CHAPTER 3: OBJECTIVES AND HYPOTHESES

3.1 OBJECTIVES

1. To determine the incidence of *B. sporothermodurans* in retail UHT milk within South Africa;
2. To determine the effect of UHT processing stresses; pre-heating, chilling, H_2O_2 and UHT re-processing on the survival of *B. sporothermodurans*;
3. To study the effect of ultra-high temperature treatment, 130 °C for 4s, on the survival, structure and chemical components of *B. sporothermodurans* spores.

3.2 HYPOTHESES

The exposure of *B. sporothermodurans* to sub-lethal stresses such as preheating, chilling, H_2O_2 and UHT re-processing will lead to an increase in heat resistance during UHT treatment because of adaptation responses to these stresses. The heat resistance of spores is influenced by many factors, before, during and after heat treatment (Scheldeman *et al.*, 2006). These factors may include the sporulation condition, heat-induced resistance and conditions of recovery from stress (Scheldeman *et al.*, 2006; Nicholson, Munakata, Horneck, Melosh & Setlow**,** 2000).

Bacillus sporothermodurans spore inactivation at UHT temperature is as a result of structural damage due to heating, resulting in the leakage of vital spore components such as dipicolinic acid and small acid-soluble proteins (Setlow *et al.*, 2006).

CHAPTER 4: RESEARCH

4.1 INCIDENCE AND SURVIVAL OF *BACILLUS SPOROTHERMODURANS* DURING UHT PROCESSING

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4.1.1 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 H_2O_2, individually and in combination on the survival of *B. sporothermodurans* was also investigated in broth. Standard plate counts were conducted for all milk samples and isolates from UHT milk were characterised using PCR. *B. sporothermodurans* vegetative cells and spores in broth were subjected to various stresses encountered during UHT processing of milk. Survival counts were conducted after all treatments. *B. sporothermodurans* were 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. sporothermodurans* spores than UHT treatment alone. H_2O_2 was also effective in eliminating *B. sporothermodurans* spores after 15 min of exposure. The adopted real time (RT) PCR with SYBR Green method was effective for the confirmation of *B. sporothermodurans*. This research is the first to be conducted with regards to the detection of *B. sporothermodurans* 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. sporothermodurans* spores during UHT processing. This research is the first in which RT PCR with SYBR Green has been used to characterise *B. sporothermodurans*.

Key words: UHT, milk, processing, *B. sporothermodurans*, spores.

4.1.2 Introduction

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

B. sporothermodurans, 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 that 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 thereby 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 sterilised 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 the 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, Sabat & Gouti, 2002). Protein denaturation and sugar modifications due to heat treatment cause UHT 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 *B. sporothermodurans* 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 & Wouters, 1999). Furthermore, a response to one stress can lead to induced-resistant responses to other stresses (Abee & 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 thereby increasing their ability to germinate as well as the induction of heat resistance (Teofila *et al.*, 1998).

H_2O_2 is used to sterilise packaging material during the UHT processing of milk. H_2O_2 is a useful chemical sterilising agent because it neither imparts an unpleasant odour to the packaged product nor does it leave residues on packaging material (Toledo, Escher & Ayres, 1973). Bacterial spores are more resistant than vegetative cells to H_2O_2. The inactivation of bacterial spores by H_2O_2 depends on various factors including the nature of the spores, a wet or dry environment, the concentration of H_2O_2 and the species of the spore former present (Toledo *et al.*, 1973).

Experiments conducted to determine the mechanism of action of H_2O_2 in the killing of spores have not been conclusive (Melly *et al.*, 2002). In one experiment, *B. subtilis* spores killed by H_2O_2 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 characterisation 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 H_2O_2

exposure, is of importance as it determines the stability of UHT milk products 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 *B. sporothermodurans* strains.

4.1.3 Materials and methods

4.1.3.1 Samples of UHT milk used in the study

(a) Retail samples

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

(b) In-process samples

Milk samples were collected at different processing steps from processor D. They include the following:

- 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; and
- 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.

(c) UHT milk directly after processing together with samples of water that have been used to rinse the filter or the sterilisers.

4.1.3.2 Microbiological counts

(a) Standard plate count (SPC)

SPCs 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 analyses were made for each milk sample by counting duplicate plates containing 30–100 colony-forming units (cfu) after incubation for 24h at 37 °C. In order to determine absolute sterility SPCs were also conducted for UHT milk that had been incubated for 15 days at 30 °C.

(b) Spore counts

Spore counts were conducted by plating serial dilutions of milk or broth samples that had 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 15 days at 37 °C. Triplicate analyses were made for each heat-treated sample by counting duplicate plates containing colony-forming units after incubation for 24h at 37 °C.

4.1.3.3 Molecular identification of isolates

(a) 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 had been washed three times by centrifugation at 13 000 × g for 3 min followed by re-suspension in distilled water. 100 µl solution of 0.1 M NaOH and 0·25% sodium lauryl sulphate (Merck, South Africa) was added to the washed pellets and heated for 17 min at 90 °C. The boiled samples were again centrifuged at 13 000 × g for 3 min and crude DNA was collected from the supernatants.

(b) PCR Procedure

The PCR reactions were conducted using *B. sporothermodurans* specific primers of 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 $MgCl_2$ and 0.2 mM of NTP mix (Fermentas, South Africa). The mixture was subjected to 30 cycles of amplification in a thermal cycler (iCycler Thermal Cycler, 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 included a final extension of 7 min at 72 °C. 10 µl of PCR products were analysed on a 1% agarose gel containing 0.5 µg/ml ethidium.

(c) 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/Bactrial DNA Kit™ (Zymo Research Corporation, USA), following the manufacturer's protocol. The concentration of extracted DNA was analysed using the DNA Nanodrop Spectrophotometer and samples were stored at -20 °C until they were used as PCR templates.

(d) 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. 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.

4.1.3.4 Treatment regimes used on B. sporothermodurans isolates

(a) Preparation of raw and pasteurised milk isolates

After plating and incubation of raw and pasteurised 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 a pure culture.

(b) 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.

(c) Preparation of control strains

A reference strain of *Bacillus sporothermodurans* (DSM No 10599), a control being 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 to determine 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 then incubated for 24h at 37 °C. A ml of each culture was centrifuged at 10 000 × 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 $BaCl_2$ to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) with physiological saline (8.5 g/L NaCl) as diluents (Andrews, 2005).

(d) 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 15 days 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 into a 1.5 ml eppendorf tube containing

sterile physiological saline. Spores were washed with double distilled water, centrifuged at 10 000 × g for 3 min and pellets were diluted to *ca*. 10^8 spores per ml with sterile physiological saline using 0.5 McFarland Standard.

(e) 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 plugs (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 for 4s or 140 ± 1 °C for 4s. The temperature equilibrium time was determined with the aid of a thermocouple mounted on a pressure tube.

(f) Simulation of the reprocessing of UHT milk

B. sporothermodurans culture suspensions 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 then followed by incubation at 37 °C for 24h. To simulate reprocessing practices in industry, 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.

(g) 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 exceeds 3 logs. In order to get a

clear response to treatments, higher levels of *B. sporothermodurans* in BHI culture were required.

(h) 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 (3 ml) were mixed with 1 ml 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.

