Phenotypic and Genotypic characterisation of *Bacillus sporothermodurans*.

By

**Alessandra Cremona**

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**Declaration**

I declare the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria, has not yet been previously submitted by myself for a degree at any other university or institution of higher education.

Alessandra Cremona
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To God for giving me the strength and allowing me to have the opportunity complete my MSc.

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Abstract

Phenotypic and Genotypic characterisation of Bacillus sporothermodurans strains and their spores

By Alessandra Cremona

Supervisor: Prof E. M. Buys

Department: Food Science

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Ultra high temperature (UHT) method is a thermal heat treatment of milk that is able to destroy non-sporoformers and sporeformers. The UHT process uses a temperature of 135-140°C for 1-2 seconds. Bacillus sporothermodurans is the only known species whose spores are able to survive the UHT treatment. With companies having the disadvantage of not being able to increase the temperatures during milk processing as a result of negative sensory properties, a better understanding of the structure of B. sporothermodurans spores and how exactly B. sporothermodurans is able to survive such high temperatures has become of great importance in the dairy industry.

The first objective of this study was to determine whether there was a genotypic difference in the structure of B. sporothermodurans spores between those strains isolated from UHT strains and those from other sources (i.e. raw milk, feed concentrate and silage). Due to the fact that B. sporothermodurans spores are becoming more widespread, other genetic tools were used to help confirm the presence of these strains and whether they have the heat resistant clonal gene, thereby confirming whether the spores are heat resistant (HRS) or not. Two recently isolated strains (i.e. F3 isolated from UHT milk in South Africa and QA1 isolated from UHT milk in Belgium) were identified using the general B. sporothermodurans method (BSPO PCR) and the more specific HRS-PCR method identifying the HRS clone. Thereafter the (GTG)_5 PCR method was used to compare 9 B. sporothermodurans strains previously obtained from UHT milk or farm sources in different countries. Results showed that the two recently isolated UHT strains, QA1 and F3, had a close association shown by the (GTG)_5 PCR patterns and were positive for the HRS clone. Results go on to show that the B. sporothermodurans strains of the HRS clone look to be primarily responsible for the production of heat resistant spores and that
(GTG)$_5$ PCR is a method that can be used to evaluate the genotypic differentiation of B. sporothermodurans strains.

The second objective was to determine the effect of heat on the structure of Bacillus sporothermodurans spores. The spores of three B. sporothermodurans strains namely F3 (HRS strain isolated from UHT milk in South Africa), DSMZ 10599 (one of the first HRS strains isolated from UHT milk in Italy) and MB 1499 (a non HRS strain isolated from a feed concentrate in Belgium) were compared using various microscopic techniques. When observing the layers, an exosporial layer was present in all three strains. The second layer, the coat, showed to have multiple layers making up the spore coat. The first layer was the outer coat layer; the middle coat was made up of 4-5 lamellae layers and the last layer was the inner spore coat layer. A short, thick appendage like structure was observed in F3 developing from the middle coat layer.

Differential scanning calorimetry was conducted on the B. sporothermodurans strain DSMZ 10599. The initial scan (thermogram from DSC) of DSMZ 10599 showed 3 transitions, one at 79°C, the second at 87°C and the third at 113°C. The initial peak was associated with the activation of the spores due to the heat. The other two endothermic peaks were most likely due to protein denaturation of the spore coat or cortex, or DNA unfolding in the spore core. The heat values were found to increase from the first to the last transition. UHT milk is heated to temperatures of up to 140°C and the DSC profile showed that spore death of strain DSMZ 10599 did not occur at this high temperature.

The third objective was to determine the effects of H$_2$O$_2$ on the structure of B. sporothermodurans spores. The strains F3, DSMZ 10599 and MB 1668 were compared in terms of susceptibility to hydrogen peroxide. After 3 minutes, the exosporial and coat layer were completely degraded with significant swelling of the spore core. Rupture of the core of strains DSMZ 10599 and F3 occurred at 6 minutes. At 9 minutes, the core outer layer of F3 and MB 1668 was completely degraded however, not fully for DSMZ 10599. At 15 minutes the core was exposed to the environment however even though DSMZ 10599 core layer was still not completely degraded, the core was still exposed to the environment.

