

Incidence and survival of *Bacillus sporothermodurans* during processing of UHT milk

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Abstract

The presence of *Bacillus sporothermodurans* in retail UHT milk along with milk from different points of a processing line was determined. The effect of chilling, pre-heating, UHT, reprocessing and H₂O₂ individually and in combination on the survival of *B. sporothermodurans* was also investigated in broth.

Design/methodology/approach - Standard plate counts were conducted for all milk samples and isolates from UHT milk were characterised using PCR. BS vegetative cells and spores in broth were subjected to various stresses encountered during UHT processing of milk. Survival counts were conducted after all treatments.

Findings - *B. sporothermodurans* was detected in retail UHT milk packs from only one processor. UHT treatment at 140 °C for 4s eliminated *B. sporothermodurans* in broth. The combination of chilling and UHT was more effective in eliminating *B.*

sporotheumodurans spores than UHT treatment alone. H₂O₂ was also effective in eliminating *B. sporotheumodurans* spores after 15 min of exposure. The adopted real time (RT) PCR with SYBR Green method was effective for the confirmation of *B. sporotheumodurans*.

Originality/Value - This research is the first to be conducted with regards to the detection of *B. sporotheumodurans* in UHT milk in South Africa and determining the effect of UHT processing stresses on their survival. These results can be used to design processing parameters so as to effectively eliminate *B. sporotheumodurans* spores during UHT processing. This research is the first in which RT PCR with SYBR Green has been used to characterise *B. sporotheumodurans*.

Key words: UHT, milk, processing, *Bacillus sporotheumodurans*, spores.

Paper type: Research paper

1. Introduction

UHT milk processing plants in South Africa sporadically experience contamination with *Bacillus sporotheumodurans*. Often, this contamination is believed to be due to the reprocessing of UHT milk; either UHT milk from packages used for quality control or UHT milk from defective packaging or inadequately processed UHT milk due to other problems that may occur during processing. It should be noted that, the main reason for reprocessing is to avoid economic losses.

B. sporotheumodurans, first detected in UHT milk in Germany in 1990, affects the stability and the shelf life of commercial UHT milk (Hammer *et al.*, 1995). This is due to the unusual thermal kinetics of the spores which allows survival at high temperatures, up to 130 °C for 4s during UHT treatment (Klijn *et al.*, 1997). These spores germinate in UHT products during storage, causing instability and reducing

shelf life. *B. sporothermodurans* spores have been found to be more resistant than other heat resistant spores of other *Bacillus* spp. at temperatures above 130 °C (Klijn *et al.*, 1997).

Based on the different identification and detection methods, *B. sporothermodurans* can be classified into heat resistant spore (HRS) formers or non heat resistant spore (non-HRS) formers. So far, the HRS group of *B. sporothermodurans* has been isolated exclusively from heat sterilized or UHT processed milk, making it difficult to predict their origin (Scheldeman *et al.*, 2002). Increasing the temperature and or the holding time in an attempt to inactivate *B. sporothermodurans* spores affects the organoleptic properties as well as nutritional quality of UHT milk (Van Boekel, 1998). The denaturation of whey protein and the accumulation of advanced products of Maillard reaction are indicators of the severity of heat treatments during UHT processing of milk (Birlouez-Aragon *et al.*, 2002). Protein denaturation and sugar modifications due to heat treatment makes UHT milk to have a “cooked” taste and Maillard reaction decreases the protein nutritional value by irreversible alteration of the lysine residue (Claeys *et al.*, 2001). An increase in the temperature of sporulation has been linked to an increase in the heat resistance of the resulting spores (Teofila *et al.*, 1998). Currently, it is still not clearly understood how the structural and chemical properties of BS spores influence their heat resistance (Klijn *et al.*, 1997).

Stress response adaptation of bacteria is of interest to the dairy industry because it influences the survival of *Bacillus* spp. during processing. After being exposed to certain stresses, *Bacillus* spp. may overcome subsequent sub lethal stresses to survive

in food systems (Abee and Wouters, 1999). Furthermore, a response to one stress can lead to induced resistant response to other stresses (Abee and Wouters, 1999).

Heat shock has been found to cause complete and partial deactivation of spores, giving rise to the disruption and relaxation of the outer membrane. These spores also release some of their dipicolinic acid (DPA) and minerals, increasing their ability to germinate as well as the induction of heat resistance (Teofila *et al.*, 1998).

H₂O₂ is used to sterilise packaging material used during UHT processing of milk. H₂O₂ is a useful chemical sterilizing agent and it does not impart unpleasant odour to the packaged product and does not leave residues on packaging material (Toledo *et al.*, 1973). Bacterial spores are more resistant than vegetative cells to H₂O₂ and the inactivation of bacteria spores by H₂O₂ depends on various factors including: the nature of the spores, a wet or dry environment, the concentration of H₂O₂ and the species of the spore former present (Toledo *et al.*, 1973).