(i) Treatment with H_2O_2

H_2O_2 (Sigma Aldrich, Midrand, South Africa) was mixed with spores in distilled water to form spore suspensions containing 30% H_2O_2 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 H_2O_2 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 1 min duration. A colourless potassium iodide starch paper indicated the complete removal of H_2O_2 while a blue-black colouration indicated the presence of active H_2O_2

4.1.4 Results

4.1.4.1 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 4.1). UHT milk packs from the other processors remained commercially sterile even after they were incubated for 15 days at 30 °C (data not shown).

4.1.4.2 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 4.1). The spore counts of UHT milk packs that were incubated for 15 days at 30 °C were higher than those that were not incubated for 15 days at 30 °C (Table 4.1). 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 15 days, did not differ. Values ranged from 2.5–3 log cfu/ml (Table 4.). The skim milk, wash water, raw fresh milk and full cream milk had SPC values that ranged from 5–7 log cfu/ml.

The SPC values for 24h raw milk, 2% fat milk and UHT milk for reprocessing ranged from 7–8 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 < raw fresh 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 4.1).

4.1.4.3 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 with 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* when considering that these isolates produced bands and melting curve similar to that of the control (Figure 4.1). The melting temperature (Tm) of the amplified segment during the RT PCR fluctuated from 86–87 °C (Figure 4.2). Isolates from fresh and pasteurised milk tested negatively as they did not produce the required band for *B. sporothermodurans*

Table 4.1 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	Chilled (4 °C)	6.06 (± 0.17)	nd
[b] 24h raw		7.31 (± 0.22)	nd
Full cream	Pasteurised	6.73 (± 0.10)	nd
Cream		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 (sterilisers)		5.27 (± 0.50)	nd
Wash water (filters)		nd	nd
UHT	Directly after processing	2.67 (± 0.16)	1.0 (± 0.33)
UHT	Incubated (15 days, 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 farms

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

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 sterilisers prior to another round of UHT processing, and spore count determination

nd = not detected for levels below 1 cfu/ml

Values = mean (± SD)

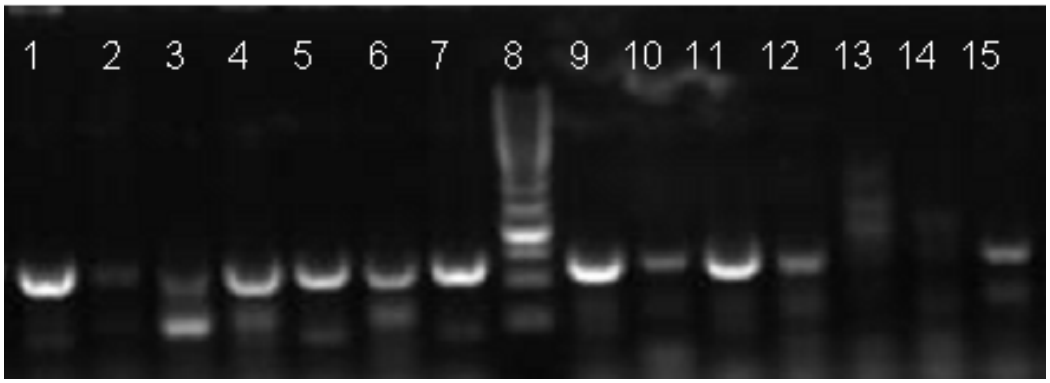

Figure 4.1 Gel photograph of PCR products confirming the identity of some of the UHT milk isolates. Lane 1 and 9, *B. sporothermodurans* DMSZ No. 10599 (Germany); lanes 3, 4, 5, 6, 7, 10, 11, 12 and 15, isolates from UHT milk detected as *B. sporothermodurans*; lanes 13 and 14, isolates that were not positive for *B. sporothermodurans*; Lane 2, *Bacillus stearothermophilus* as negative control; Lane 7, 100 bp DNA ladder (Fermentas, South Africa)

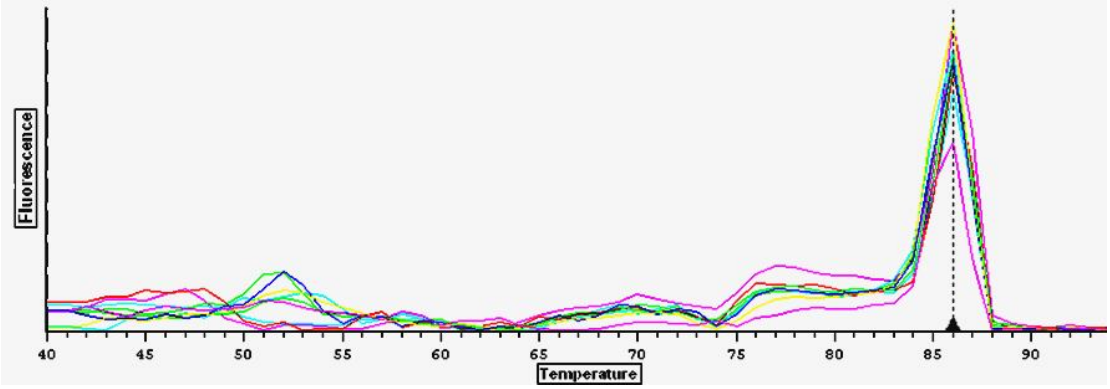

Figure 4.2 Real time PCR melting curve (Tm= 86) specific for *Bacillus sporothermodurans* using the BSPO-F2 and BSPO-R2 primers

4.1.4.4 Effect of UHT only and UHT reprocessing

The counts of *B. sporothermodurans* after heat treatment at 120 for 4s were significantly different ($p \leq 0.05$) from those heated at 140 °C for 4s (Table 4.2). *B. sporothermodurans* were significantly ($p \leq 0.05$) reduced by 4 log cfu/ml, but were not completely eliminated after heat treatment at 120 °C for 4s; from log cfu/ml 6.86

to 2.11 and log cfu/ml 5.78 to 1.81 for DMS10599 and UP20A respectively. After heating at 140 °C for 4s, no *B. sporothermodurans* were detected. The two *B. sporothermodurans* strains were not affected differently by the two heat treatments with respect to 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* were detected for the 140 °C heated cultures (Table 4.2).

4.1.4.5 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 4.3). However, after subsequent UHT treatment no growth was detected for either strain. Similarly no growth was detected for either strain when cultures were subjected to UHT treatment (Table 4.3).

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

There were significant differences ($p \leq 0.5$) in the survival of spores after the two treatments of UHT treatment with and without prior chilling (Table 4.4). The effect of 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 4.1). 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 in reducing the *B. sporothermodurans* counts as both strains were inactivated to undetectable levels.

Table 4.2 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]
	[α]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]

α = 10% reprocessing, the addition of an overnight UHT treated culture to a fresh culture 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 1 cfu/ml

Values = mean (± SD)

Table 4.3 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 1 cfu/ml

Values = mean (± SD)

4.1.4.7 Effects of 30% H_2O_2 on the survival of B. sporothermodurans spores

The exposure time to H_2O_2 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 4.5). With each 3 min increase in the exposure time to 30% H_2O_2 the *B. sporothermodurans* level decreased linearly (Figure 4.3). The reduction in *B. sporothermodurans* spore survivals within the first 15 min was preceded by an initial abrupt decrease for both strains for the first 3 min. There was a constant decrease and significant decrease in the survival of spores after each exposure time (Table 4.3). By using the regression equation, the D30% of H_2O_2 was calculated to be 3.33 min (Figure 4.3).