It was concluded that (GTG)$_5$ PCR is a method that can be used to evaluate the genotypic differentiation of B. sporothermodurans strains. When observing B. sporothermodurans
phenotypically, for the first time, short appendage like structures were present protruding from the spore coat which may explain attachment to steel pipe surface resulting in post and cross contamination in UHT milk. A further investigation would be interesting to determine whether this species is able to produce a biofilm. It was also concluded that *B. sporothermodurans* spore structures do contribute to the resistance of heat and chemicals. It would be interesting to determine whether the damage done to the spore by heat is reversible.
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Chapter 1: Introduction & Problem statement

Ultra high temperature (UHT) method is a thermal heat treatment that is able to destroy non-sporeformers and sporeformers. The UHT process uses a temperature of 135-140°C for 1-2 seconds (Jay et al., 2005). As a result, one is able to store the UHT milk without refrigeration as there should be destruction of any vegetative cells and endospores present in the milk (Pettersson et al., 1996). Bacillus sporothermodurans is the only known species whose spores are able to survive UHT treatment.

B. sporothermodurans is a facultative thermophile which produces endospores and is often found in UHT milk (Montanari et al., 2004; Pettersson et al., 1996). B. sporothermodurans were first detected in UHT milk from Germany in the 1990s’ (Scheldeman et al., 2006); however detection of B. sporothermodurans spores has become more widespread and has been noted in countries such as France, Spain as well as South Africa (Pettersson et al., 1996; Tabit & Buys, 2010). Other contaminated products include whole, skimmed and reconstituted milk, UHT cream, chocolate milk, milk powder, silage and feed concentrate (Klijn et al., 1997). The presence of B. sporothermodurans spores may cause future problems in the milk, such as a decrease in shelf life of flavoured milk products if not stored under the correct conditions (Klijn et al., 1997; McKillip, 2000).

Due to the fact that B. sporothermodurans spores are becoming more widespread, other genetic tools are used to help confirm the presence of these strains and whether they have the heat resistant clonal gene. These tools will confirm whether the spores are heat resistant (HRS) or not. A number of PCR methods have been used to characterize B. sporothermodurans such as random amplified polymorphic DNA analysis, repetitive extragenic palindromic (REP)-PCR and 16S ribosomal DNA (rDNA) sequence analysis and HRS-PCR (Herman et al., 1998). HRS-PCR is a method used to determine whether B. sporothermodurans strains have the HRS clone (Herman & Heyndrickx, 2000). (GTG)₅ PCR is a fairly new technique used to differentiate and characterize bacterial organisms such as lactobacilli and enterococci and has not been used on B. sporothermodurans. This method may be potentially beneficial as these primers are easily obtained internationally therefore becoming a more simple tool for strain identification between laboratories (Wiid et al., 1994).
Although genotypic characteristics of *B. sporothermodurans* spores play a role in heat resistance, phenotypic characteristics may also play a role. The main components of the *B. sporothermodurans* spore are the exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and central core (Driks, 2002). A less common layer, an outer loosely fitted exosporium layer is present in certain species such as *Bacillus cereus* (Appendix 1) and *B. sporothermodurans*. Other structures such as appendages are present in *B. cereus* spores (Wrath, 1987). Appendages have been observed to protrude from the exosporium such as from *B. cereus* and *B. thuringiensis*, whereas certain appendages emerge from the coat layer. Appendages attach themselves to a surface allowing for germination to occur and the growth of vegetative cells (Panessa-Wareen, 2007). Studies have shown that spores of *B. cereus* adhere better to hydrophobic than hydrophilic surfaces. It has been suggested that spores adhere to surfaces by penetrating the potential barriers and increasing the hydrophobicity of the spore surface (i.e. adhered stainless steel surfaces found in processing equipment) (Husmark & Ronner, 1990). The degree of hydrophobicity and the presence of appendages have not yet been determined for *B. sporothermodurans* spores.

Microscopes such as atomic force microscopy (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are powerful tools for studying natural surfaces of biological material. Several studies of spore surface morphology suggest that closely related species may be differentiated by their surface structures (Zolock et al., 2006). Therefore using various microscopic techniques may give a better insight into the structure of the *B. sporothermodurans* spores.

