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REVIEW ARTICLE

Bacillus sporothermodurans and other highly heat-resistant spore formers in milk

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Abstract

A recent example of a micro-organism causing undesired growth in consumer milk is *Bacillus sporothermodurans* producing highly heat-resistant spores (HRS) which may survive ultra-high temperature (UHT) treatment or industrial sterilization. Molecular typing showed a heterogeneous group of farm isolates (non-HRS strains), but a clonal group of UHT isolates from diverse European countries and other continents (HRS-clone) suggesting a common source. During a survey of Belgian dairy farms for the presence of potentially highly heat-resistant spore formers, high numbers of these spores were detected in filter cloth, green crop and fodder samples. The strain collection showed a high taxonomic diversity with 18 potentially new species and with *Bacillus licheniformis* and *Geobacillus pallidus* as predominating species overall. Seventeen *B. sporothermodurans* isolates were identified, mainly originating from feed concentrate. Heat resistance studies showed the UHT resistance of *B. sporothermodurans* spores present in industrially contaminated UHT milk, but a lower heat resistance of laboratory-grown strains (HRS and non-HRS). Hydrogen peroxide, used as sanitizer in the dairy industry, was found to induce higher heat resistance of laboratory-grown *B. sporothermodurans* strains to a certain level. This indicates that sublethal stress conditions may affect the heat resistance. By transmission electron microscopy, structural differences at the spore level were found between HRS and non-HRS strains. The data indicate that the attainment of extreme heat resistance is rather multifactorial.

Introduction

Importance of aerobic spore formers in food

Aerobic spore-forming bacteria are important in the food industry for several reasons (Andersson *et al.* 1995; Heyndrickx and Scheldeman 2002). First, the ubiquitous nature of these spore formers makes it basically impossible to prevent their presence in raw food and ingredients. When deficiencies occur in the filling apparatus or the sterilization of the packaging material, contamination can easily occur. Secondly, commonly used pasteurization processes, although adequate in inactivating vegetative cells, fail to kill spores. Surviving spores, experiencing little or no competition from faster growing vegetative cells, may germinate and proliferate rapidly in the product. Thirdly, the adhesive characteristics of some spores facilitate their attachment to the surfaces of pipelines and processing equipment, leading to the formation of biofilms (e.g. Andersson and Ronner 1998). And last but not least, there is a growing concern about the (increasing) tolerance or resistance of spores or vegetative cells to conditions or treatments generally presumed to stop growth (low temperatures and low pH), or to inactivate all living material, such as sterilization and ultra-high temperature (UHT) processing. These 'super bugs', either adapted or new organisms with high intrinsic tolerance or resistance properties, might be selected for by the use of new feed

or food ingredients and processing or packaging technologies. In the food industry, problems arising form aerobic spore-forming bacilli are basically twofold. A first and increasing concern is the pathogen *Bacillus cereus*, which can cause serious food-borne intoxications. Food products frequently contaminated by *B. cereus* include, among others, milk, dairy products, dry foods, rice dishes, egg and legume products (Granum 2002). A second concern is food spoilage caused by spore formers during production, storage and distribution (Huis in't Veld 1996). Food spoilage, generally defined as any change that renders a product unsuitable for human consumption, often results in considerable economic losses despite modern manufacturing techniques. Some examples are given in Table 1. Related nonspoilage problems caused by aerobic spore formers are nonsterility of UHT-treated and sterilized milk.

Aerobic spore formers in milk

An overview of the psychrotolerant, mesophilic and thermophilic aerobic spore-forming flora in raw milk, and at several stages in milk processing, was given by Heyndrickx and Scheldeman (2002). Total aerobic spore counts in raw milk are subjected to seasonal variation, with usually a higher incidence observed in the winter period, when cows are housed indoors (Sutherland and Murdoch 1994). Generally, the average counts are in the range of $10–10^2$ CFU ml^{-1} (Waes 1976; te Giffel *et al.* 2002). Despite some regional, seasonal and methodological differences, there is mostly a predominance of *Bacillus licheniformis* in raw milk (Phillips and Griffiths 1986). *Bacillus cereus* often is the most common psychrotolerant species, especially in the summer period.

In pasteurized milk, obtained by a conventional bulk treatment at 61–66°C for 30 min or by a flash pasteurization (also called high temperature short time) at 71·7°C for at least 15 s (but usually 30–40 s), (most) vegetative cells are killed but spores remain. Because pasteurized milk has to be stored at low temperatures, the psychrotolerant spore formers, defined as those micro-organisms that can grow at 7°C or less (irrespective of their optimal growth temperature), are of particular concern. With the longer refrigerated storage before processing, higher pasteurization conditions, reduction of postpasteurization contamination and prolonged shelf-life combined with the fact that pasteurization often activates germination of spores (Hanson *et al.* 2005), the particular presence of psychrotolerant spore formers is of increasing importance. Not only is there the potential of food-borne intoxications as a result of *B. cereus* outgrowth, but also many spoilage defects caused by enzymatic activity may also occur (reviewed by Heyndrickx and Scheldeman (2002). The possible sources of *B. cereus* contamination in both raw milk and processing plants were also reviewed by the same authors.

In UHT-processed milk, obtained by a treatment at minimally 135°C for 1 s (but usually between 135 and 150°C for 1–8 s) in a continuous flow and subsequent packaging in presterilized containers, virtually all micro-organisms including spores are killed. The same applies to sterilized milk, obtained by a preheating for 1–60 s at 120–135°C followed by a sterilization after bottling at 110–120°C for 10–20 min. Both products are 'commercially sterile' (i.e. not more than one potential spoiled 1-l container per 1000 and usually 1 per 10 000 or less) and have a long shelf-life (more than 6 months) without refrigeration. In Europe, UHT milk is mainly consumed in amongst others France, Germany and Belgium. To meet the legal requirements established by the European Union Hygiene directive 92/46, the colony count at 30°C of unopened packages after 15 days of incubation at 30°C must be below 10 CFU per 0·1 ml (Anonymous 1992). Spoilage infrequently occurs because of recontamination during filling and is mostly caused by proteolytic activity of some *Bacillus* species. The presence of aerobic spore formers (including *Bacillus sphaericus*, *B. licheniformis* and *Brevibacillus brevis*) below the EC directive level was, for instance, shown in 30% of fresh Sardinian UHT milk samples (Cosentino *et al.* 1997).