Table 4.4 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 1 cfu/ml

Values = mean (± SD)

Table 4.5: Effect of 30% H_2O_2, on *B. sporothermodurans* spores, at different exposure times

H_2O_2 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)

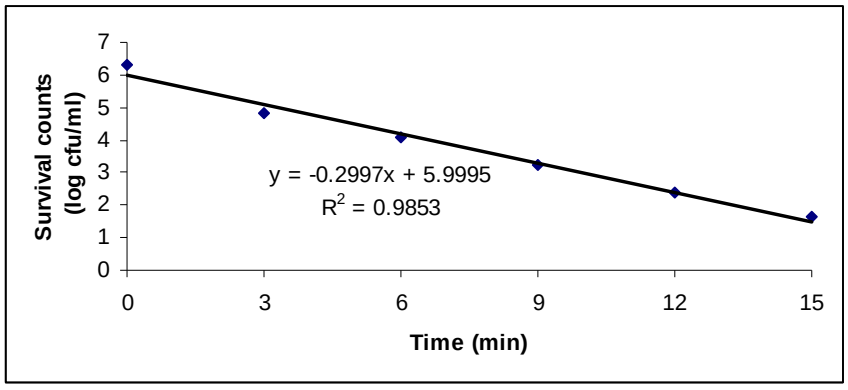

Figure 4.3 Regression equation of the survival of *Bacillus sporothermodurans* spores following exposure to 30% H_2O_2 at different time ($D_{30\%}$ = 3.33)

4.1.5 Discussion

4.1.5.2 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 of 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 a high bacterial load in fresh milk (Muir, 2007). Similarly, the pasteurised 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 pasteurised milk. This is similar to the level endorsed by the Dairy Standard Agency (DSA) in South Africa and 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*, the incidence of this highly heat-resistant spore-producing bacteria in the local dairy environment is confirmed. UHT milk packages of other processors remained sterile even after incubation for 15 days at 30 °C. This implies that *B. sporothermodurans* contamination is not widespread or extensive in the South African milk industry.

Although the SPC of the contaminated UHT milk packages were higher than the 10 cfu/0.1 ml maximum stipulated by the European Council Directives 85/397/EEC in 1985, they were less than 10^5 cfu/ml recorded for 37% of contaminated Italian UHT milk packages analysed over a period of two years (Montanari, Borsari, Chiavari, Ferri, Zambonelli & Grazia, 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 15 days at 30 °C (Montanari *et al.,* 2004). 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 15 days at 30 °C and those that were not. This could be due to growth restraint associated with *B. sporothermodurans*, taking into consideration that 10^5 cfu/ml is the maximum *B. sporothermodurans* load in UHT that has been noted in different publications 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 for UHT treated milk that was to be reprocessed is of great concern. It is inevitable that spores and vegetative cells originating from the previous UHT treatment would be re-introduced into the UHT processing line. This practice has grave implications, as it is likely to result in enhanced resistance and proliferation of *B. sporothermodurans* in UHT milk packs during further 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 & 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. The reason is that 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. The resistance of spores of a *Bacillus* spp. has been linked to different stages in spore development with researchers noting that the older the spore the higher its resistance to heat (Knott, Russell & Dancer, 1995).

The SPC of the wash-water of the sterilisers was high. This could be due to the presence of spores, considering that pockets of biofilm containing high concentrations of spores located within the sterilisers may have been washed off during the rinsing

process. In addition, vegetative cells are likely to have been killed during UHT processing of milk (Brown, 2000).

4.1.5.2 Identification of B. sporothermodurans using two different methods

The adoption of the RT PCR methodology used in this research to detect *B. sporothermodurans* in place of the PCR methodology of Schelderman *et al*. (2002) was successful. In addition, this is the first time that *B. sporothermodurans* have been detected using RT PCR with SYBR Green along with the primers of 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 as 32 out of 33 isolate tested positive after a single run. The fluctuation of the Tm between 86–87 °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 in a study involving the melting curve analysis for the identification of *Plum poxvirus* strains (Varga & James, 2005).

4.1.5.3 Effect of different treatment regimes on B. sporothermodurans isolates

The significant difference in *B. sporothermodurans* survival following heating at 120–140 °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 is possible *B. sporothermodurans* survived this treatment or revived from injury (Gonzalez, Lopez, Mazas, Gonzalez & Bernardo, 1995). Heat treatment at 140 °C for 4s was severe enough to completely inactivate spores and vegetative cells for both strains. This finding is contrary to the finding of Huemer *et al.* (1998) where spores of *B. sporothermodurans* were found to be more resistant at temperatures above 130 °C. Huemer's finding was based on *B. sporothermodurans* spores that were harvested from a 7-day-old ONA^{+} agar plate culture. The *B. sporothermodurans* culture used in this study could only possibly have contained spores that were 24h old. The older the spore, the higher is its 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 on the duration of exposure to sub-lethal temperatures (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 practice of reprocessing milk often occurs in order to avoid milk wastage when a producer realises that packages of a particular batch are defective or if it is observed that samples kept for shelf-life determination are defective. This may contribute to the recontamination of UHT milk with *B. sporothermodurans* during processing. However, this study has indicated that there were no recontamination impediments associated with the 10% reprocessing level applied during the study at temperatures of 120–140 °C as no detection of an increase of the *B. sporothermodurans*' load in the final product was detected. More research needs to be done with higher levels of *B. sporothermodurans* recontamination during re-processing to ascertain the behaviour of *B. sporothermodurans* with regard to heat resistance.

The pre-heating of broth culture before UHT treatment did not influence the survival of *B. sporothermodurans* 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 an abrupt structural change in individual spores occurs. 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, which is a

prerequisite characteristic for heat resistance to occur in spores. In this experiment, heat exposure was brief so as to reflect some practices associated with UHT milk processing.

4.1.6 Conclusions

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

4.2 THE EFFECTS OF WET HEAT TREATMENT ON THE STRUCTURAL AND CHEMICAL COMPONENTS OF *BACILLUS SPOROTHERMODURANS* SPORES

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4.2.1 Abstract

The objective of this research was to study the rate of structural damage and survival of *Bacillus sporothermodurans* spores following treatment at high temperatures by determining the amount of Dipicolinic acid (DPA) and soluble protein leakage over time. A reference strain of *B. sporothermodurans* (DSM 10599) and a South African strain (UP20A) isolated from UHT milk were used to determine the survival of spores heated at 130 °C for 4, 8 and 12 min. To check the viability of spores, plate counts were determined. Structural damage was determined using transmission electron microscopy (TEM). The filtrate of the heated spore suspension was analysed for the amount of DPA and soluble protein release due to heating. The amount of DPA released was quantified by HPLC analysis while the amount of soluble protein released from heated spores was quantified using the Bradford method. The log values of spore counts, released DPA and soluble proteins from triplicate experiments were analysed.

The results of this study indicate that inactivation of *B. sporothermodurans* spores during wet heat treatment is due to the penetration of hot moisture into the spore which then moistens the spore components and inactivates enzymes. Because of the high water pressure, vital spore components such as proteins and DPA in solution leak out of the spore. Interestingly, the vast majority of heated spores were inactivated before a significant amount of DPA was released. This research is the first to determine the effect of high temperature wet heat treatment on the structure of *B. sporothermodurans* spores and gives an insight regarding the mechanisms of destruction of *B. sporothermodurans* spores by wet heat.