A number of conditions such as chemicals and heat may affect the structure of the spore. Due to noticeable changes in sensory and nutritional quality in the milk due to high temperature and long time processing, this treatment would not be viable in the dairy industry. For one to determine the safety of a product, one needs to determine the heat resistance of spores in the product in question (Palop et al., 1999). Research of *B. sporothermodurans* spores has shown that the spore core and its genetic content do play a role in heat resistance however it has not been demonstrated what the extent of heat resistance is, that spore layers impart. The level of spore heat resistance may depend on a number of factors such as the amount of various heat-labile macromolecules important for germination and outgrowth, and the intrinsic stability of these macromolecules as well as the environment in which the spores are formed (Lindsay et al., 1990). One way to determine at what temperature the spore coat layers are affected and how much energy is needed to denature the proteins, is the use of differential scanning
calorimetry (DSC). The effect of heat treatment has been determined using differential scanning calorimetry (DSC) on a number of bacterial spores such as *Bacillus subtilis* and *Bacillus megaterium*. The DSC measures heat absorbed or lost from matters at specific temperatures. When a vegetative bacterial cell is placed in a DSC, a number of disturbances occur to the specific heat capacity of the cell. This is recorded as an exothermic or endothermic transition (Belliveau *et al.*, 1992). Endothermic peaks are often related to protein denaturation as well as DNA and RNA unfolding, depolymerization of complex cell structures and denaturation-induced aggregation of macromolecules. Depolymerization and aggregation reactions are more complex and can either be endothermic or exothermic (Lepock *et al.*, 1988). The large exothermic peak is often associated with spore death. The transitions were irreversible which was most likely due to the unfolding and the denaturation of the protein (Belliveau *et al.*, 1992). The peaks also play a role in the DSC scan profiles. The higher the peak, the more energy is needed to denature the proteins. Therefore, at each peak, there was either a high protein concentration or a number of different proteins with peaks at the same temperature. Albett *et al.* (1999) gave two reasons for endothermic events occurring. The first reason is due to enthalpy of the peak increase with an increase in moisture content. The second reason is that temperature peaks increase by annealing sample. The DSC profile of *B. cereus* showed the first endothermic peak occurred at 56°C. Another two endothermic peaks occurred at 95°C and 103°C and an exothermic peak at 105°C (Maeda *et al.*, 1978), whereas the DSC profile of *B. megaterium* had three major endothermic peaks at 56, 100 and 114 °C (Belliveau *et al.*, 1992). The degree of protein denaturation differs at different temperatures. Even though the activation temperatures were the same, *B. megaterium* seemed to show protein denaturation of the spore coat at a higher level thereby concluding that *B. megaterium* spores are able to withstand higher temperatures than *B. cereus* spores. Maeda *et al.* (1978) also concluded that the thermodynamic process mainly occurred in the spore since this coat is made up of proteins and disulfide bonds that are mainly denatured at 56 °C. One can observe from these results that every spore from different species seem to be unique in terms of their ability to withstand high temperatures.

Bacterial spores are resistant to a number of chemicals however there are only certain chemicals which are effective in spore killing (e.g. formaldehyde, nitrous acid, alkylating agents) (Russell, 1990; Setlow, 2006). Spore killing by chemicals is mainly by DNA damage as a result of survivors accumulating mutations which in turn will sensitize the spores to these chemical agents. The precise damage to the inner membrane however is not known (Setlow, 2006).
Packaging material is often sterilized by a number of different methods such as heat, hydrogen peroxide, irradiation, infrared light, etc. With the use for example of hydrogen peroxide (H₂O₂), the chemical is seen as an indirect sterilant of food or milk (Ansari & Datta, 2003; Russell, 1990). H₂O₂ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivates the proteins (Melly et al., 2002). The main target for spore killing by H₂O₂ is at the exterior of the spore’s inner membrane (Melly et al., 2002). Tabit & Buys (2010) have shown that the *B. sporothermodurans* spore concentration decreased significantly after an increase in time in H₂O₂, however viable spores were still present after treatment. The extent of damage of the *B. sporothermodurans* spore after H₂O₂ treatment has yet to be determined microscopically.