Experiments conducted to determine the mechanism of action of H₂O₂ in the killing of spores have not been conclusive (Melly *et al.*, 2002). In one of such experiments, *B. subtilis* spores killed by H₂O₂ maintained their permeability barrier which prevented the core contents, in particular DPA from leaking out but there was no indication as to whether the heat resistance of spores was affected (Melly *et al.*, 2002). Until now, no study has been conducted on the presence and the characterization of *B. sporothermodurans* in the South African dairy environment. Furthermore, understanding how vegetative cells and spores of *B. sporothermodurans* are influenced by processing such as pre-heating, chilling, reprocessing and H₂O₂ exposure is of importance as it determines the stability of UHT milk product during storage. Therefore, the aim of this study was to determine the incidence of *B.*

sporothermodurans in retail milk in South Africa and the effect of UHT processing stresses on the survival of isolated strains.

2. Materials and methods

2.1 Samples of UHT milk used in the study

2.1.1 Retail sample

Two packages of UHT milk from 6 processors (A to F) were bought from local supermarkets.

2.1.2 In process sample

Milk samples were collected at different processing steps from processor D, they include:

- Fresh raw milk that had just been received from the farms.
- 24h raw fresh milk that had been in the storage tank for 24h at 4 °C.
- 2 % fat pasteurised milk.
- Pasteurised skim milk.
- Pasteurised cream
- UHT milk from defective packages removed from the processing line, kept for reprocessing, to be mixed with fresh milk to a 10 % v/v final concentration and subjected to another round of UHT treatment.
- UHT milk directly after processing
- Wash water that had been used to rinse the filter or the sterilizers

2.2 Microbiological count

2.2.1 Standard plate count (SPC)

SPC were carried out by plating serial dilutions (1:10) of the milk samples. UHT milk samples were plated on BHI agar plates while the pasteurised and raw milk samples were plated on nutrient agar. Triplicate analysis were made for each milk sample by counting duplicate plates containing 30 to 100 colony forming units (CFU) , after incubation for 24h at 37 °C. In order to determine absolute sterility SPC were also conducted for UHT milk that has been incubated for 15d at 30 °C.

2.2.2 Spore counts

Spore counts were conducted by plating serial dilutions of milk or broth samples that have been heat treated at 95 °C for 30 min. Spore counts were conducted by counting colony forming units on BHI agar plates after incubation for up to 15d at 37 °C. Triplicate analysis were made for each heat treated sample by counting duplicate plates containing colony forming units after incubation for 24h at 37 °C.

2.3. Molecular identification of isolates

2.3.1 Preparation of DNA for PCR

The isolation of genomic DNA for PCR with agarose gel detection was conducted as described by Scheldeman *et al.* (2002), with some modifications. DNA templates were extracted from 3-day-old pure cultures from BHI agar plates, which have been washed three times by centrifugation at 13 000 x g for 3 min followed by re-suspension in distil water. 100 µl solution of 0.1 mol NaOH and 0.25 % sodium lauryl sulphate (Merck, South Africa) was added to the washed pellets and was heated for 17 min at 90 °C. The boiled samples were again centrifuged at 13 000 x g for 3 min and crude DNA was collected from the supernatants.

2.3.2 PCR Procedure

The PCR reactions were conducted using *B. sporothermodurans* specific primers, BSPO-F2 (5'-ACG GCT CAA CCG AG-3') and BSPO-R2 (5'-GTA ACC TCG CGG TCT A-3') as described by Schelderman *et al.* (2002). Primers prepared by Iqaba biotechnical, Pretoria, South Africa were used to amplify fragments of about 664 base pairs from the 16S rDNA gene. For the PCR detection with agarose gel electrophoresis, 100 ng of genomic DNA was used as template for the PCR reaction in a total reaction volume of 25 µl, using 1.25 U Taq DNA polymerase, 200 nM of each primer, 50 mM KCl, 10mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ and 0.2 mM of NTP mix (Fermentas, South Africa). The mixture was subjected to 30 cycles of amplification in a thermal cyclar (iCycler Thermal Cyclar, BIO-RAD, South Africa). The first cycle was preceded by an initial denaturation step of 2 min at 94 °C. Subsequently, each cycle consisted of a denaturation step of 20s at 94 °C, an annealing step of 45s at 45 °C and an elongation step of 60s at 72 °C. The last cycle went along a final extension of 7 min at 72 °C. 10 µl of PCR products were analyzed on a 1% agarose gel containing 0.5 µg/ml ethidium.

2.3.3 Preparation of DNA for RT PCR

DNA for Real Time PCR was extracted from 3 day old pure cultures on BHI agar plates. About 5 or 6 colonies were dissolved in 300 µl of double distilled water and pure DNA was extracted and purified using the ZR Fungal/Bactria DNA KitTM (Zymo Research Corporation, USA), following the manufacturer's protocol. The concentration of extracted DNA was analyzed using the DNA Nanodrop Spectrophotometer and samples were stored at -20 °C until they were used as PCR templates.