Key words: *Bacillus*, spores, Dipicolinic acid, spore protein, TEM, UHT

4.2.2 Introduction

Bacillus sporothermodurans, first detected in UHT milk in Germany in 1990, affects the stability and the shelf life of commercial UHT milk (Pettersson *et al*., 1996). This is due to the unusual thermal kinetics of *B. sporothermodurans* spores that enable them to survive at high temperatures (130 °C for 4s) of UHT treatment (Huemer *et al*., 1998). Spores germinate during storage in UHT products causing instability due to their proteolytic activities thereby reducing shelf life and consumer acceptability. Spoilage due to *B. sporothermodurans* growth can be in the form of a slight change in the colour of the milk, 'off flavours' and destabilisation of casein micelles (Klijn *et al*., 1997). Increasing the temperature or the holding time in an attempt to inactivate *B. sporothermodurans* spores affects the organoleptic and nutritional qualities of UHT milk (Claeys *et al*., 2001). Severe heating will lead to protein denaturation, Maillard reactions and lactose isomerisation. Protein denaturation and sugar modification are responsible for the 'cooked' taste, while the Maillard reactions induce a decrease of the protein nutritional value by irreversible alteration of the lysine residue (Claeys *et al*., 2001). The mechanisms in which the structural and chemical components of *B. sporothermodurans* spores are influenced during wet heat inactivation are currently not clear (Klijn *et al*., 1997).

Bacterial spore layers consist of the following layers: exosporium, spore coats, outer membrane, cortex, germ cell wall, inner membrane and central core (Setlow, 2006). The exosporium and the spore coats have not been associated with wet heat resistance of spores. Removal of the outer spore membrane along with coat proteins also did not produce a major effect on spore resistance to wet heat (Nicholson *et al*., 2000). The cortex, which is made up of peptidoglycan (PG), has been linked to the production of dormant spores with low core water content, however, the mechanism of dormant spore production is still unclear (Setlow, 2006). The core is the innermost layer of the spore and is comparable to the protoplast of the vegetative cell. The spore core consists of spore enzymes, DNA, ribosomes, tRNA, divalent cations, small acid

soluble proteins (SASP) and dipicolinic acid (DPA) (Setlow, 2006). The major factors that contribute to the wet heat resistance of bacterial spores are the core water content, mineralisation of the spore core due to the accumulation of high levels of divalent cations and DPA and the presence of high levels α/β-type SASP in the spore core (Melly *et al*., 2002). An increase in the mineralisation of spores due to high levels of divalent cations and DPA increases the wet heat resistance (Paidhungat *et al.*, 2000). SASP binds to spore DNA protecting it from wet heat and other treatments such as chemicals, radiation, and so forth (Setlow, 2006). The SASP consist of α/ß-type 56 and γ-type with the average molecular weight ranging from 5–10 kD. In *B. subtilis* spores these SASP types are only synthesised in developing spores late in sporulation (Hathout *et al*., 2003).

It has been reported that the killing of spores by wet heat is not through DNA damage but rather by the rupturing of the spore's inner membrane permeability barrier and the inactivation of core enzymes (Warth, 1980; Setlow, 2000). Nevertheless, the exact target for lethal damage by wet heat and the mechanism behind it have not been ascertained. Therefore, there is a need to understand the structural integrity of *B. sporothermodurans* spores following UHT treatment in relation to their survival. The objective of this research was to study the survival of *B. sporothermodurans* spores and structural damage as affected by wet heat.

4.2.3 Materials and methods

4.2.3.1 B. sporothermodurans strains

A reference strain of *Bacillus sporothermodurans* DSM 10599 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany while *B. sporothermodurans* UP20A was isolated from UHT milk in South Africa. 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 (BHI) broth and incubated for 24h at 37 °C.

One ml of each culture was centrifuged at 10 000 × g for 3 min and pellets were subsequently diluted to approximately 0.5 McFarland Standard (Andrews, 2004).

4.2.3.2 Spore preparation

From an overnight pure culture of each strain, 1 ml of culture was collected and spread on BHI agar plates and incubated for 14 days 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 into a 1.5 ml eppendorf tube containing sterile physiological saline. Spores were washed with double distilled water, centrifuged at 10 000 × g for 3 min and pellets were diluted to about 10^8 spores per ml with sterile physiological saline using 0.5 McFarland Standard.

4.2.3.3 Heat treatment of spores

Heating was conducted, with some modifications, as described by Huemer *et al.* (1998). Spore suspensions were heated in sterile pressure tubes with a threaded type B plug (Sigma Aldrich, Midrand, South Africa) with an outer diameter of 25 mm and wall thickness of 2 mm in an oil bath. The tubes were filled with 3 ml of spore suspension, closed with stoppers and submerged in an oil bath set at 130 °C. To check the viability of spores, standard plate counts were conducted by plating 10 times serial dilutions of heated spore on BHI agar plates and incubated at 37 °C for 4 days.

4.2.3.4 Transmission Electron Microscopy (TEM) analyses of spores

TEM was conducted with heated spores, as described by Van der Merwe and Coetzee (1992) with some modifications. Spores were centrifuged at 3 000 × g for 3 min and pellets were re-suspended in a solution containing 3 ml of 2.5% gluteraldehyde, 15 ml 0.15 M sodium phosphate buffer and 30 ml double distilled water and incubated for 24 hr at room temperature and centrifuged. The pellets were washed by resuspension

in 50% 0.15 M sodium phosphate buffer for 15 min followed by centrifugation at 3 000 × g for 3 min. This procedure was repeated three times. After washing, pellets were dissolved in 1% 100 OsO_4 and allowed to stand for 1h prior to another round of washing in 50% 0.15 M sodium.

The pellets were dehydrated by washing once in 50% ethanol for 15 min followed by centrifugation at 3 000 × g for 3 min. This procedure was repeated in 70% and 80% ethanol in succession. The resulting pellets were washed 3 times in 100% ethanol and passed through 33%, 66% and 100% resin (29.8% Quetol, 44.6% Nadic Methyl Anhydride, 16.6% Dodenyl Succinic Anhydride, 2% Araldite RD2, 1% 2, Dimethylaminoethane), prior to polymerisation. Thin sections of resin-polymerised spores were made with a microtome and placed on grids and stained with uranyl acetate and lead citrate. Micrographs were taken using the Philips EM301 transmission electron microscope (Eindhoven, Netherlands).

4.2.3.4 Analysis of soluble protein leakage from heated spores

Analysis of leaked soluble protein was conducted with the filtrate obtained from filtering the 3 ml spores suspension (10^8 spores/ml) of *B. sporothermodurans* that had been heated at 130 °C for 0, 4, 8 or 12 min. Filtration was through a 2 µm filter and the filtrates were used to estimate the concentration of leaked soluble protein using the method determined by Bradford (1976).

4.2.3.5 HPLC analysis of leaked dipicolinic acid

The filtrate of the heated spore suspension described above was used to analyse DPA. DPA analysis was conducted using the Breeze HPLC System equipped with the following: Waters 2487 detector, Waters 1525 HPLC Pump with a 250 x 4.6 mm, 5 µm particle sizes, C12 reverse phase HPLC column (Waters Corporation, Milford, USA). Elution was with 1.5% tert-amyl alcohol in 0.2 M K phosphate at pH 1.8 that had been filtered with a 0.2 µm membrane filter with a flow rate of 1.5 ml per min at 25 °C. Chromatograms were generated following UV absorbance at 271 nm (Warth,

1979). Commercial dipicolinic acid (Sigma Aldrich, Midrand, South Africa) was used as standard. The peak area, which is a reflection of the DPA concentration, was generated using a HPLC analyser.