In this study the morphological characteristics of heat resistant and non-heat resistant *B. sporothermodurans* spore strains were compared. With investigations having been done on the spore surface morphology of *Bacillus* spp. such as *B. cereus, B. thuringiensis, B. anthracis* as well as *B. globigii*; there is little information on the surface morphology of *B. sporothermodurans* spores found in UHT milk and farm sources. The effect of H₂O₂ on the structure of *B. sporothermodurans* spores was also determined as well as at what temperature the structure of *B. sporothermodurans* spores were most affected.
Chapter 2: Literature Review

2.1. Introduction

Milk is made up of 87% water, 3.5% protein, 3.9% fat, 4.9% carbohydrate and vitamin B. Due to the high nutrient content of milk, it becomes an ideal growth medium for microorganisms (Jay et al., 2005). Contamination may occur from Gram-negative bacteria that contaminate the milk after heat treatment or from Gram positive bacteria that are able to survive heat treatment (such as UHT processing) (i.e. Bacillus spp. such as B. subtilis, B. sporothermodurans and B. cereus) (Ternstrom et al., 1993). Survival of heat treatments are due to certain bacterial species such as Bacillus sporothermodurans producing highly heat resistant spores, hence its original designation as a heat resistant spore former or HRS (Scheldeman et al., 2006). It may occur that these HRS spores survive the UHT treatment thereby passing into the final product. These spores will begin to germinate and grow in the processed milk due to little or no competition from the faster growing vegetative cells (Pettersson et al., 1996; Scheldeman et al., 2006).

Contamination of milk from B. sporothermodurans not only occurs post heat treatment but may also occur at the farm. A contamination source is cattle feed whereby spores are able to survive in the digestive track of cattle and are as a result, shed in the faeces (Varewijck et al., 2001). However past research has shown that farm isolates do not have the HRS clone and are therefore easily killed during heat treatment (Scheldeman et al., 2002). Therefore the nature and origin of these spores is of great importance as this information may contribute to reduction of contamination and therefore improvement in the quality of dairy products (Varewijck et al., 2001).
2.2. Milk processing

2.2.1. Raw milk

Generally, the milk is sterile in the mammary gland of the milk; however contamination sources from the farm, worker hygiene and in factories may cause contamination of the milk (Smigic et al., 2012). Sources of contamination from the farm may be from the udder, the milker or the environment (Table 1). Soiling of the udder may play a large role in the contamination of raw milk with spores. Another form of contamination source is cattle feed whereby spores are able to survive in the digestive track of cattle and as a result shed in the faeces (Varewijk et al., 2001).

Human outbreaks from the consumption of raw milk are frequently reported from Campylobacter species and Salmonella species. The only possible benefit in consuming raw cow milk is nutritionally (i.e. proteins, fats, vitamins and minerals) because heat processing tends to reduce nutritional contents such as heat sensitive vitamins (Claeys et al., 2013).

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2.2.2. Pasteurisation & Ultra High Temperature processing

Pasteurisation is either a low temperature-long time heat treatment, which occurs at a temperature of 63°C for 30 minutes or a high temperature-short time heat treatment occurring
at 72°C for 15 sec (De Buyser et al., 2001). Pasteurised milk presents minimal hazards; however outbreaks linked to pasteurisation, post-pasteurisation contamination or storage temperatures have nevertheless occurred. The main aim of pasteurisation is to kill any disease causing microorganisms such as *Bacillus cereus*, *Clostridium botulinum* and *Clostridium sporogenes* and to increase the shelf-life of dairy products (Scheldeman et al., 2006).

The ultra high temperature (UHT) processing method is a thermal heat treatment that is able to destroy non-sporeforming and sporeforming organisms. The UHT process uses a temperature of 135-140°C for 1-2 seconds (Neuman et al., 2010). As a result, one is able to store the UHT milk without refrigeration as there should be destruction of any vegetative cells and endospores present in milk (Pettersson et al., 1996).

