quite a large species diversity was observed. While the THRS counts in raw milk were usually lower than 10^2 CFU ml^{-1}, 159 raw milk isolates were assigned to 20 different species of spore-forming bacteria, of which *B. licheniformis* far outnumbered the other species. Even more different species (26) were detected in the 256 fodder isolates. From the often relatively high numbers of spores recovered from fodder after a 30-min 100°C heat treatment, *B. pallidus* and *B. subtilis* group were most frequently isolated. Remarkable was the finding of 15 *B. sporothermodurans* isolates from fodder (approximately 6% of isolates), with feed concentrate as the principal isolation source (11 isolates), thereby confirming previous findings (47).

Although fewer isolates were recovered from the green crop samples, still, members of 15 different species were identified within the 43 isolates. Concerning the milking equipment, the highest diversity was found in the clusters and the filter cloth with 16 and 15 different species, respectively. This finding is in line with the observations that these two parts showed the largest THRS counts at 37 and 55°C. These two parts of the mechanical milker can therefore be regarded as possible important reservoirs of spores, even after the heat-cleaning step.

It should be mentioned that with the method of isolation applied here, not all but only representative colonies were isolated from each sample according to morphological appearance. For this reason, species with high morphological variation may have been overrepresented. Similarly, since samples were incubated at three different temperatures (20, 37, and 55°C), organisms displaying a wide growth temperature range, such as *B. subtilis* group and *B. licheniformis*, may have been somewhat overrepresented. In addition, for species whose spores occur in large numbers in a given sample (e.g., *B. licheniformis* [5]), isolates are more likely to be recovered after the 30-min 100°C heat treatment than isolates of less abundant but equally heat-resistant species.

Influx of potentially highly heat-resistant spores in raw milk. Possible points of entry of potentially highly heat-resistant spores from several sources into raw milk were investigated. Representatives of *A. thermoaerophilus*, *Brevibacillus borstelensis*, the novel *Bacillus* species B and D, and *Paenibacillus* sp. nov. J were recovered solely from both raw milk and fodder samples, with feed concentrate as the primary source. Twelve out of the 20 different spore-forming species found in raw milk were also detected in feed concentrate. It is not unlikely that high levels of spores in feed (Table 2) may lead to large quantities of spores in the feces, which in turn can contaminate the udder and teats of lactating cows. When not removed by udder and teat washing, these contaminating spores can gain access to the milk during milking (24).

Although no exclusive presence of certain species in both raw milk and green crop samples was observed, this does not exclude the possibility that green crop can attribute to the possibly highly heat-resistant spore flora in raw milk. After all, six species, which mainly originated from hay or straw, were still observed in common between both sample categories.

Contamination also seems to be able to accumulate in the milking apparatus, where 10 species which also occur in raw milk were isolated, 3 of which were detected exclusively in both raw milk and the milking equipment. These findings, combined with the large number of potentially heat-resistant spores observed, particularly in the clusters and the filter cloth even after the heat-cleaning step, indicate that the milking apparatus might act as a reservoir and entry point of potentially heat-resistant spores into the raw milk, possibly by the formation of biofilms in areas difficult to access for cleaning.

Of course, the presence of members of the same species is only an indication for possible contamination sources of these potentially highly heat-resistant spores in raw milk. Further molecular typing could provide proof of the given contamination routes, as shown for silage (46). Yet again, members of five spore-forming species were detected solely in the raw milk (mainly *Paenibacillus* and *Virgibacillus* spp.), suggesting that there are still other possible entry points of potentially highly heat-resistant spores into raw milk.

Concluding remarks. In this study, at the dairy farm level, several spore-forming species, whose presence in an industrial setting has been previously reported, were found. More precisely, *B. sporothermodurans* (15, 30), *P. lactis* (36), *Brevibacillus borstelensis* (9), *B. sphaericus*, *B. licheniformis*, and *Brevibacillus brevis* (4) spores were previously isolated from UHT milk. *A. thermoaerophilus* was previously isolated from an Austrian sugar beet factory (26), and representatives of the *B. subtilis* group, *B. thermoamylovorans*, *Brevibacillus agri*, and *Brevibacillus borstelensis* were isolated in a gelatin production process, where severe heat treatments are also used (6, 8).

However, this does not necessarily imply that the isolates found at the dairy farm level form the primary contamination source. Indeed, molecular typing of *B. sporothermodurans* strains from both dairy farm and industrial samples clearly showed a distinction based on the isolation source (15). Further molecular typing is needed to prove that the potentially highly heat-resistant spores found here in raw milk would act as an important point of entry in the dairies.

The large numbers of isolates of certain species recovered in this study, similarly, do not necessarily imply that the spores are indeed highly heat resistant, as this also depends on the initial counts of a given spore former in a given sample. However, it is clear that by the selective heating at 100°C, a different flora is observed compared to the flora of similar samples after the classical heat treatment for 10 min at 80°C. This result suggests that the spore formers isolated in this study indeed tend to be more heat resistant. To what extent these spores are indeed highly heat resistant still needs to be determined by individual heat resistance studies.

Nevertheless, this study showed that high spore counts can occur at the dairy farm and that the feed and the milking equipment can act as reservoirs or entry points for potentially highly heat-resistant spores into raw milk. Lowering this spore load by good hygienic measures (e.g., thorough cleaning of the teats and the milking equipment) 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. Other parameters, however, such as the equipment and packaging materials in the factory have also been shown to play a role (see, e.g., references 11 and 34) and should be studied in more detail to control spores in food production processing from the raw materials to the final products.

This study revealed a large diversity of spore-forming species which are able to survive heating for 30 min at 100°C, with an important fraction belonging to as-yet-undescribed species. New species also imply unknown properties of resistance, spoilage potential, or health risks. A good characterization of these novel species is therefore indispensable to be able to react fast and adequately to newly arising microbial problems in dairy products.

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