4.2.3.5 Statistical analysis

The effects of the main factors which were the strain and time on the log values of spore survival counts and values of the amount of released DPA and soluble proteins, each from triplicate experiments, were determined using ANOVA (STATISTICA, Statsoft Inc., Tulsa, OK). Least square means were also used to determine significant difference.

4.2.4 Results

4.2.4.1 TEM analyses of spores

As seen on Figure 4.4a and from the core outward, a *B. sporothermodurans* spore consists of four noticeable layers, namely: cortex, spore coats, exosporium and surface layer. The core is a centralised dense structure of approximately 500 nm in diameter and surrounded by the cortex, which is close to 62 nm wide. The coat is also surrounded by the exosporium, which is about 187 nm wide. The exosporium is the widest of all the layers and surrounded by the surface layers. All measurements were estimated using the measurement lengths on micrographs. Looking at the differences in intensity of the different layers on the TEM micrographs, the density of the layers are in this order; core > coats > cortex > exosporium (Figure 4.4a). The spore coat consists of two layers; the outer spore coat and the inner spore coats with a loose space between the outer and inner spore coats (Figure 4.4b). Between the inner spore coat and the cortex, lies the cortical membrane while the inner exosporial membrane is located between the outer spore coat and the exosporium (Figure 4.4b). The surface layer also consists of a surface membrane that is underlined by coat-like structures (Figure 4.4b). Both strains have similar TEM profiles.

Structural damage was observed when *B. sporothermodurans* spores were heated at 130 °C for 4 min (Figure 4.5a–d). Structural damage (marked X) within the cortical membrane region, beneath the spore coats was clearly visible. This depicts a pull away of the coat structures from the cortex within the cortical membrane after heating for 4 min. However, some spores in the same crop did not show any visible sign of structural damage at this stage (Figure 4.5d).

After heating for 8 min, the damage in the cortical membrane widened (marked X) and some of the cortical materials were lost at this stage, rendering it less dense (marked Z) (Figure 4.6a, b, c and d). Similarly, the exosporium lost some of its content and was compressed (marked Y) on Figure 4.6b and c. The spore core had also started losing some of its components (Figure 4.6d).

The exosporium and the cortex were severely damaged and had lost most of their components after heating for 12 min and there was a reduction in the density of the core. The surface layers, exosporium and spores coats collapsed on each other at this time (marked X) on Figure 4.7a, b, c, when compared to that of unheated spores (Figure 4.6a and b). The spore core at this stage was severely damaged and most of its contents had been washed away (Figure 4.7a, b, c and d).

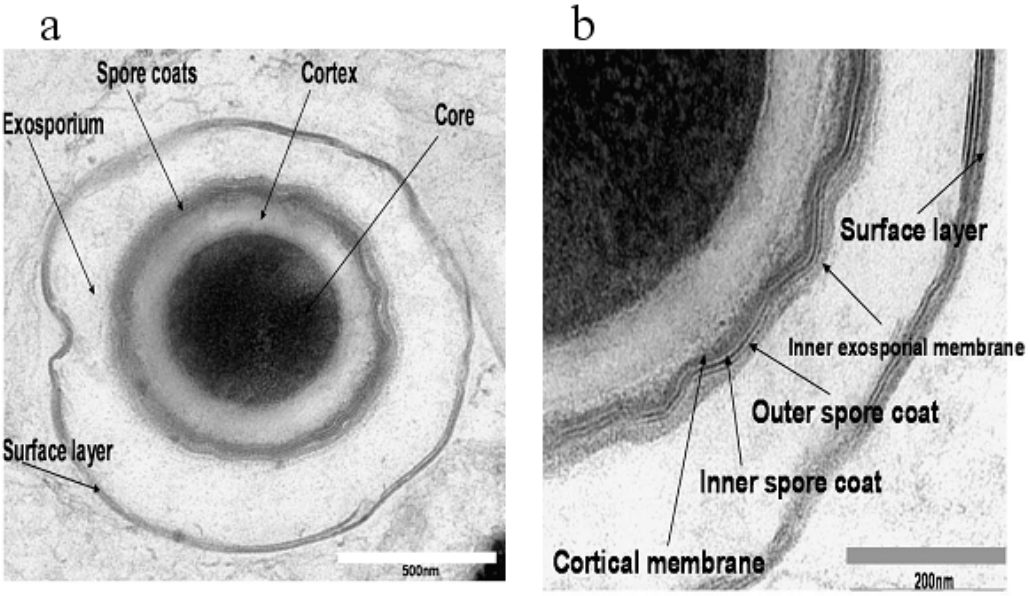

Figure 4.4 a & b: Micrographs generated from TEM analysis, showing the detail structures of unheated spores of *Bacillus sporothermodurans* UP20A, harvested from 14 days old BHI agar plates. NB: The spore structures of both strains as revealed by TEM analysis were similar.

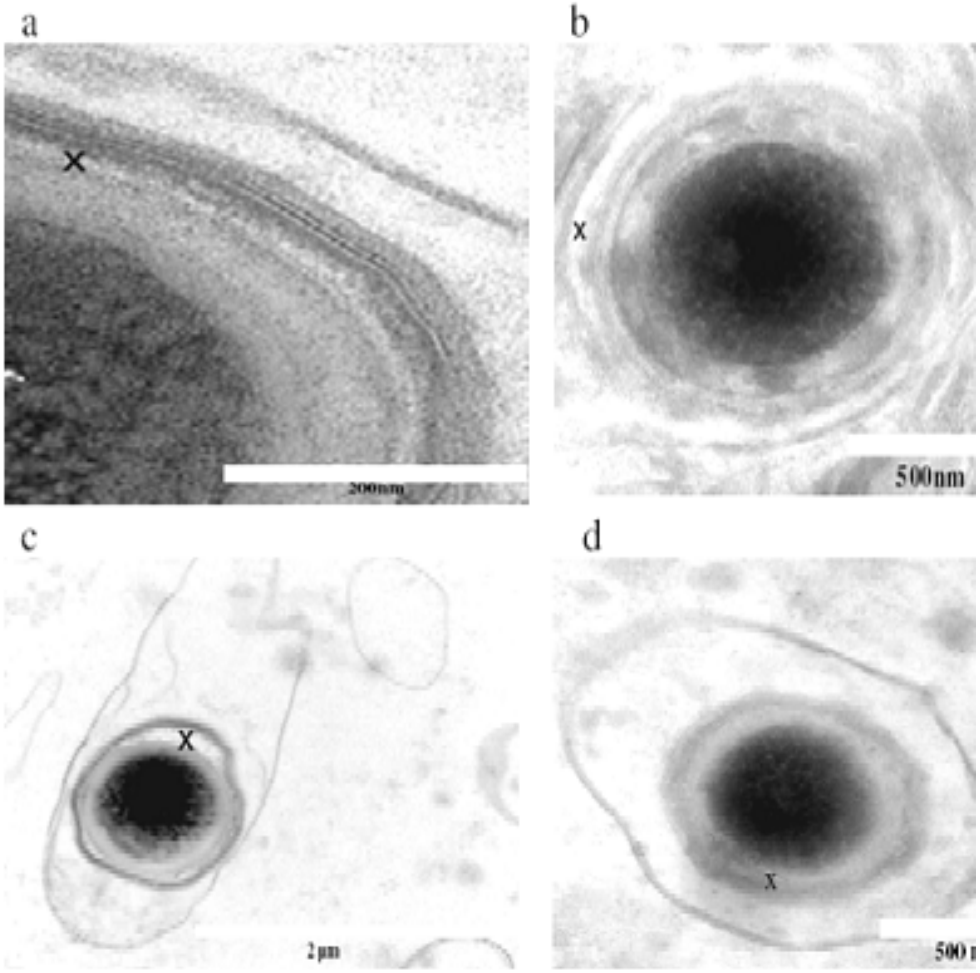

Figure 4.5 a, b, c & d: Micrographs generated from TEM analysis, showing the structure of the spore of *Bacillus sporothermodurans* UP20A, heat treated at 130 °C for 4 min. NB: The spore structures of both strains as revealed by TEM analysis were similar.