2.3.4 RT PCR procedure

For the Real Time PCR, 1 ng of pure genomic DNA was mixed with iQTM SYBR[®] Green Supermix (Bio-rad, South Africa), primers and sterile distilled water to a total of 25 µl reaction volume and the reaction was conducted using the DNA Engine[®] Peltier Thermal Cycler, incorporated with a Chromo 4 Real-Time PCR detector (Bio-rad, South Africa). Primer concentrations and reaction conditions were the same as that of the PCR detection with agarose gel electrophoresis.

2.4 Treatment regimes used on *B. sporothermodurans* isolates

2.4.1 Preparation of raw and pasteurised milk isolates

After plating and incubation of raw and pasteurized samples, about five individual colonies that displayed the morphology of a *Bacillus* colony were harvested and transferred into different eppendorf tubes containing BHI broth for the establishment of pure culture.

2.4.2 Preparation of UHT milk isolates

Similarly, four individual colonies were randomly picked from each BHI plate spread with samples from different UHT milk packs and transferred into separate eppendorf tubes containing BHI broth to establish pure cultures of UHT milk isolates.

2.4.3 Preparation of control strains

A reference strain of *Bacillus sporothermodurans* (DSM No 10599), a control was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany and a local *B. sporothermodurans* isolate from UHT milk in South Africa, were used for determining the effect of UHT processing stresses on their survival. In order to prepare pure cultures, a single colony of each isolate was collected from BHI agar plates and transferred into 1.5 ml eppendorf tubes containing

1 ml of freshly prepared Brain Heart Infusion broth and was incubated for 24h at 37 °C. A ml of each culture was centrifuged at 10 000 x g for 3 min and pellets were subsequently diluted to approximately 0.5 McFarland Standard prepared by adding 0.5 ml of 0.048 M BaCl₂ to 99.5 ml of 0.18 M H₂SO₄ (1% w/v), with physiological saline (8.5 g/L NaCl) as diluents (Andrews, 2005).

2.4.4 Preparation of spore suspension of test isolates

From an overnight pure culture of each strain, 1 ml of culture was collected and spread on BHI agar plates and incubated for 15d at 37 °C. Growths on each plate were checked for sporulation using a light microscope, until *ca.* 100% sporulation was attained. Spores were harvested by scraping the dense growth on the surface of agar plates with a sterile loop and transferred in a 1.5 ml eppendorf tube containing sterile physiological saline. Spores were washed with double distilled water, centrifuged at 10 000 x g for 3 min and pellets were diluted to *ca.* 10⁸ spores per ml with sterile physiological saline using 0.5 McFarland Standard.

2.4.5 UHT treatment process

Heating was conducted with some modifications as described by Huemer *et al.* (1998). UHT treatment was conducted with sterile pressure tubes with threaded type B plug (Sigma Aldrich, Midrand, South Africa) with an outer diameter of 25 mm and wall thickness of 2 mm and an oil bath. The tubes were filled with 3 ml spore suspension and 1 ml of BHI broth. The tubes were closed with stoppers and were submerged in an oil bath for either 120 ± 1 °C, 4s or 140 ± 1 °C, 4s. The temperature equilibrium time was determined with the aid of a thermocouple mounted on a pressure tube.

2.4.6 Simulation of the reprocessing of UHT milk

B. sporothermodurans culture suspension of both isolates, 3 ml, were mixed with 1 ml of BHI broth and subjected to UHT treatment at 120 °C and 140 °C for 4s individually, followed by incubation at 37 °C for 24h. To simulate reprocessing practices in industries, the incubated culture suspension was mixed with fresh milk to a 10 % v/v total and subjected to another round of UHT treatment at 120 °C and 140 °C for 4s.

2.4.7 Pre-heating treatment

Pre-heating treatment was conducted to determine the effect of double heat processes on the survival of *B. sporothermodurans* during UHT processing. *B. sporothermodurans* culture suspensions of both isolates, 3 ml, were mixed with 1 ml of BHI broth and were either subjected to pre-heating (78 °C for 15s) then UHT heating (120 °C for 4s) or pre-heating only or UHT only.

It should be noted that BHI cultures instead of milk cultures were preferred because *B. sporothermodurans* growth in milk culture rarely exceed 3 logs. In order to get a clear response to treatments, higher levels of *B. sporothermodurans* in BHI culture were required.

2.4.8 Chilling treatment

Chilling treatments were conducted to determine the effect of chilling followed by immediate UHT treatment on the survival of *B. sporothermodurans* spores. *B. sporothermodurans* spore suspensions of both strains, 3ml, were mixed with 1ml of BHI broth and were subjected to chilling (24h at 7 °C), UHT heating (120 °C for 4s) or a combination treatment of chilling plus UHT.