![Flow diagram for the production of UHT milk](image)

**Figure 1: Flow diagram for the production of UHT milk** (Jay et al., 2005)

UHT milk processing can be carried out with two methods namely direct and indirect heating (Fig. 1). With the indirect method, milk is treated in tube or plate heat exchangers with high operating pressures used to prevent boiling of the milk. With direct heating, milk is preheated...
using a regenerative heat exchanger then heated up to 140°C with superheated steam by injection or infusion (Jay \textit{et al.}, 2005).

2.3. \textit{Bacillus}

2.3.1. Introduction

Bacilli are facultatively thermophilic Gram positive sporeforming rods and depending on the strain, can grow at mesophilic and thermophilic temperatures. Examples of \textit{Bacillus} spp. are \textit{B. coagulans}, \textit{B. sporothermodurans} and \textit{B. subtilis}. The sporeforming \textit{Bacillus} species are often reported to be found in raw milk. \textit{Bacillus} species are also frequently found in pasteurised milk, UHT processed milk, milk powder, cheese and yoghurt (Klijn \textit{et al.}, 1997).

One of the main problems that arise is that certain \textit{Bacillus} spp. produce hydrophobic spores, which are able to adhere to steel. \textit{Bacillus} spp. are also involved in biofilm formation in various dairy processes. This poses a problem in the food industry due to the species strong adherence to surfaces and resistance to cleaning procedures. Another problem that may occur is that the bacteria detach from the steel and cross contaminate products during processing which may be problematic (Faille \textit{et al.}, 2010). The only positive aspect with regards to the presence of thermophilic bacilli found in milk products is that it may provide an advantage to processors as they give an indication of the hygiene of the processed product (Burgess \textit{et al.}, 2010).

2.4. \textit{Bacillus sporothermodurans}

2.4.1. Introduction

\textit{B. sporothermodurans} are facultative thermophiles that produce endospores and are most often found in UHT milk. \textit{B. sporothermodurans} are rod shaped cells with an average length of 10μm and width of 0.5μm. The spores of this strain were cylindrical and had an average width of 1.1-1.2μm and width of between 0.7-0.8μm (Montanari \textit{et al.}, 2004). The \textit{B. sporothermodurans} colonies are beige in colour and appear as a string of pearls when viewed under a light microscope (Montanari \textit{et al.}, 2004; Pettersson \textit{et al.}, 1996). The \textit{B. sporothermodurans} spores may also appear as pinpoint translucent colonies on plate count agar when incubated at 37°C. Vegetative cell growth was $10^5$ and $10^3$ for spores respectively after incubation at 37°C for 15 days in an unopened UHT milk package (Montanari \textit{et al.}, 2004).
B. sporothermodurans was first detected in UHT milk from Germany in the 1990s’ (Scheldeman et al., 2006). However, detection of B. sporothermodurans spores has become more widespread in UHT milk from countries such as France, Spain as well as South Africa (Pettersson et al., 1996; Tabit & Buys, 2010). A South African B. sporothermodurans strain, namely UP20A, was examined under TEM by Tabit & Buys (2010). The core was approximately 500 nm in diameter, surrounded by the cortex, which was 62 nm wide, and the coat was 187 nm wide (Tabit & Buys, 2010). B. sporothermodurans is not only found in milk but also in silage and feed concentrate. Varewijck et al. (2001) found that feed concentrate might be one of the sources of contamination for raw milk. However, the strains differ genetically as the HRS clone is present in UHT milk strains and not feed concentrate strains (Scheldeman et al., 2006).

2.4.2. Spore layers of B. sporothermodurans

The spore structure and its chemical composition plays an important role in the spores’ heat resistance. The main differences between the bacterial species are mainly the number of spore coat layers, the type and the numbers of proteins present (Burgess et al., 2010). The main components of the spore are the exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane and central core (Fig. 2).