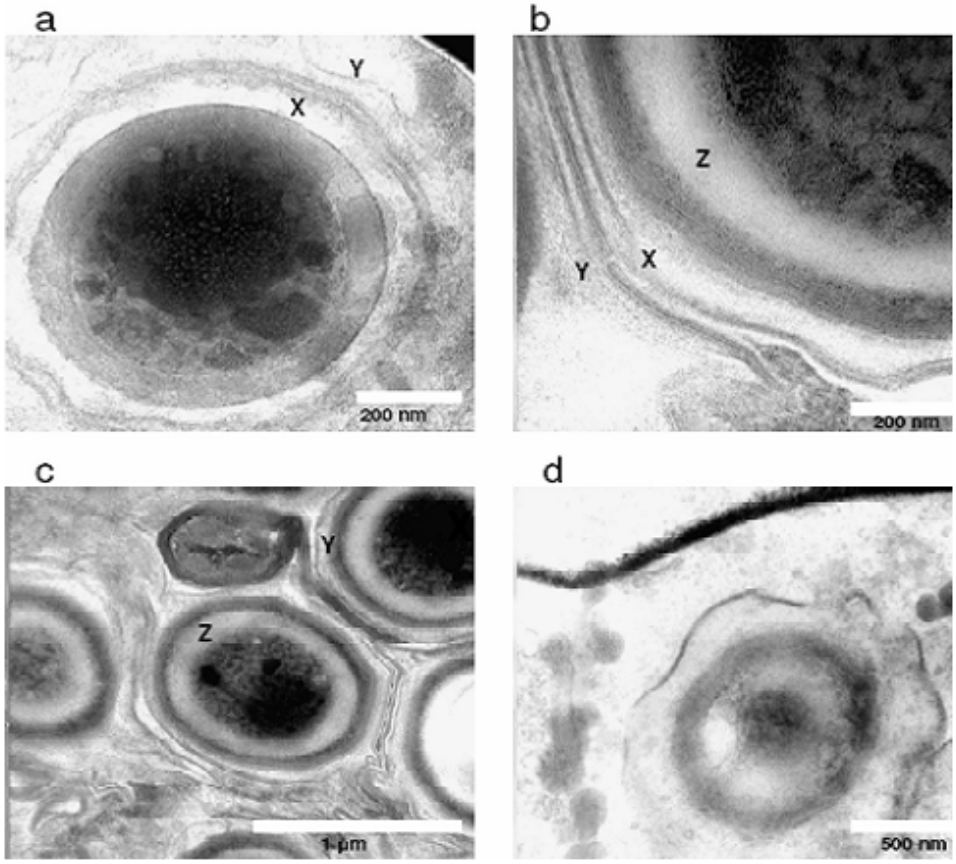

Figure 4.6 a, b, c & d: Micrographs generated from TEM analysis, showing the structure of *Bacillus sporothermodurans* UP20A spores, heat treated at 130 °C for 8 min. NB: The spore structures of both strains as revealed by TEM analysis were similar.

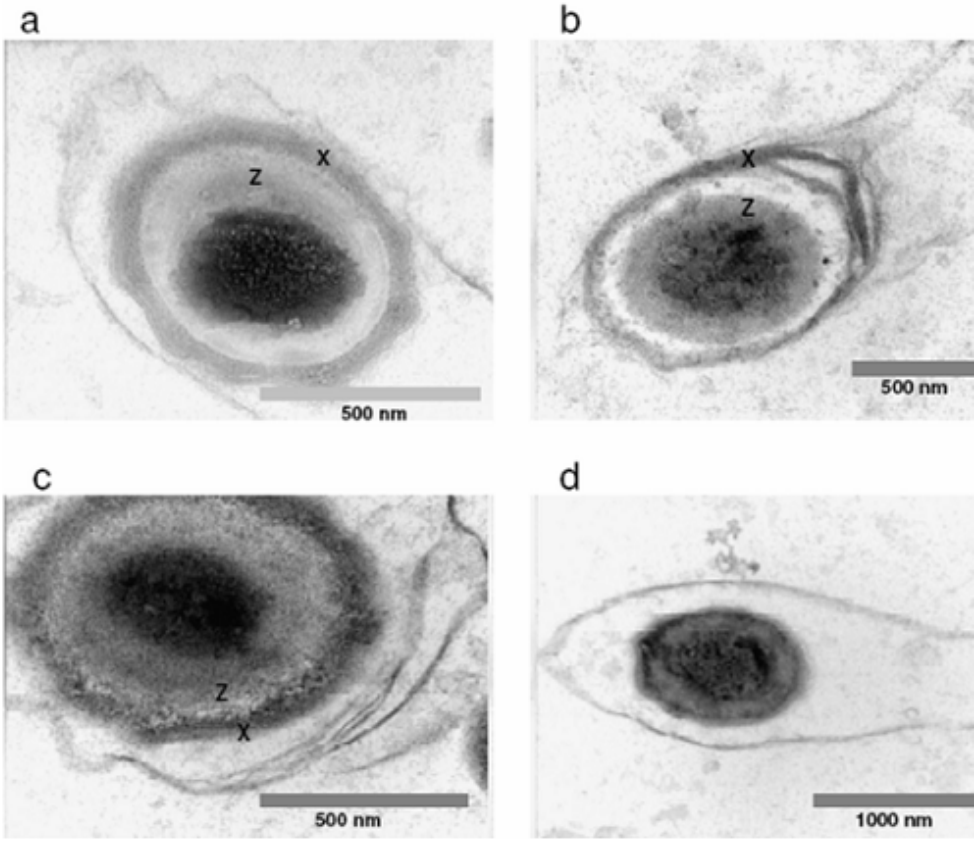

Figure 4.7 a, b, c & d: Micrographs generated from TEM analysis, showing the structure of *Bacillus sporothermodurans* UP20A spores, heat treated at 130 °C for 12 min. NB: The spore structures of both strains as revealed by TEM analysis were similar.

4.2.4.2 Spore viability following treatment at 130 °C

Spores from both strains reacted in a similar way to heat treatments, considering that neither the strain effect nor the strain-time interaction effect on the survival count were not significant at $p = 0.36$ and $p = 0.84$ respectively. Conversely, the time effect on the survival count was significant at $p \leq 0.05$. The effect of time is shown by the 7.5 log reduction in the survival of spores after heating for 4 min for both strains. Spores of neither strain survived heating for 8 min (Figure 4.8).