Table 1 *Bacillus* species causing spoilage in foods

Species	Food products affected	Type of spoilage	References
Alicyclobacillus acidoterrestris	Fruit juices, acidic beverages	Off-flavours	Splittstoesser *et al.* (1994), Yamazaki *et al.* (1996), Jensen (1999)
Bacillus cereus	Pasteurized milk, dairy products	Bitty cream, sweet curdling, off-flavours	Griffiths *et al.* (1981), Cousin (1982), Meer *et al.* (1991)
Geobacillus stearothermophilus *Bacillus coagulans* *Bacillus licheniformis*	Evaporated milk, low-acid canned vegetable tomato juice	Flat sour	Montville (1982), Kalogridou-Vassiliadou (1992)
Bacillus subtilis	Bread	Ropy bread	Rosenkvist and Hansen (1995)

Bacillus sporothermodurans

Contamination of UHT and sterilized milk

Massive contaminations of entire commercial lots of UHT- and sterilized milk with a then unknown mesophilic aerobic spore former were first reported in Italy and Austria in 1985 and in 1990 also in Germany (Hammer *et al.* 1995). This organism was provisionally called a 'highly heat-resistant spore former' (termed HHRS or HRS), as the causative organism could be isolated from a bypass directly after the heating section of an indirect UHT-heating device. Contrary to post-heat treatment contamination, this problem seemed to be caused by survival of the UHT process by the HRS and occurred more frequently in indirect UHT than in direct UHT processing. The problem subsequently spread to countries in and outside Europe (Hammer *et al.* 1995; Guillaume-Gentil *et al.* 2002). Affected milk products included whole, skimmed, evaporated or reconstituted UHT milk, UHT cream and chocolate milk in different kinds of containers and also milk powders (Hammer *et al.* 1995; Klijn *et al.* 1997). The HRS organism appeared as small, pinpoint colonies on plate count agar incubated at 30°C, usually reaching a maximum of 10^5 vegetative cells and 10^3 spores ml^{-1} milk after a 15-day incubation at 30°C of unopened packages of consumer milk according to the EC regulation. These densities do not affect the pH of the milk, and usually do not alter the stability or sensory quality (Klijn *et al.* 1997). However, this contamination level far exceeds the sterility criterion of 10 CFU per 0·1 ml according to the EC regulation. Recently, higher bacterial loads in 37% of Italian contaminated UHT milk samples exceeding 10^5 CFU ml^{-1} have been reported (Montanari *et al.* 2004). Several HRS strains were tested and showed no pathogenic potential (Hammer and Walte 1996). The HRS organism was taxonomically described as the new species *B. sporothermodurans* based on isolates solely from UHT milk (Pettersson *et al.* 1996). Despite its poor growth characteristics in milk, UHT milk can be regarded as a new ecological niche for *B. sporothermodurans* because of the lack of competition from other organisms in this product. The organism has also been isolated from contaminated UHT-treated coconut cream (F. Priest, pers. comm. and M. Heyndrickx, unpubl. data).

Detection and ecology

Isolation of *B. sporothermodurans* from UHT- or sterilized milk is best performed on brain heart infusion (BHI) plates supplemented with vitamin B_{12} and incubation at 37°C, whereas growth on milk plate count agar, as used by most dairies to test the milk downstream heat processing, is poor. The isolation of *B. sporothermodurans* from raw milk or other farm sources, however, is not evident because of the high competitive background microflora. The best selection method is autoclaving for 5 min or heating the sample at 100°C for 30–40 min and subsequent plating on supplemented BHI. Phenotypic identification of *B. sporothermodurans* is hampered by its poor growth characteristics and its negative reactions in many of the standard and API 50CHB tests (with the exception of esculin hydrolysis) (Pettersson *et al.* 1996). Moreover, some positive characters seem to be variable between strains or between authors (Pettersson *et al.* 1996; Klijn *et al.* 1997; Montanari *et al.* 2004), most probably because of different media used. The presence of other, not yet described potentially highly heat-resistant spore formers in raw milk (see later) may also complicate unequivocal confirmation of *B. sporothermodurans*.

Phylogenetically, the closest relatives of *B. sporothermodurans* are *Bacillus oleronius*, *Bacillus lentus*, *Bacillus firmus* and *Bacillus benzoevorans*. However, operon heterogeneity could result in reading difficulties in direct cycle-sequencing of the V1 and V2 regions of the 16S rRNA genes of *B. sporothermodurans* for identification purposes (Pettersson *et al.* 1996; Klijn *et al.* 1997). A PCR detection method for *B. sporothermodurans*, with primers derived from a unique sequence, obtained after subtractive hybridization of *B. sporothermodurans* DNA with DNA of a closely related raw milk strain, proved to be specific for a subset of *B. sporothermodurans*. This subset encompassed all UHT isolates and only a few farm isolates (Herman *et al.* 1998; Guillaume-Gentil *et al.* 2002), and consequently this PCR was referred to as HRS-PCR. This PCR detection method can be used in conjunction with a chemical pretreatment of a raw milk sample followed by a heat activation and cultivation of the spores on supplemented BHI to detect the HRS organism in the presence of a background flora, without the need to isolate the strain, which can be problematic as described above (Herman *et al.* 1998). A second, broader PCR-identification test was also constructed on the basis of the 16S rDNA sequence (Scheldeman *et al.* 2002). This PCR test not only detects the subset of UHT isolates, but also all farm isolates (Guillaume-Gentil *et al.* 2002). A similar approach and range of detection was also achieved by using a DNA probe prepared from a cloned 16S-23S rDNA spacer region (de Silva *et al.* 1998). Several typing methods have been used for *B. sporothermodurans* including random amplified polymorphic DNA (Klijn *et al.* 1997), repetitive element palindromic PCR (REP-PCR) with separation on agarose (Klijn *et al.* 1997) or on polyacrylamide gels (Herman *et al.* 1998) and ribotyping (Guillaume-Gentil *et al.* 2002). Especially the two latter techniques provide the highest discrimination level (see later).

With the above-described detection, identification and typing techniques, the ecology of *B. sporothermodurans* has been investigated in order to find the most probable contamination and spreading routes for consumer milk and milk products. A first source was, logically, sought in raw milk. Only a temporal, local occurrence at a very low contamination level was found in raw milk using the above-described HRS-PCR detection method (Herman *et al.* 2000); however, as this PCR method is more specific for UHT isolates, other strains may have been missed. Still, one strain isolated from raw milk after a treatment of 30 min at 100°C was identified and reported as the first raw milk isolate of *B. sporothermodurans* (Scheldeman *et al.* 2002); this remains the only raw milk isolate up to now. At the dairy farm level, *B. sporothermodurans* spores were occasionally reported in feed concentrate, silage, soy, pulp and compost (de Silva *et al.* 1998; Vaerewijck *et al.* 2001; Scheldeman *et al.* 2002; Zhang *et al.* 2002). Most of the dairy farm isolates have been obtained from feed concentrate at incubation temperatures ranging from 20 to 55°C, but the majority at 37°C (Scheldeman *et al.* 2002). This indicates feed concentrate as most probable primary source with the other positive farm samples probably resulting from contamination cycles on the farm. However, it must be noted that the presence of *B. sporothermodurans* on the farm is relatively rare, as only 17 isolates of this species were obtained on a total collection of about 700 potentially highly heat-resistant isolates, which will be discussed later. Finally, contamination could also result from reprocessing of contaminated lots of UHT milk in the dairy factory or from processing of contaminated milk powder (Hammer *et al.* 1995; Herman *et al.* 1998).