![TEM images of a B. sporothermodurans spore](image)

Figure 2: TEM images of a B. sporothermodurans spore (Tabit & Buys, 2010)

The sporulation process of B. sporothermodurans spores occur when the forespore (internal compartment) is formed within the bacterial cell. Before sporulation can occur, the mother cell must first sense a number of complex parameters such as the nutrient level or optimal temperate
conditions (Burgess et al., 2010; Driks, 2002). Once the cell has committed to sporulate (i.e. under stressful conditions), it forms a septum on the side of the cell compartment producing a smaller forespore (Driks, 2002). Thereafter the forespore is engulfed by the mother cell resulting in the formation of two membranes (Palop et al., 1999). The germ cell wall and the cortex are formed between the membranes. At this point, calcium and other minerals accumulate in the forespore. Other compounds such as dipicolinic acid (DPA) are synthesised in the mother cell and transported to the forespore (Palop et al., 1999). Thereafter a thick protein shell, the core, encases the forespore (Driks, 2002). Maturation occurs after coat assembly and cortex formation in which mineralization and dehydration of the spore takes place, which leads results in it becoming heat resistant. The mother cell then lyses and releases the spore (Palop et al., 1999).

Most often, the spore coat layer makes the first contact with the environment however in certain cases such as with B. sporothermodurans spores, the exosporium layer is present (Nishihara et al., 1980). Starting with the outermost structure, the exosporium is a loosely fitted layer, which is not found in all spores of Bacillus spp. (Plomp et al., 2005). The exosporium is made up of a paracry stalline basal layer surrounded by a hair like nap. These hair like naps are made up of a number of collagen-like glycoproteins and have not been observed on the exosporium of B. sporothermodurans as of yet (Faille et al., 2010). The exosporium layer is made up of glycoproteins and is said to be an extension of the outermost coat layer. The function of this structure is not really known however, it is said to be important in spore interaction with the target organisms (Setlow, 2006).

The spore coat is made up of several layers containing more than 50 different proteins with a smaller amount of carbohydrates and lipids in species such as B. subtilis, which are spore-specific, and gene specific (Russell, 1990; Setlow, 2006). The functions of these coat proteins are not known however, they may play a role in spore coat and exosporium assembly (Setlow, 2006). Without the exosporial layer, most Bacillus spp. have ridges and bumps on the coat layer. The ridges are usually due to dehydration causing folds in the spore layers. The patterns of these ridges are found to differ according to species. For example, ridges of B. anthracis spores run along the long axis of the spore whereas B. cereus ridges run in a zig zag pattern (Chada et al., 2003).
Appendages have been observed to protrude from the exosporium such as from *B. cereus* and *B. thuringiensis*, whereas certain appendages emerge from the coat layer. Appendages attach themselves to a surface allowing for germination to occur and the growth of vegetative cells (Panessa-Wareen, 2007). Studies have shown that spores of *B. cereus* adhere better to hydrophobic than hydrophilic surfaces. It has been suggested that spores adhere to surfaces by increasing the hydrophobicity of the spore surface (i.e. adhered stainless steel surfaces found in processing equipment) (Husmark & Ronner, 1990). There are a number of aspects that affect the adherence of *B. cereus* spores to surface. Firstly, added ethanol decreases the hydrophobic effect. Secondly, altering the pH negatively affects the adherence. This is due to the electrostatic repulsion between the spore and the glass surface, which are both negatively charged (Husmark & Ronner, 1990). The degree of hydrophobicity and the presence of appendages have not yet been determined for *B. sporothermodurans* spores.

These attached cells may also produce biofilms, thereby becoming more resistant to sanitizers. Biofilms are defined by Costerton *et al.* (1987) as “a functional consortium of microorganisms attached to a surface and is embedded in the extracellular polymeric substances (EPS) produced by microorganisms”. These biofilms are a menace in the dairy industry because they can reduce the heat flow across the surface, increase the fluid frictional resistance at the surfaces and increase the corrosion rate at the surface resulting in product loss (Ganesh Kumar & Anand, 1998). The potential for *B. sporothermodurans* to form biofilms has not yet been determined.

The next layer is the spore coat, which plays a large role in resistance of physical and chemical stresses and protection of the spore core containing the DNA (Nicholson *et al.*, 2000; Plomp *et al.*, 2005). The middle layer of the coat is made up of 3-6 lamellae layers protected by an inner and outer coat layer. The thick spore coat contains a number of different proteins and protects the peptidoglycan cortex from lytic enzymes and chemicals such as gluteraldehyde (Melly *et al.*, 2002). The thickness of the coat layers varies with the strains and species (Henriques & Moran, 2007).