2.4.9 Treatment with H₂O₂

H₂O₂ (Sigma Aldrich, South Africa) was mixed with spores in distilled water to form a spore suspensions containing 30% H₂O₂ concentration. The suspensions were incubated for 0, 3, 6, 9 or 15 min at room temperature. After each interval, 1 ml was collected from each of the incubated suspensions and added to 9 ml of catalase solution (0.4 mg per ml distilled water), allowing it to stand for 1 min for the complete removal of active H₂O₂ prior to viable spore count. The effective removal of catalase was tested by dipping a potassium iodide starch paper (MACHEREY-NIGEL GmbH & Co KG, Germany) into the mixture after the one minute duration. A colourless potassium iodide starch paper indicated the complete removal of H₂O₂; while a blue-black colouration indicated the presence of active H₂O₂.

3. Results

3.1 Bacterial quality of UHT milk at retail level

Of all the retail UHT milk samples analysed from processors A - F, UHT milk from only processor D was positive for bacterial growth (Table 1). UHT milk packs from the other processor remained commercially sterile even after they were incubated for 15d at 30 °C (data not shown).

3.2 Bacterial quality of milk from processor D processing line.

Similar to the retail UHT milk, UHT milk obtained immediately after processing from processor D was not commercially sterile (Table 1). The spore counts of UHT milk packs that were incubated for 15d at 30 °C were higher than those that were not incubated for 15d at 30 °C (Table 1). The SPC of all the contaminated UHT milk packs, from the retail level and UHT milk from the processing line of processor D, with or without incubation for 15d, did not differ, values ranged from 2.5 to 3 log

cfu/ml (Table 1). The skim milk, wash water, fresh raw milk and full cream milk had SPC values that ranged from 5 to 7 log cfu/ml. The SPC values for 24h raw milk, 2% fat milk and UHT milk for reprocessing ranged from 7 to 8 log cfu/ml. The pasteurised cream had the worst quality, with SPC levels above 8 log cfu/ml. The bacterial quality of the milk samples decreased in this order: UHT milk < pasteurised skim milk < fresh raw milk < pasteurised full cream milk < 24h raw milk < 2 % fat pasteurised milk < UHT milk for reprocessing < pasteurised cream.

The spore counts of fresh UHT milk after heat treatment at 90 °C for 30 min were at the limit of detection (1 cfu/ml), while those of incubated UHT milk were close to 20 times higher (Table 1).

3.3 identification of UP20A isolates using PCR

From a total of 74 UHT milk isolates obtained from different batches of contaminated UHT milk packages, 29 tested positive PCR with agarose gel electrophoresis. By contrast, 32 isolates out of 33 tested positive using RT PCR coupled with SYBR Green. Some isolates that displayed a negative result using PCR, tested positive when RT PCR was used.

By comparison to positive control samples, all contaminated UHT milk packages from processor D contained *B. sporothermodurans* considering that these isolates produced bands and melting curve similar to that of the control (Figure 1; Figure 2). The melting temperature (T_m) of the amplified segment during the RT PCR fluctuated between 86 and 87 °C. Isolates from fresh and pasteurized milk tested negative as they did not produce the required band for *B. sporothermodurans*

3.4 Effect of UHT only and UHT reprocessing

The counts of *B. sporothermodurans* after heat treatment at 120 °C for 4s were significantly different ($p \leq 0.05$) from those heated at 140 °C for 4s (Table 2). *B. sporothermodurans* was significantly ($p \leq 0.05$) reduced by 4 log cfu/ml, but were not completely eliminated after heat treatment at 120 °C for 4s. After heating at 140 °C for 4s no *B. sporothermodurans* was detected. The two *B. sporothermodurans* strains were not affected differently by the two heat treatments, regarding the survival counts. After reprocessing, the *B. sporothermodurans* levels for the 120 °C heated cultures were similar to those recorded after initial heating at 120 °C and no *B. sporothermodurans* was detected for the 140 °C heated cultures (Table 2).

3.5 Effects of pre-heating (78 °C for 15s), UHT (120 °C for 4s) and a combination of pre-heating and UHT treatments, on the survival of *B. sporothermodurans*

Strain UP20A was affected more by pre-heating than the DMS10599 strain (Table 3). However, after subsequent UHT treatment no growth was detected for either strain. Similarly no growth was detected for both strains when cultures were subjected to UHT treatment (Table 3).

3.6 Effects of UHT treatment with and without chilling on the survival of *B. sporothermodurans* spores following UHT treatment.

There were significant differences at $p \leq 0.5$, in the survival of spores after the two treatments, which were, UHT treatment with and without prior chilling (Table 4). The effect of the chilling on the survival of spores was significant at $p \leq 0.5$. However, the strain effect and the strain-time interaction effects on the survival of *B. sporothermodurans* spores were not significant at $p \leq 0.5$, (Table 4). UHT treatment alone caused a 5 log reduction in the survival count of spores for both strains while

chilling combined with UHT treatment was more effective on reducing the *B. sporothermodurans* counts as both strain were inactivated to undetected levels.