4.2.4.3 Protein released from Bacillus sporothermodurans spores following treatment at 130 °C

The time effect and the strain effect on the release of soluble protein were significant at $p \leq 0.05$. Similarly, the time-strain interaction effect on the release of soluble protein was also significant at $p \leq 0.05$. Looking at the trend of soluble protein (µg/ml) released from both strains it can be seen that there was a significant increase in the amount of soluble protein released after each heating period, which explains the time effect on the amount of protein released (Figure 4.9).

The release of soluble protein was similar for both strains after heating for 4 min but differed significantly after heating for 8 min during which the protein released from DSM 10599 spores was higher. However, by 12 min the protein released from the DSM 10599 spores slowed to a value which was not significantly different from that released from the UP20A spores.

4.2.4.4 DPA from Bacillus sporothermodurans spores following treatment at 130 °C

The time effect and the time-strain interaction effect on the release of DPA from heated spores were significant at $p \leq 0.05$. On the other hand, the strain effect on the release of DPA was not significant at $p = 0.59$. Looking at Figure 4.10, it can be seen that there was a non-significant increase in the release of DPA after heating for 4 min for both strains. After 8 min of heating there was a significant increase in the DPA amount of UP20A, while for DSM 10599 the increase was not significant.

A reverse trend was observed after heating for up to 12 min in which the amount of DPA released from UP20A was not significant while that for DSM 10599 was significant. Looking at the overall trend, there was a gradual increase in the release of DPA over time, explaining the strain effect. Between 4–12 min of heating, both strains reacted differently over time, explaining the time-strain interaction effect (Figure 4.10).

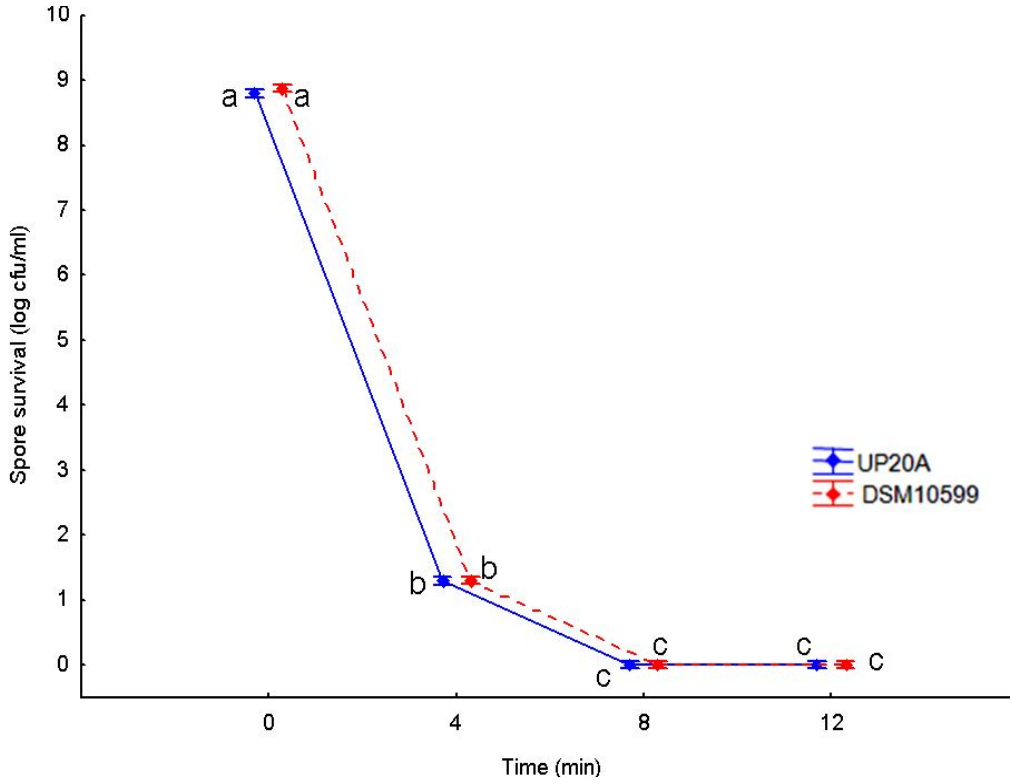

Figure 4.8 Inactivation trend of spores of two strains of *B. sporothermodurans*: DSM 10599 and UP20A, following heat treatment at 130 °C at different times. Different letters a, b and c denote statistical differences at $p \leq 0.05$ (n = 3). Log cfu/ml of 0 represent counts ranging from 1 to 0 spore/ml (n = 3).

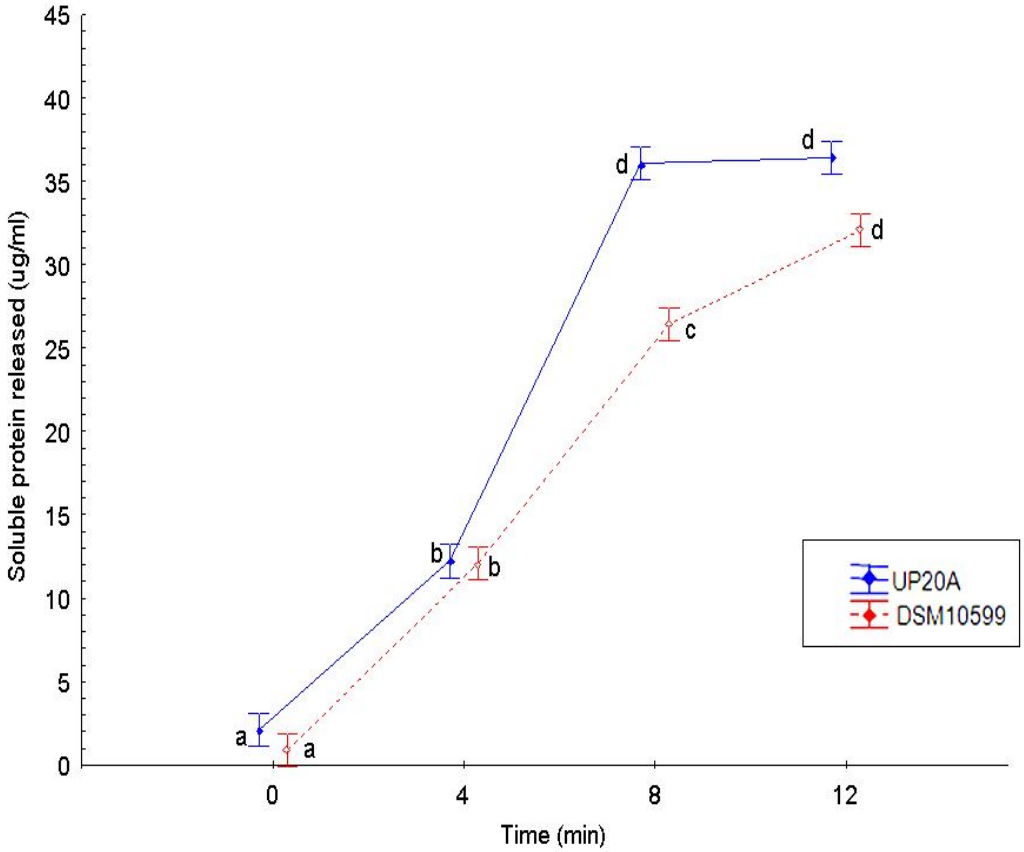

Figure 4.9 Protein released (µg/ml) from spores of two strains of *Bacillus sporothermodurans*: DSM 10599 and UP20A, following heat treatment at 130 °C at different times. Different letters a, b and c denote statistical differences at $p \leq 0.05$ (n = 3). Log cfu/ml of 0 represent counts ranging from 1 to 0 spore/ml (n = 3).