The relative importance of these contamination sources has been illustrated by different typing studies (Herman *et al.* 1998; Guillaume-Gentil *et al.* 2002). The latter was the most extensive study with the typing of UHT milk isolates from different countries and continents (Europe, South America, Asia) and of farm isolates with a combination of REP-PCR and ribotyping using two restriction enzymes. Both cluster analysis and three-dimensional scaling (Fig. 1) of the combined fingerprints revealed a very compact cluster or group composed of most of the UHT isolates. This suggests a clonal origin of these UHT isolates, referred to as HRS clone, which is remarkable as they were obtained from UHT- and sterilized milk samples produced on three different continents. In contrast to the homogeneity found for the majority of the UHT isolates, the combined typing data showed that the farm isolates (feed concentrate, silage, soy, raw milk), including two HRS-PCR-positive strains, formed a genetically very diffuse group, different from the group of UHT isolates (Fig. 1). These data indicate that there is no 100% concordance between a positive result in the HRS-PCR and an HRS clone pattern in polyphasic molecular typing. These data also show that only a few clones, with a predominance of the HRS clone, have been (and still are occasionally) responsible in the mid-1990s for the regular contamination of UHT- and sterilized milk and milk

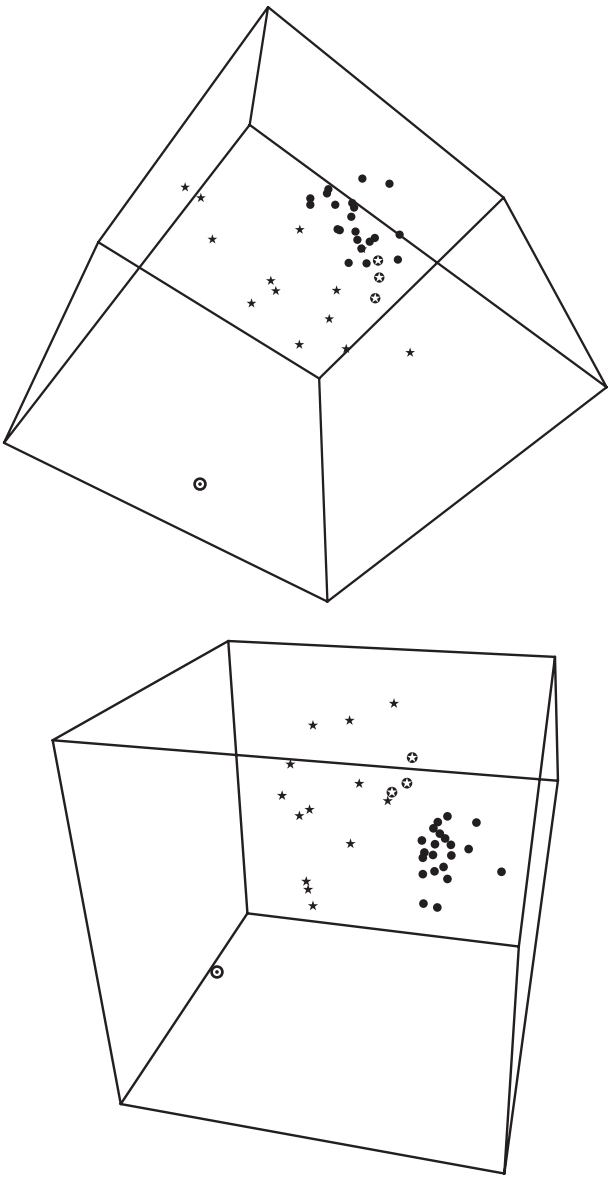

Figure 1 Three-dimensional representation (modified from Guillaume-Gentil *et al.* 2002) of the genetic relatedness between *Bacillus sporothermodurans* strains based on a combined clustering analysis (Pearson correlation) of ribotyping patterns (*Eco*RI and *Pvu*II) and REP-PCR patterns. Black spots represent the HRS-clone. ● 22 *B. sporothermodurans* strains from ultra-high temperature (UHT) products; ✪ 3 *B. sporothermodurans* strains from German UHT products; ★ 13 *B. sporothermodurans* strains from dairy farms; ⊙ *B. oleronius* strain from raw milk.

products due to the production of highly heat-resistant spores. Probably, the spread of the HRS clone has been caused by reprocessing and circulation of contaminated milk within and between UHT production units. Occasionally, a new genetic type, as exemplified by the German UHT isolates, is introduced in a UHT plant, probably via raw milk. The spread to other continents may be explained by the use of contaminated milk powder to reconstitute milk for UHT processing. The question remains of whether the extreme heat resistance of spores to UHT and sterilization temperatures is restricted to the subset of UHT isolates or whether it is a property more widespread in *B. sporothermodurans*. The typing data seem to indicate a restriction of this property to particular clones with a possible common ancestor. Furthermore, the genetic diversity of *B. sporothermodurans* as demonstrated in the above study is not represented in the original description of the species (Pettersson *et al.* 1996), which was only based on the phenotypically very unreactive UHT isolates and inadequate media for phenotypic characterization. An emended description of this industrially important species may thus be necessary, including some phenotypically more reactive farm isolates (N. Logan, pers. comm.).

Other highly heat-resistant spore formers in milk

Besides *B. sporothermodurans*, a few other spore-forming species have also been reported to sporadically contaminate UHT- or sterilized milk with direct or indirect evidence for the production of highly heat-resistant spores: *Geobacillus* (formerly *Bacillus*) *stearothermophilus* (see references in Rombaut *et al.* 2002), *Br. brevis* and/or *Brevibacillus borstelensis* (de Silva *et al.* 1998; Rombaut *et al.* 2002), *B. sphaericus*, *B. licheniformis* and *Br. brevis* (Cosentino *et al.* 1997) and *Paenibacillus lactis* (Scheldeman *et al.* 2004a). The production of spores with high resistance at 120–121°C is well known for *G. stearothermophilus* (Huemer *et al.* 1998; Brown 2000), but as this organism is a thermophile, spoilage problems may only occur in packs held at elevated temperatures. *Brevibacillus brevis* has been shown to produce spores heat resistant at 130°C (Rombaut *et al.* 2002). The best documented recent event is a periodical but tenacious contamination of UHT milk packages from one dairy plant with spores of *P. lactis*. Indirect evidence that this new species, described by Scheldeman *et al.* (2004a), can produce highly heat-resistant spores comes from the fact that the contaminated UHT milk packages came from different processing lines (direct and indirect UHT) and were co-contaminated with *B. sporothermodurans*. Interestingly, *P. lactis* strains had also been isolated on different dairy farms from raw milk, milking apparatus and filter cloth. One of these farm strains, isolated from a cluster of the milking apparatus, showed the same REP-PCR patterns as the UHT isolates. In this case, there seems to be for the first time plausible evidence of a direct link of contamination with highly heat-resistant spores from the raw milk on the dairy farm to the heat-treated milk in the dairy.