3.7 Effects of 30 % H₂O₂ on the survival of *B. sporothermodurans* spores

The exposure time to H₂O₂ significantly affected the *B. sporothermodurans* spore level for both strains at $p \leq 0.05$ and as the exposure time increased the *B. sporothermodurans* level decreased (Table 5). With each 3 min increase in the exposure time to 30% H₂O₂, the *B. sporothermodurans* level decreased linearly (Figure 3). The reduction in *B. sporothermodurans* spore survivals within the first 15 min was preceded with an initial abrupt decrease for both stains.for the first 3 min. There was a constant decrease and significant decrease in the survival of spores after each exposure time (Table 5) Using the regression equation, the D_{30%} of H₂O₂ was calculated to be 3.33 min (Figure 3).

4. Discussion

4.1 Incidence of *B. sporothermodurans* in South African milk

The SPC values of raw milk from Processor D were higher than the 300 000 (log 5.48) cfu/ml maximum acceptable SPC level for raw milk prior to pasteurisation as stipulated by the European Council Directives 85/397/EEC, 1985. This is a reflection a poor fresh milk handling sanitation and herd health conditions (Muir, 2007). Export of UHT milk with this quality will therefore not be permitted. Ineffective cooling, poor maintenance of milking equipment and poor water quality are often the cause of high bacteria load in fresh milk (Muir, 2007). Similarly, the pasteurized milk packages had SPC higher than 50 000 (log 4.70) cfu/ml which is the maximum SPC level stipulated by the European Council Directives 85/397/EEC, 1985, for pasteurized milk which is similar to the level endorsed by the Dairy Standard Agency

(DSA) in south Africa and this is a reflection of the poor quality of the raw milk that was used.

The results of the PCR reactions indicated that, isolates from contaminated UHT milk packages were *B. sporothermodurans*. Considering that at least one isolate from each of the UHT milk packages of different batches from processor D tested positive for *B. sporothermodurans*. This confirmed the incidence of this highly heat resistant spore producing bacteria in the local dairy environments. Considering that UHT milk packages of other processors remained sterile even after incubation for 15d at 30 °C. This implied that *B. sporothermodurans* contamination is not wide spread or extensive in the South African milk industry.

Although the SPC of the contaminated UHT milk packages were higher than the 10 cfu/0.1ml maximum stipulated by the European Council Directives 85/397/EEC in 1985. However, these SPC were, less than that (10^5 cfu/ml) obtained from 37% of contaminated Italian UHT milk packages analyzed over the period of two years (Montanari *et al.*, 2004). It should be mentioned that, this value is the maximum *B. sporothermodurans* load that has been reported so far for contaminated UHT milk packages after incubation for 15d at 30 °C.

The SPC of contaminated UHT milk packages did not show a difference that would have been expected between UHT milk packages that were incubated for 15d at 30 °C and those that were not. This could be due to growth restraint that is associated with *B. sporothermodurans*, taking into consideration that, 10^5 cfu/ml is the maximum *B. sporothermodurans* load in UHT that has been published so far (Montanari *et al.*,

2004). This also explains the similarities in the SPC values of successive batches of UHT milk packs.

The fact that high *B. sporothermodurans* counts were determined when UHT treated milk to be used for reprocessing was analysed is of great concern. This is because of the inevitable re-introduction of spores and vegetative cells originating from spores that survived the previous UHT treatment, into the UHT processing line. This practice has grave implications; it is likely to result in enhanced resistance and proliferation of *B. sporothermodurans* in UHT milk packs during processing and storage. Furthermore, spores of *B. stearothermophilus* that survived a sub-lethal heat treatment have been found to exhibit a heat-induced resistance to subsequent heat treatment (Etoa and Michiels, 1988). Therefore, spores of *B. sporothermodurans* that may have survived a previous UHT treatment and have not germinated could acquire a heat-induced resistance.

The spore counts of the contaminated UHT milk from the retail level and from the end of the processing line, without prior incubation could barely reach detectable levels because most of the vegetative cells of *B. sporothermodurans* had not yet undergone sporulation or if already sporulated, spore at this stage could not support the 95 °C for 30 min heating, considering that the resistance of spores of a *Bacillus* spp. have been linked to the different stages in spore development, the older the spore the higher the resistance to heat (Knott *et al.*, 1995).

The SPC of the wash-water of the sterilizers was high; this could be due to the presence of spores, considering that pockets of biofilm containing high concentration of spores located within the sterilizers may have been washed off during the rinsing

process, taking into account that vegetative cells are likely to have been killed during heating (Brown, 2000).