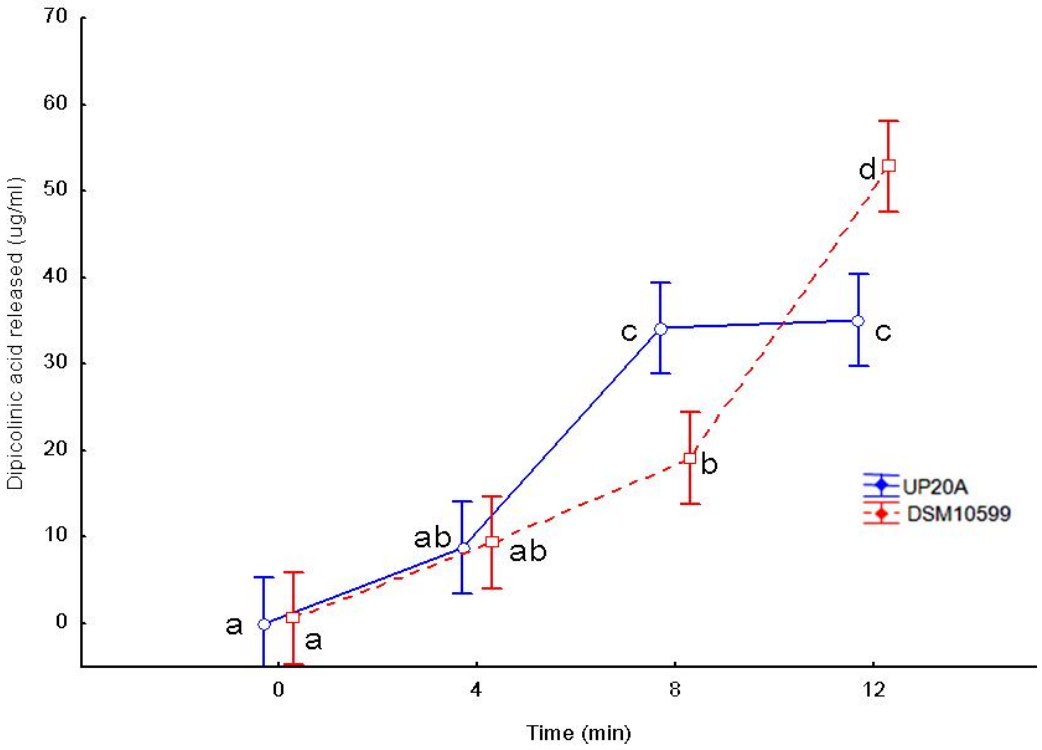

Figure 4.10 DPA released (µg/ml) from spores of two strains of *Bacillus sporothermodurans*: DSM 10599 and UP20A, following heat treatment at 130 °C at different times. Different letters a, b and c denote statistical differences at $p \leq 0.05$ (n = 3). Log cfu/ml of 0 represent counts ranging from 1 to 0 spore/ml (n = 3).

4.2.5 Discussion

The TEM did not reveal any structural differences between the unheated spores and treated spores of both strains. This could be attributed to structural similarities between the two strains. On the contrary, the release of protein (µg/ml) was significantly ($p \leq 0.05$) different between the two strains. This could be attributed to structural difference in protein composition of both strains considering that the composition of the exosporium and coat structures of *Bacillus* spores have been found to be species and strain specific (Henriques & Moran, 2007). However, the release of DPA throughout the heating times was not significantly different between the two strains. This is in agreement with similarities observed in the inactivation pattern and structural damage of both strains at different heating times.

From the TEM, spores began losing their structural components after 4 min of heating. The cortical membrane was the area first affected by wet heat, which was where the first visible signs of structural damage appeared. The reason for this early damage is due to the washing away of peptidoglycan materials, which are the main constituent of the cortex (Setlow, 2006). Perhaps heat penetrated the spore layers and entered the spore core resulting in the release of more protein than DPA. This finding is in agreement with findings of Coleman, Chen, Li, Cowan and Setlow (2007) on *Bacillus subtilis* spores in which DPA release was accompanied by a large amount of protein damage.

The fact that a small proportion of spores did not show visible structural damage after 4 min of heating could be that damage is not acquired at the same rate by all spores. This is in agreement with findings of a similar study in which *B. stearothermophilus* spores were found to be inactivated at different rates following heating at UHT temperatures (Feeherry, Munsey & Rowley, 1987). The more dehydrated spores are, the more their resistance to wet heat treatment increases (Setlow, 2006).

The majority of spores were inactivated after 4 min of heating during which the amount of DPA released was very small. This result is in agreement with findings in which the rate of DPA release was found to be slower than the rate of spore death following heating at UHT temperature (Mallidis & Scholefield, 1985). The appearance of DPA was a clear sign of a breach in the protective barrier of spores following wet heat treatment and the onset of spore inactivation. More research needs to be done to determine the exact amount of DPA to be released for inactivation to take place.

After 8 min of heating there was total inactivation of spores. The cortical membrane lost most of its components at which point the structural damage became clearer on the micrographs. This is due to the fact that continued heat induces damage, which results in the washing away of more peptidoglycan from the cortex. Similarly, the spore core and exosporium also lost some of their components after 8 min but the damage was not severe compared to that of the cortex. The reason why the cortex

degraded faster than the exosporium and the spore core could be due to differences in their components. This implies the peptidoglycan matrix is more susceptible to wet heat treatment.

When compared to the small amount of DPA released after 4 min when the majority of spores were inactivated, a significant ($p \leq 0.05$) amount of DPA was released only after total inactivation. This finding is in agreement with studies conducted on *B. subtilis* spores (Coleman *et al.*, 2007). Therefore, DPA release is an indication that considerable destruction of spores has taken place and is not linked to spore resistance.

By 12 min, there was complete destruction of spore components with the cortex almost washed off completely leaving behind a reduced and less dense spore core surrounded by a loosely attached coat and surface layer. This is due to the total destruction of spores after prolonged heating which resulted in the flushing out of a considerable amount of soluble protein from spores. Levelling off of protein release occurs between 8–12 min of heating. Contrary to soluble protein, the amount of DPA release at this stage did not level off. As cited earlier, this is attributed to the fact that DPA is located exclusively in the spore core which was the last area in which visible structural damage was noted during the entire heating time as seen on the TEM (Kuwana, Kasahara, Fujibayashi, Takamatsu, Ogasawara & Watabe, 2002).

By integrating the TEM, protein and DPA analyses, the mechanism of *B. sporothermodurans* spore inactivation with time can be summarised. Heat penetrates into the spore core. Hot moisture follows thereafter and rehydrates the spore. Finally, spore structures are destroyed resulting in a massive inactivation of spores (small amounts of DPA leak out) to total inactivation of spores (large amounts of DPA leak out).

4.2.6 Conclusion

The onset of DPA release during wet heat treatment coincides with visible signs of structural damage and significant inactivation of spores. Visible signs of spore structural damage emanate at different rates. The amount of protein released seems to be strain specific.