Only a few studies have addressed the presence of highly heat-resistant spores at the dairy farm (Table 2), which can be the original source for spores causing spoil-

Table 2 Previous isolations of highly heat-resistant spores at the dairy farm

Spore-forming species	Source	Heat treatment applied	Reference
Bacillus sporothermodurans *Brevibacillus borstelenis* *Bacillus licheniformis* Three new species* Two unassigned isolates	Grass silage	60 min 100°C	de Silva *et al.* (1998)
Bacillus licheniformis *Bacillus subtilis* *Bacillus oleronius/ sporothermodurans* *Paenibacillus* *Brevibacillus* *Aneurinibacillus*	Grass & maize silage	120–130°C	te Giffel *et al.* (2002)
Bacillus cereus *Bacillus subtilis* group *Bacillus sporothermodurans* *Bacillus amyloliquefaciens* *Bacillus oleronius* *Bacillus pallidus*	Grass silage Feed concentrate	30 s 125°C 30 min 100°C	Vaerewijck *et al.* (2001)

*One species was subsequently described as *Bacillus siralis* (Pettersson *et al.* 2000).

age, poisoning or contamination of heat-treated milk upon germination and growth in the final product with long shelf-life. The most comprehensive study of potentially highly heat-resistant spore formers was performed recently by Scheldeman *et al.* (2005) investigating raw milk, milking equipment after the heat-cleaning procedure (teat cups, clusters, connection points, filter cloth, collection tank), green crop (silage, maize, hay/straw) and fodder (feed concentrate, pulp, cereals) in the winter period at 17 dairy farms in geographically different locations in Belgium. The notation 'potentially highly heat-resistant spore formers' was used because the selective heat treatment of the samples (30 min at 100°C) could not only select for spores with a high intrinsic heat resistance but also for spores with a lower heat resistance which were very abundant in the sample. Depending on the incubation temperature, high average counts of potentially highly heat-resistant spore formers were found in filter cloths (10^2–10^3 CFU g^{-1} at 37 and 55°C), green maize (10^2–10^3 CFU g^{-1} at 37°C), hay/straw (>10^3 CFU g^{-1} at 37°C) and feed concentrate samples (10^2–10^3 CFU g^{-1} at 20, 37 and 55°C). It is noteworthy that 10–20% of the concentrate and self-made mixtures contained >10^2 CFU g^{-1} of psychrotolerant spore formers, which are of main interest in the cold milk chain. After a polyphasic taxonomic characterization of the potentially highly heat-resistant spore-forming isolates, a very large taxonomic diversity was found covering as much as seven genera (*Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, *Paenibacillus*, *Ureibacillus* and *Virgibacillus*). In Fig. 2 a neighbour-joining tree is shown to reflect the phylogenetic diversity of these spore formers. Eighteen previously unknown taxa were found, covering 23% of all isolates, of which five have been described as a result of this study: *Bacillus farraginis*, *Bacillus fortis* and *Bacillus fordii* (Scheldeman *et al.* 2004b), *P. lactis* (Scheldeman *et al.* 2004a) and *Bacillus ruris* (Heyndrickx *et al.* 2005). Overall, *B. licheniformis* and the thermophile *Geobacillus* (formerly *Bacillus*) *pallidus* were the most frequently isolated species. Besides these two species, also *B. farraginis* and members of the *Bacillus subtilis* group were the most widely spread species across the sampled farms. In raw milk, 20 different species were found, of which *B. licheniformis* far outnumbered the other species. All investigated samples, and especially feed concentrate, hay and straw, silage, teat cups, clusters and filter cloth, were identified as possible entry points for potentially highly heat-resistant spores into raw milk. Sixty per cent of the different species found in raw milk were also recovered from feed concentrate, but some species such as *P. lactis* and *Ureibacillus thermosphaericus*, were only recovered from the milking equipment. Nevertheless, for five species, other entry points must exist as they were only found in raw milk in the course of this study.

Heat resistance

Heat resistance of spore formers

By subjecting raw materials to drastic heat treatments, even extremely heat-resistant *B. sporothermodurans* spores would be rendered inactive. Unfortunately, severe heat treatments are not well tolerated by milk because of negative organoleptic and nutritional effects, e.g. a considerable increase in lactulose content exceeding 400 mg kg^{-1}. Therefore, a heat treatment process of milk has to be designed to ensure a safe product with acceptable organoleptic and nutritional properties. To evaluate the safety of commonly applied heat treatments in the dairy industry, it is important to know the heat resistance of spores. The heat resistance of a micro-organism or spores is determined by heat inactivation studies in function of time. The current official methods to calculate sterility of thermally processed foods are based on the assumption that microbial heat inactivation follows a first-order kinetics. Hence, the decimal reduction time or '*D*-value', which is the time needed to reduce the size of the treated population by a factor of 10, can be used as a measure of the organism's or spore's heat resistance at the corresponding temperature. It is also assumed that the temperature dependence of *D* is log linear, which produces the '*z*-value', i.e. the temperature interval at which *D* will decrease (or increase) by a factor of 10. Although there is growing evidence that the isothermal semi-logarithmic survival curves of micro-organisms and spores are more of a nonlinear nature, this discussion is beyond the scope of this review and we continue to use the widely accepted *D* and *z*-concept here. We refer to others for a review on the mathematical properties of nonlinear semi-logarithmic survival curves (Geeraerd *et al.* 2000) as well as for non-isothermal inactivation patterns of *B. sporothermodurans* spores as occurring in industrial thermal processes (Periago *et al.* 2004; Peleg *et al.* 2005).

Classically, the wet heat resistance of spores is determined by heating spores in a medium with the glass capillary tube method in an oil bath or in a pilot UHT installation. While the first method is relatively simple to perform, it is only reliable for temperatures up to 125°C (Huemer *et al.* 1998); the latter method is preferred for measurements in the UHT region (130–150°C), but it requires large volumes of milk spiked with spores. Recently, a rapid optical assay to monitor wet heat resistance of spores based on the measurement of the release of dipicolinic acid as a function of heating time and temperature was applied on *B. sporothermodurans* spores (Kort *et al.* 2005).

The heat resistance of spores is influenced by many factors, before, during and after the heat treatment such as