4.2 Identification of *B. sporothermodurans* using two different methods

The adoption of the PCR methodology of Schelderman *et al.* (2002) for to the RT PCR methodology used in this research to detect *B. sporothermodurans* was successful and this is the first time in which *B. sporothermodurans* have been detected using RT PCR with SYBR Green using the primers; BSPO-F2 (5'-ACG GCT CAA CCG AG-3') and BSPO-R2 (5'-GTA ACC TCG CGG TCT A-3'). The adopted RT PCR methodology was efficient, considering that 32 out of 33 isolate tested positive after a single run. The fluctuation of the T_m between 86 and 87 °C could be attributed to minor variations in the nucleotide sequences within the amplified segment of the 16S rRNA gene. A similar fluctuation was observed by in a study involving the melting curve analysis for the identification of *Plum pox virus* strains (Varga and James, 2005).

4.3 Effect of different treatment regime on *B. sporothermodurans* isolates

The significant difference in *B. sporothermodurans* survival following heating 120 and 140 °C is understandable, considering that 140 °C is a more rigorous heat treatment than 120 °C. Heat treatment at 120 °C for 4s did not eliminate *B. sporothermodurans* as counts were obtained when treated cultures were incubated for 24h. It could be that *B. sporothermodurans* survived this treatment or revived from injury (Gonzalez *et al.*, 1995). Heat treatment at 140 °C for 4s was severe enough to completely inactivate spores and vegetative cells for both strains. This finding is in contrary to the finding of Huemar *et al.* (1998), where spores of *B. sporothermodurans* were found to be more resistant at temperatures above 130 °C.

However, Huemar's finding was on *B. sporothermodurans* spores that were harvested from a 7 days-old ONA⁺ agar plate culture, while the 24h *B. sporothermodurans* culture used in this study could only possibly contained spores which were 24h old and are fully mature to display the high heat resistance taking in to consideration that, the older the spore the higher the resistance to heat (Knott *et al.*, 1995).

Recontamination of *B. sporothermodurans* culture with a UHT treated culture of *B. sporothermodurans* did not influence the survival of *B. sporothermodurans* spores following a second UHT treatment. This may indicate that, spores and vegetative cells from the heat treated *B. sporothermodurans* culture used for recontamination of the fresh *B. sporothermodurans* culture did not acquire heat induced resistance. Only sub-lethal heat treatment of spores has been associated with induced heat resistance of spores and the resistance was found to be dependent of the duration of exposure to sub-lethal temperature (Teofila *et al.*, 1998). More research needs to be done to assess the effect of high levels of recontamination during reprocessing (repeated UHT treatment), on the survival of *B. sporothermodurans* following UHT treatment.

The reprocessing practice is often done to avoid milk wastage, after realising that packages of a particular batch are defective or the reprocessing of samples kept for shelf life determination. This is often indicated to contribute to the recontamination of UHT milk with *B. sporothermodurans* during processing. However, this study indicated that there was no recontamination impediments associated with the 10% reprocessing level applied during this study at temperature of 120 and 140 °C, considering that there was no enhancement of the *B. sporothermodurans* load in the final product. More research needs to be done with higher levels of *B.*

sporoothermodurans recontamination during re-processing to ascertain the behaviour of *B. sporoothermodurans* with regard to heat resistance.

The pre-heating of broth culture before UHT treatment did not influence the survival of *B. sporoothermodurans*, contrary to the findings of Teofila *et al.* (1998), where pre-heating was found to induce heat resistance in spores of *Bacillus* spp. This may be attributed to the fact that spores did not attain the required level of induction, given that heat induced resistance is relative to the duration of exposure to sub lethal temperature (Teofila *et al.*, 1998). They also found that the activation of dormant spores is a process that progresses with time until there is an abrupt structural change in individual spores. Heat induced resistance only becomes effective at the point where there is an expansion of the cortical peptidoglycan against an intact coat resulting in more water in the cortex and less water in the protoplast, a prerequisite characteristic for heat resistance in spores. In this experiment, heat exposure was brief so as to reflect some practices associated with UHT milk processing.

5. Conclusions

B. sporoothermodurans is present in UHT milk but the incidence is not extensive or widespread. Heat treatment at 140 °C unlike 120 °C eliminates *B. sporoothermodurans* in broth whereas chilling renders *B. sporoothermodurans* spores more susceptible to UHT treatment. The PCR detection protocol adopted for RT PCR is effective in confirming *B. sporoothermodurans*.

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Table 1: Bacterial quality of milk collected from retail and during processing of processor D

Milk sample	Treatment	SPC (log cfu/ml)	Spore count (spores/ml)
^a Fresh raw		6.06 (± 0.17)	nd
^b 24h raw	chilled (4 °C)	7.31 (± 0.22)	nd
Full cream		6.73 (± 0.10)	nd
Cream	pasteurised	8.85 (± 0.08)	nd
2 % fat		7.80 (± 0.13)	nd
Skim		5.67 (± 0.14)	nd
^c UHT	from defective packs	7.92 (± 0.08)	nd
^d Wash water (sterilizers)		5.27 (± 0.50)	nd
Wash water (filters)		nd	nd
UHT	Directly after processing	2.67 (± 0.16)	1.0 (± 0.33)
UHT	Incubated (15d, 30 °C)	3.01(± 0.12)	20 (± 2.73)
UHT	Retail level	2.96 (± 0.21)	nd

a = milk that has just been received from the farms.

b = fresh raw milk that had been in the storage tank for 24 hrs at 4 °C.

c = UHT milk from defective packages removed from the processing line to be mixed with fresh milk to a 10 % v/v final concentration and subjected to another round of UHT treatment.

d = water that had been used to rinse the filter or the sterilizers prior to another round of UHT processing, and spore count determination.

nd = not detected for levels below 1 cfu/ml.