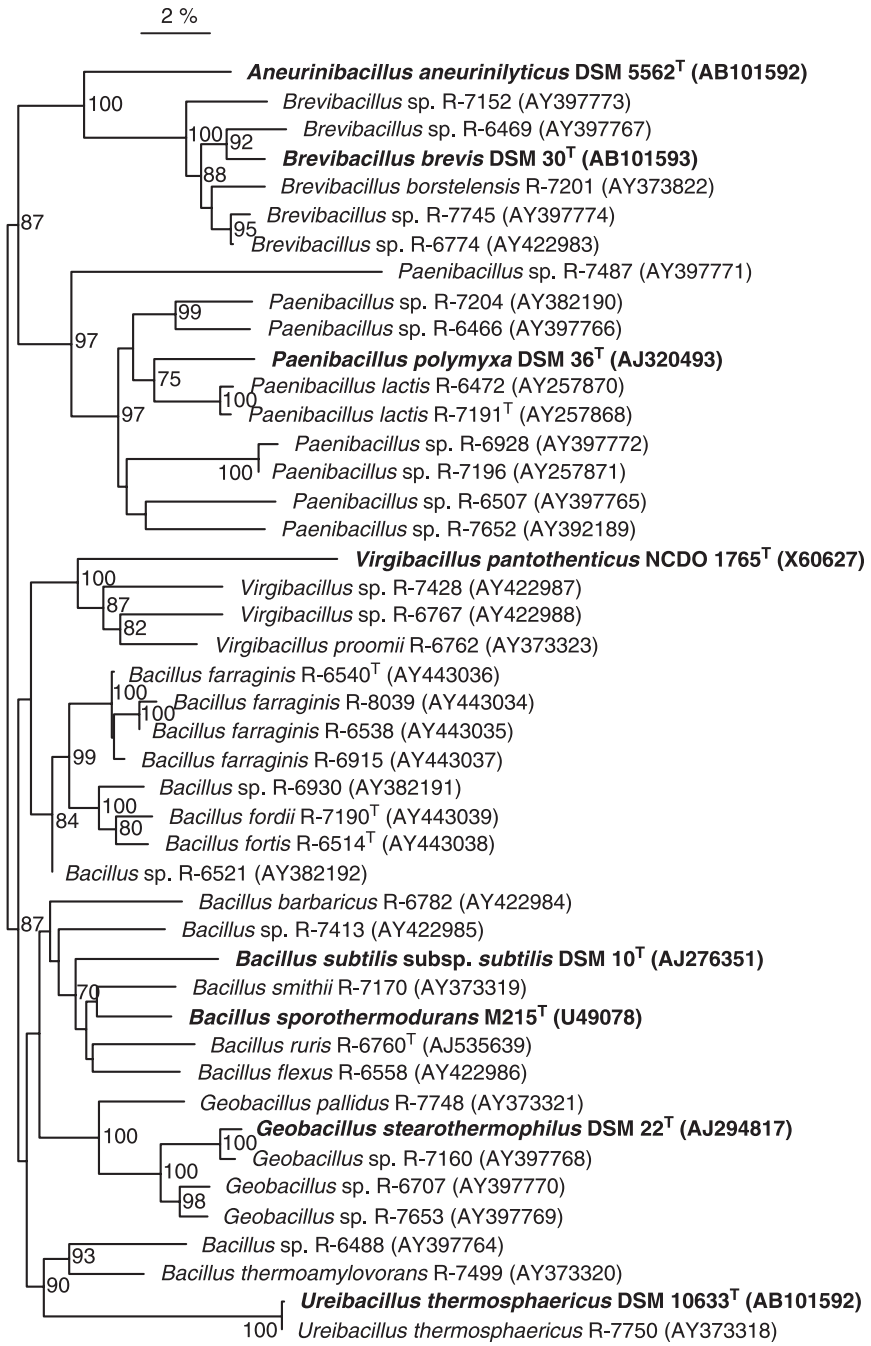

Figure 2 Neighbour-joining tree from nearly complete 16S rDNA sequences, showing the phylogenetic positions of the potentially highly heat-resistant dairy farm isolates and a few type species of *Bacillus* and related genera (in bold face). GenBank accession numbers are given in parentheses and bootstrap values (expressed as percentages of 1000 replications) are shown at branch points when higher than 70%. R, Research collection Laboratory of Microbiology (Ghent University). Unknown bases were not considered for the analysis.

sporulation conditions (sporulation temperature and medium), the physiological state of the organism (e.g. heat-induced resistance after sublethal heat treatment of spores or sporulating cells), composition of the heating medium (e.g. pH) and recovery conditions for enumeration of heated bacterial spores. This means that heat resistance data can only be interpreted within the same study or between studies with comparable methodology. Published data on the heat resistance of *B. sporothermodurans* spores are very scarce, probably due to the diffi-

culty in obtaining a sufficient quantity of *B. sporothermodurans* spores for the heat resistance determination. An overview of heat resistance determinations is given in Table 3. Hammer *et al.* (1995) showed the wide variation in *D*- and *z*-values obtained by different laboratories and the difficulty to draw clear conclusions based on these results. In a broader study, Huemer *et al.* (1998) reported the extreme heat resistance of *B. sporothermodurans* spores from UHT milk isolates with $D_{140°C}$-values varying between 3·4 and 7·9 s , determined in spiked milk. Compared with *G. stearothermophilus* spores, this means that *B. sporothermodurans* spores are equally or even less heat resistant as *G. stearothermophilus* at sterilization temperature (121°C), but are exceptionally heat resistant at ultra high temperatures. This author also reported that the heat resistance of *B. sporothermodurans* spores from the original stock culture were twice as high as for spores from a culture that underwent 10 culture passages, indicating a loss of natural heat resistance under laboratory cultivation conditions. Scheldeman (2004) compared the heat resistance of *B. sporothermodurans* spores from different origins (heat-treated milk products *vs* dairy farm strains) and a different history (industrial *vs* laboratory grown spores) as well as of spores of potentially highly heat-resistant spore formers isolated in the dairy farm study. Species other than *B. sporothermodurans* could display other general features (toxigenic, pathogenic, proteolytic or lipolytic activity, antibiotic resistances, etc.), each with their own implications for the food industry. Heat resistance determinations with spiked or industrially contaminated milk in a UHT pilot installation and with spore suspensions in milk or Ringer solution heated at 100°C were used; the latter method is a simple test system allowing a quicker detection of different levels of heat resistance with possible relevance for UHT resistance. From these heat resistance determinations at 100°C, large differences were observed between the *B. sporothermodurans* strains investigated (Table 3). Surprisingly, the highest $D_{100°C}$-value was obtained for feed concentrate and soy isolates. The lowest $D_{100°C}$-value was obtained for the raw milk isolate. The UHT milk isolate, cultivated in the lab, showed either a comparable or a lower heat resistance at 100°C compared with some feed strains, depending on the batch of spores used (within a same batch *D*-values were reproducible). Also spores present in industrially contaminated semi-skimmed UHT milk were tested directly for their heat resistance at 100°C without prior isolation and cultivation in the lab: a very high $D_{100°C}$-value of approx. 800 min was observed, exceeding the heat resistance of all other spores tested. Upon isolation and cultivation of these 'industrial' spores, it was seen that the resulting isolate displayed a positive HRS-PCR and a HRS clone pattern in REP-PCR, but a much lower $D_{100°C}$-value of 165 or 262 min (depending on the culture medium), which is comparable with the heat resistance of the other UHT milk isolate under lab cultivation conditions. These findings corroborate a previous observation that laboratory cultivation causes gradual loss of heat resistance of the spores together with a decreased capacity of the strain to grow in milk (Huemer *et al.* 1998) as well as a recent observation that the relatively high heat resistance of spores is not always maintained after germination and culturing under nutrient-rich conditions (Kort *et al.* 2005).

For the determination of the heat resistance at 100°C for spores of potentially highly heat-resistant isolates from the dairy farm screening, predominantly raw milk isolates were chosen (Table 3). The $D_{100°C}$-values varied from 14·1 to 111·2 min for respectively *P. lactis* MB 1871^T and *Virgibacillus proomii* MB 1865. Also, the heat resistance of the raw milk isolates belonging to the same species varied strongly (Table 3). Most of the spores of raw milk isolates had comparable heat resistances with $D_{100°C}$-values between 14 and 32 min, which are significantly higher than the spores of a *B. cereus* isolate from mastitis milk. For a *B. licheniformis* and a *V. proomii* raw milk isolate, even higher $D_{100°C}$-values of approx. 100 min were obtained. As this is in the same range as observed for lab-cultivated UHT milk isolates of *B. sporothermodurans* and of *P. lactis*, this may indicate that spores of these raw milk strains have the potential to survive higher heat treatments. Further UHT experiments are necessary to prove this.

For the determination of UHT resistance pilot scale experiments were performed with *B. sporothermodurans* spores spiked in raw milk or directly in contaminated UHT milk, so called 'industrial' spores (Scheldeman 2004; Table 3). From Fig. 3, it can be seen that at the lower temperatures activation of the spores is observed, implying that a heat shock of 10 min 80°C or 30 min 100°C is not sufficient to obtain complete germination and thus counting of the initial spore population. The 'industrial' spores from contaminated UHT milk can clearly be distinguished by their extreme heat resistance in the UHT temperature range (135–140°C). The obtained $D_{140°C}$-value of 4·7 s for these 'industrial' spores corresponds well with the $D_{140°C}$-values determined by Huemer *et al.* (1998). These *D*-values can be considered reference values for UHT-resistant spores. The feed concentrate strains showed a similar activation at 120–125°C as the 'industrial' spores, but were killed quickly at UHT temperatures. The gradation in *D*-values at ultra high temperatures for the tested spores corresponded well with the ones of the $D_{100°C}$-values, indicating that $D_{100°C}$-values can be a good indicator of potential UHT resistance (Table 3). It is noteworthy that strains belonging to the same, genetically homogeneous HRS

Table 3 Overview of the *D*-values for spores of *Bacillus sporothermodurans* strains of other highly heat-resistant species and of some reference species

Species	Source and/or strain*	*D*-value (min) 100°C†	*D*-value (s)‡ 120–121°C	125°C	130°C	135°C	140°C	*z*-value (°C)	Reference
B. sporothermodurans	UHT milk		135 (79)§		42·2 (23·9)	14·3 (6·7)	5·0 (1·6)	13·1 (14·2)	Huemer *et al.* (1998)
	'Industrial spores' in naturally contaminated UHT milk	±800		45·8	28·9	7·5	4·7	14	Scheldeman (2004)
	MB 372 from UHT milk	160/85		13					Scheldeman (2004)
	MB 1188 from UHT-based vanilla drink	77		5					Scheldeman (2004)
	MB 1316 from feed concentrate	468			7·2	1·3			Scheldeman (2004)
	MB 1317 from feed concentrate	132			2·3				Scheldeman (2004)
	MB 385 from raw milk	23/14		2					Scheldeman (2004)
	MB 1501 from feed concentrate	476							Scheldeman (2004)
	MB 1503 from soy	570							Scheldeman (2004)
	MB 1504 from pulp	194							Scheldeman (2004)
	MB 1505 from silage	126							Scheldeman (2004)
G. stearothermophilus	NIZO B469		191				0·9	9·1	Huemer *et al.* (1998)
B. cereus	MB 1632 from mastitis milk	6/8							Scheldeman (2004)
B. oleronius	Raw milk		2				<0·8		Klijn *et al.* (1997)
	MB 397 from raw milk	18							Scheldeman (2004)
B. licheniformis	MB 1882 from raw milk	20							Scheldeman (2004)
	MB 1880 from raw milk	103							Scheldeman (2004)
B. fordii	MB 1878^T from raw milk	22							Scheldeman (2004)
B. ruris sp. nov	MB 1873 from raw milk	27							Scheldeman (2004)
Paenibacillus lactis	MB 1871^T from raw milk	14							Scheldeman (2004)
	MB 2035 from raw milk	64							Scheldeman (2004)
	MB 1868 from milking apparatus	80							Scheldeman (2004)
	MB 1928 from UHT milk	103							Scheldeman (2004)
Paenibacillus sp. nov.	MB 1870 from raw milk	16·5							Scheldeman (2004)
Brevibacillus sp.	Raw milk		5				<0·8		Klijn *et al.* (1997)
	R-6469 from raw milk	14							Scheldeman (2004)
Virgibacillus proomii	MB 1866 from raw milk	33							Scheldeman (2004)
	MB 1865 from raw milk	111							Scheldeman (2004)
Virgibacillus sp. nov	MB 1864 from raw milk	27							Scheldeman (2004)

*MB, culture collection of the Department of Animal Product Quality, Melle (Belgium); R, Research Collection of the Laboratory of Microbiology, Ghent University (Belgium).

†Spores were obtained on agar slants with sporulation medium (25 g l^{-1} nutrient broth, 7 mg l^{-1} $MnSO_4{\cdot}H_2O$, 1 g l^{-1} $CaCl_2{\cdot}2H_2O$, pH 6·8) incubated at 37°C until sufficient sporulation (monitored by phase contrast microscopy). The heat resistance of the harvested endospores at 100°C was tested in Ringer solution by heating in a boiling water bath in function of time. At each time point, a heated Eppendorf tube with spore suspension was placed on ice and a decimal dilution series in Ringer solution plated in duplicate on brain heart infusion (BHI) agar supplemented with 1 mg l^{-1} vitamin B_{12}. After incubation at 37°C for 48 h, colony-forming units were counted at each time point and used for calculation of the *D*-value. *D*-values are only given when (i) they were reproducible in repeated experiments for the same batch of spores and (ii) survivor curves had a correlation coefficient (R^2) higher than 0·90. If two values are given, these refer to a different spore batch. 'Industrial' spores were heated directly in the contaminated UHT milk for heat resistance determination.

‡Heat resistance experiments at temperatures above 121°C were carried out in a UHT pilot installation (APV Junior, Silkeborg, Denmark) in direct operation mode with 20 l raw milk spiked with spores obtained as described above. The milk was prewarmed to 65°C with a plate heat exchanger, heated to the desired temperature by steam injection for 5 s and then flash-cooled to 65°C, homogenized and cooled to room temperature for aseptic filling in sterilized glass bottles. One ml of milk and decimal dilutions in Ringer were plated on large diameter (14 cm) BHI plates with a vitamin B_{12} supplement; surviving colonies were enumerated after 48 h incubation at 37°C and used for calculation of the *D*-value at the respective heating temperature using the initial spore number determined after a heating for 5 s at 120°C (see also Fig. 3).

§Values in parentheses were determined after 10 culture passages of the original stock culture.