Values = mean (± SD).

Table 2: Effect of heating at 120 or 140 °C for 4s, and repeat UHT with 10% reprocessing on the survival of two strains of *Bacillus sporothermodurans*

UHT temp (° C)	Treatment	Strain	Survival (log cfu/ml)
120	Before UHT	DMS10599	6.86 (±0.67) ^a
		UP20A	5.78 (±0.92) ^a
	After UHT	DMS10599	2.11 (±0.01) ^b
		UP20A	1.81 (±0.27) ^b
	^a 10 % reprocessing	DMS10599	1.65 (±0.45) ^b
		UP20A	1.31 (±0.54) ^{bc}
140	Before UHT	DMS10599	6.43 (±0.41) ^a
		UP20A	5.58 (±0.84) ^a
	After UHT	DMS10599	nd
		UP20A	nd
	10 % reprocessing	DMS10599	nd
		UP20A	nd
p-values	p = 0.68 ¹	p = 0.53 ²	p = 0.89 ³

^a = 10% reprocessing = the addition of an overnight UHT treated cultures to fresh milk to a 10 % v/v final concentration and subjected to another round of UHT treatment.

Superscripts: a, b and c if similar denote statistical significant difference at $p \leq 0.05$, otherwise statistical similarity (n = 3).

1 = treatment effect, 2 = strain effect and 3 = strain-treatment interaction effect.

nd = not detected for levels below 1cfu/ml

Values = mean (± SD).

Table 3: Effect of pre-heating (78 °C for 15s), UHT (120 °C for 4s) and a combination of pre-heating and UHT, on the survival of *Bacillus sporothermodurans*.

Treatment	Strain	Survival (log cfu/ml)
Before treatment	DMS10599	5.50 (± 0.11) ^a
	UP20A	5.78 (± 0.92) ^a
Pre-heating only	DMS10599	1.50 (± 0.46) ^c
	UP20A	0.57 (± 0.51) ^b
UHT only	DMS10599	nd
	UP20A	nd
Combined pre-heating and UHT	DMS10599	nd
	UP20A	nd
	$p \leq 0.05$ ¹	$p = 0.31$ ²
		$p \leq 0.05$ ³

Superscripts: a, b and c if similar denote statistical significant difference at $p \leq 0.05$, otherwise statistical similarity (n = 3).

1 = treatment effect, 2 = strain effect and 3 = strain-treatment interaction effect.

nd = not detected for levels below 1cfu/ml

Values = mean (\pm SD).

Table 4: Effect of UHT treatment (120 °C/4s), with and without chilling (24h at 7 °C), on spores of *B. sporothermodurans* strains.

Treatment	Strain	Survival (log cfu/ml)
Before UHT treatment	DMS10599	7.40 (±0.01) ^a
	UP20A	7.24 (±0.10) ^a
After UHT	DMS10599	2.25 (±0.10) ^b
	UP20A	2.25 (±0.16) ^b
Combined chilling and UHT	DMS10599	nd
	UP20A	nd
$p \leq 0.05$ ¹	$p = 0.18$ ²	$p = 0.56$ ³

Superscripts: a, b and c if similar denote statistical significant difference at $p \leq 0.05$, otherwise statistical similarity (n = 3).

1 = treatment effect, 2 = strain effect and 3 = strain-treatment interaction effect.

nd = not detected for levels below 1cfu/ml.

Values = mean (± SD).

Table 5: The effect of 30% H₂O₂, on *B. sporothermodurans* spores, at different exposure times.

H₂O₂ Exposure (min)	Strain	survival (log cfu/ml)
0	DMS10599	6.31 (±0.03) ^a
	UP20A	6.23 (±0.11) ^a
3	DMS10599	4.84 (±0.06) ^b
	UP20A	4.84 (±0.05) ^b
6	DMS10599	4.08 (±0.03) ^c
	UP20A	3.99 (±0.12) ^c
9	DMS10599	3.25 (±0.05) ^d
	UP20A	3.28 (±0.10) ^d
12	DMS10599	2.36 (±0.07) ^e
	UP20A	2.36 (±0.07) ^e
15	DMS10599	1.67 (±0.08) ^f
	UP20A	1.64 (±0.21) ^f
$p \leq 0.05$ ¹	$p = 0.36$ ²	$p = 0.84$ ³

Superscripts: a, b and c if similar denote statistical significant difference at $p \leq 0.05$, otherwise statistical similarity ($n = 3$).