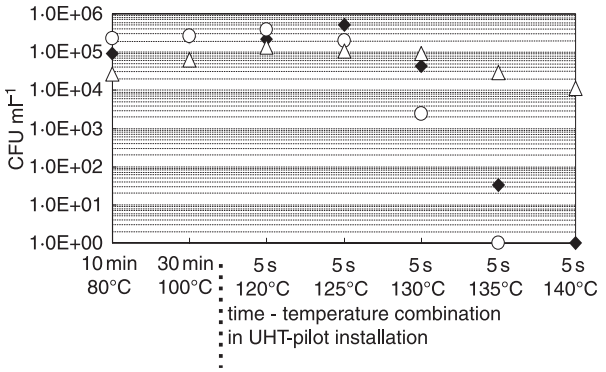

Figure 3 Survival of *Bacillus sporothermodurans* spores from different origins in a pilot ultra-high temperature (UHT)-installation (direct mode). ◆, Feed concentrate isolate MB 1316; ○ Feed concentrate isolate MB 1317; △ industrial spores.

clone display such different spore heat resistance characteristics. The observed gradual decrease in heat resistance combined with the fact that the few *B. sporothermodurans* strains at the dairy farm level are not extremely heat resistant, suggests that certain parameters or conditions may induce higher heat resistance.

Induction of higher heat resistance

The observations on heat resistance of highly heat-resistant spores described above suggest that certain environmental- and/or sublethal stress conditions may affect the heat resistance of *B. sporothermodurans* spores. It has indeed been observed previously that spores or sporulating cells imposed to sublethal stress conditions or treatments such as sublethal heat treatment of spores (e.g. Movahedi and Waites 2000), acid shock (Lee *et al.* 2003) and ethanol, puromycin or cold shock treatment (Movahedi and Waites 2002) during the sporulation process of *B. subtilis* induce an increased heat resistance of the resulting spores. In aseptic packaging systems used in the dairy industry, packaging materials are sterilized by various methods in order to kill micro-organisms contained in the packaging during forming and transport through the machine prior to filling. Hydrogen peroxide, with concentrations up to 30%, temperatures of up to 80°C and contact times up to 15 s, has been found to be a successful sterilization method for inactivation of micro-organisms during inline aseptic filling (Ansari and Datta 2003). Scheldeman (2004) compared the heat resistance of spores of a *B. sporothermodurans* UHT strain before and after a sublethal H_2O_2 treatment. The heat resistance was determined at 100°C on the spores generated by incubation at 37°C immediately after the H_2O_2 treatment of the initial spore suspension. The results are summarized in Table 4. After a single treatment with H_2O_2 during 30 min at room temperature, followed by resporulation on supplemented BHI or sporulation medium (SP), a significant increase of the heat resistance at 100°C was observed. This was also observed at 121°C when autoclaving H_2O_2-treated spores for 4 min (data not shown). This heat resistance induction effect was dependent on the H_2O_2 concentration applied and was highest after sporulation on sporulation medium. The application of successive H_2O_2 treatments (with each time a sporulation phase in between treatments) led to the following observations: (i) after a second H_2O_2 treatment approximately the same heat resistance was found as after a single H_2O_2 treatment, indicating no cumulative effect and (ii) the heat resistance induced by a first H_2O_2 treatment,

Table 4 Heat resistance of spores of *Bacillus sporothermodurans* UHT milk isolate MB 372 after different sublethal H_2O_2 treatments for 30 min at room temperature (Scheldeman 2004)

	$D_{100°C}$ (min)	
Treatment*	BHI†	SP†
Untreated	–	91
Single		
10% H_2O_2	175	217
5% H_2O_2	138	–
Successive		
1st 10% H_2O_2	175	–
2nd 10% H_2O_2	162	–
1st 10% H_2O_2	175	–
2nd 150 min 100°C	93	–
3rd 10% H_2O_2	177	–
Combined		
150 min 100°C/2% H_2O_2	105	–
150 min 100°C/5% H_2O_2	154	–

*Treatment of spores with H_2O_2 was essentially as described by Sagripanti and Bonifacino (1996). In short, vegetative cells were killed by heating for 10 min at 80°C and the resulting spore suspension divided in 50-μl aliquots. In single treatments, 50 μl H_2O_2 double-concentrated solution was added to 50 μl spore suspension to give the indicated final H_2O_2 concentrations. Control experiment consisted of adding 50 μl water instead. After 30 min exposure at room temperature (giving at least 3 log reduction of the initial spore population), 900 μl ice cold BHI broth was added and the resulting volume was spread entirely on large diameter BHI or SP agar plates. Plates were incubated at 37°C until surviving colonies showed a sufficient sporulation degree (monitored by phase contrast microscopy). These resulting spores were harvested and suspended in Ringer solution for the subsequent heat inactivation study, as described in the second footnote to Table 1. In successive treatments, a second H_2O_2 treatment was executed on the spores obtained after the first treatment as described above. In combined treatments, the same spore suspension was first heat treated and then H_2O_2 treated, as indicated.

†Medium on which spores were obtained after H_2O_2 treatment and incubation at 37°C: BHI, brain heart infusion agar supplemented with vitamin B_{12}; SP, sporulation agar (see second footnote to Table 1).

dropped to approximately initial values after an interim heat treatment, and increased again following a second H_2O_2 treatment. When sublethal heat and peroxide treatments were combined (successively on the same spore suspension), no additional induction effect was observed. The peroxide-induced heat resistance effect also had only a temporary character as spores obtained from a stress-free sporulation after a sublethal H_2O_2 treatment returned to the initial heat resistance (data not shown). It thus seemed that the sporulation characteristics and the heat resistance of spores of a *B. sporothermodurans* UHT strain are greatly affected by a sublethal hydrogen peroxide treatment in a H_2O_2 concentration (and to a lesser extent also temperature)-dependent manner. The heat resistance observed with 'industrial' spores at 100°C was, however, never achieved by the tested H_2O_2 sublethal treatments or by combination with a sublethal heat treatment. To evaluate the possible universality of the phenomenon of increased heat resistance, the heat resistance before and after a sublethal H_2O_2 treatment was also determined for some other *B. sporothermodurans* strains and spore-forming species (Scheldeman 2004). Remarkably, no heat resistance induction effect was observed for *B. sporothermodurans* strains from other sources than UHT milk, nor for a *P. lactis* strain from UHT milk, but a very slight induction effect was observed for a *B. cereus* strain (data not shown). It can be concluded that hydrogen peroxide can induce heat resistance induction effects for some spores, but other unknown stress conditions and/or factors (environmental conditions) and the genetically determined physico-chemical spore properties probably act together for inducing some spores to the extreme UHT heat resistance observed in practice.

Spore structure

Scheldeman (2004) determined the mineral composition of *B. sporothermodurans* spores of strains of different origin and for spores of other species (*B. cereus*, *P. lactis*) sporulated on sporulation medium. There appeared to be a correlation between heat resistance and the amount of calcium. The $D_{100°C}$-value varied between 5·21 min for a *B. cereus* strain and 400 min for a feed concentrate isolate of *B. sporothermodurans*, while the amount of calcium in the spores of these strains was 22·24 and 189·44 μg mg^{-1} spores respectively. Spores with intermediate heat resistances also had intermediate calcium levels (77–117 μg mg^{-1} spores). A correlation between mineralization and wet heat resistance of spores has been shown previously (Nicholson *et al.* 2000). It is speculated that mineralization as well as other factors exert their effect indirectly through modulation of the spore core water content. The latter is clearly a major factor determining the spore wet heat resistance, as an inverse correlation has been observed over a wide range of core water contents in spores of different species between core water content and heat resistance (Beaman and Gerhardt 1986). It is thought that reduced water content decreases the amount of water associated with spore proteins, thereby stabilizing these to thermal denaturation.

Electron microscopy revealed structural differences between spores of *B. sporothermodurans* isolates from different origin and of other species (Fig. 4) (Scheldeman 2004). In spores of the UHT strains either belonging to *B. sporothermodurans* or *P. lactis*, the core was very compact and the surrounding cortex comparatively large. In the spores of the *B. sporothermodurans* raw milk isolate and of *B. cereus*, the core was proportionally larger (less compact) in relation to the cortex size. The spores of the *B. sporothermodurans* feed concentrate isolate showed intermediate properties. A compact core could be attributed to a more complete dehydration, an essential factor in defining the heat resistance of bacterial spores (e.g. Nicholson *et al.* 2000). In a recent study (Novak *et al.* 2003), transmission electron microscopic shots of several *Clostridium perfringens* spores indeed revealed a negative correlation of the average core size with *D*-values obtained at 100°C. Another observation of a less electron dense, lighter coloured cortex for the UHT isolates, also seems to be correlated with higher levels of heat resistance (S. Foster, pers. comm.).

Conclusions

Highly heat-resistant spores have appeared as a problem in the dairy industry only relatively recently. It can be assumed that these spores were and still are initially introduced as the cause of important changes in dairy farming with the change from land-own crops to the extensive use of new feeds and feed ingredients such as concentrate. This feed component may contain ingredients such as manioc, coconut meal and citrus pulp, which probably harbour new and unknown spore-forming species. Fortunately, the load of these highly heat-resistant spores in raw milk is low and the occurrence of some of them such as *B. sporothermodurans* is highly infrequent in the dairy farm environment. Therefore, the carry-over from raw milk to the dairy plant is very limited. Several findings suggest that it is not very likely that the extreme heat resistance at ultra high temperatures is a natural property. On the contrary, they support the hypothesis that highly heat-resistant spores were adapted by sublethal stress conditions in the industrial process and selected for by the heating step. As a result, considerable problems may occur through recirculation in the international dairy industry environment, leading to contaminated lots of

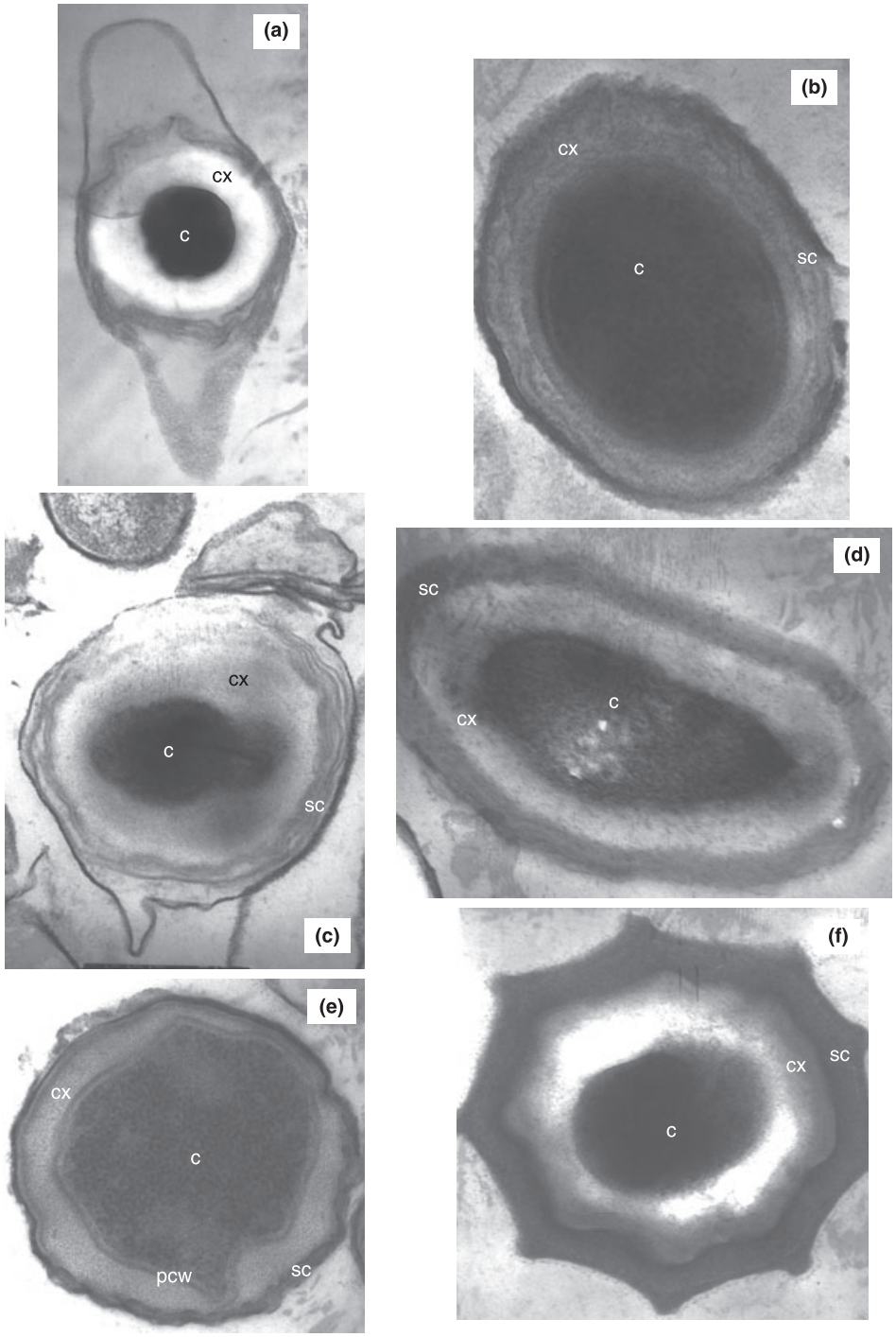

Figure 4 Transmission electron microscopic images of spores obtained on sporulation medium. (a) *Bacillus sporothermodurans* German ultra-high temperature (UHT)-isolate (MB 372), magnification 78 000×, (b) *B. sporothermodurans* raw milk isolate (MB 385), 21 0000×, (c) *B. sporothermodurans* UHT milk isolate (MB 1313, HRS-clone), 104 000×, (d) *B. sporothermodurans* feed concentrate isolate (MB 1317), 146 000×, (e) *B. cereus* mastitis milk isolate (MB 1632), 146 000×, (f) *Paenibacillus lactis* UHT milk isolate (MB 1928), 210 000×. c, spore core; cx, spore cortex; sc, spore coat; pcw, primordial cell wall.

milk and milk products. There are also indications that this adaptation is restricted to some species and maybe even some clones within species (e.g. HRS clone).

Further research on the influence and the nature of stress conditions on the heat resistance of spores and on the molecular mechanisms behind them is of great

importance to develop preventive measures and to accommodate industrial heat treatments.

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