1 = treatment effect, 2 = strain effect and 3 = strain-treatment interaction effect.

Values = mean (± SD).

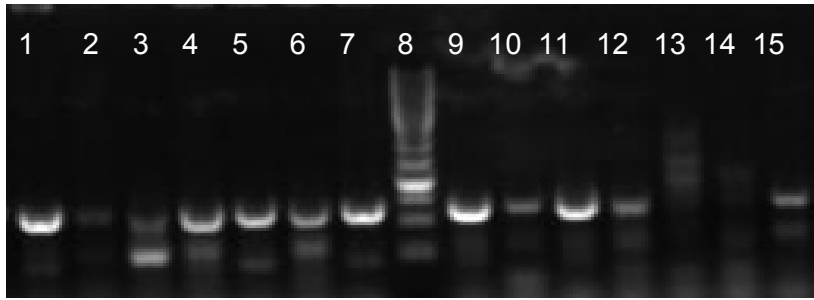


Figure 1: A gel photograph of PCR products confirming the identity of some of the UHT milk isolates. Lane 1 and 9, *B. sporothermodurans* DMSZ No. 10599 (Germany); lanes 3, 4, 5, 6, 7, 10, 11, 12 and 15, isolates from UHT milk detected as *B. sporothermodurans*; lanes 13 and 14, isolates that were not positive for *B. sporothermodurans*; Lane 2, *Bacillus stearothermophilus* as negative control; Lane 7, 100 bp DNA ladder (Fermentas, South Africa).

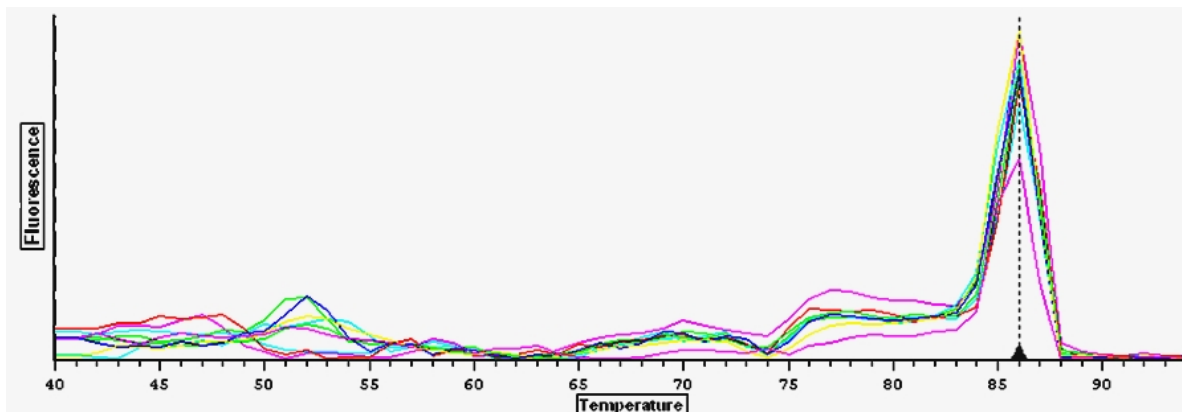


Figure 2: Real time PCR melting curve ($T_m= 86$) specific for *Bacillus sporothermodurans* using the BSPO-F2 and BSPO-R2 primers.

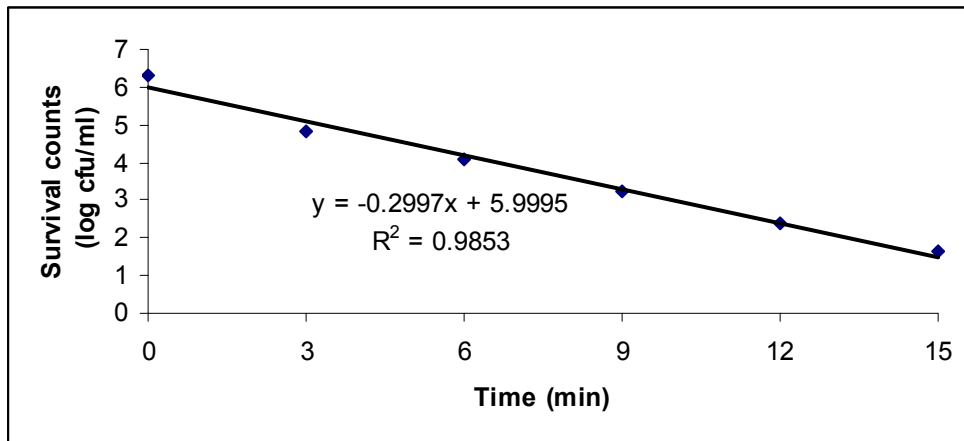


Figure 3: Regression equation of the survival of *Bacillus sporothermodurans* spores following exposure to 30 % H₂O₂ at different time ($D_{30\%} = 3.33$).