Then there is the outer membrane, which does not have a clear function although it may play a role in spore formation. The removal of the outer membrane provides no noticeable resistance to heat, radiation and certain chemicals. Compared to the outer membrane, the inner membrane is less permeable, more compressed and plays a big role in the resistance against chemicals. The inner membrane appears to be condensed in the dormant spore (Setlow, 2006).
The next layer is the cortex layer, which consists of peptidoglycan (PG) and has a structure similar to that of vegetative cells however with spore specific components (Atrih et al., 1996; Setlow, 2006). This cortical peptidoglycan layer consists of amino acids and sugar constituents similar to those found in the vegetative cell wall peptidoglycan (Atrih et al., 1996). The cortex is important for the formation of a dormant spore and for the reduction of water in the spore core (Setlow, 2006). The main role of the spore cortex is suggested to be involved in the maintenance of the dormant state of the spore and the role of heat resistance. Dormancy is most likely due to the ability of the cortex to prevent rehydration of the spore core. Very little is known about this cortical layer and the features that assist in dormancy and heat resistance (Atrih et al., 1996).

2.4.2.1. Spore core content

In the spore core of *Bacillus* spp., the small acid soluble proteins (SASP) have been found to be directly associated with the DNA in vivo. There are three major SASPs called, α, β and γ. All three of these SASPs are synthesized during sporulation and degraded during spore germination (Mason & Setlow, 1986). Spores that lack these SASPs are much more sensitive to heat than ones that have these proteins present (Setlow & Setlow, 1995). SASPs are synthesized late during sporulation and make up a high percentage (3-6%) in the spores total protein content. The α/β SASPs, in the spore core, saturate the spore DNA as well as play a significant role in the spore resistance to heat, chemicals and to UV (Setlow, 2006).

Dipicolinic acid (DPA) is also present within the spore core. DPA is synthesized late during sporulation in the mother cell compartment of the sporulating cells and thereafter transported to the forespore (Slieman & Nicholson, 2001). The highest concentration of DPA is present in the spores’ core and is most likely chelated with divalent cations such as Ca²⁺ that comprises
10% of the spores’ dry weight (Plomp et al., 2005; Slieman & Nicholson, 2001). Once spore germination occurs, DPA is immediately released along with the divalent cations (Paidhungat et al., 2000). The accumulation of a large amount of DPA in the core plays a role in the reduction of core water content (Setlow, 2006).

**2.4.3. Genotypic characterisation of *B. sporothermodurans***

As the presence of spores in milk do not affect the pH, stability, or sensory quality of milk, contamination is not often detected. It is however important to detect the presence of *B. sporothermodurans*. Phenotypic tests have been used for the identification of *B. sporothermodurans* however it is time consuming because of the poor growth characteristics (Scheldeman et al., 2002). More rapid tests have been developed by Herman et al. (1997), such as a sensitive and rapid polymerase chain reaction (PCR) test (Scheldeman et al., 2002). A number of PCR methods have been used to characterize *B. sporothermodurans* such as random amplified polymorphic DNA analysis, repetitive extragenic palindromic (REP)-PCR and 16S ribosomal DNA (rDNA) sequence analysis (Herman et al., 1998). Survival of UHT treatments is due to *B. sporothermodurans* producing highly heat resistant spores, hence its original designation as heat resistant spore formers or HRS. The more general *B. sporothermodurans* method (called BSPO PCR) is used to determine whether strains are in fact *B. sporothermodurans* strains, whereas HRS-PCR is a method used to determine whether *B. sporothermodurans* strains have the HRS clone that determines whether the strains are heat resistant or not (Herman & Heyndrickx, 2000).

*B. sporothermodurans* have not only been isolated from UHT milk but also from raw milk, feed concentrate and silage. This was confirmed with research from Scheldeman et al. (2002) where they confirmed the presence (i.e. MB 1317) of a *B. sporothermodurans* strain in feed concentrate, which was the major source. It was also observed that MB 385, isolated from raw milk, was negative for the HRS clone (Herman et al., 1998) and that most strains from silage and feed concentrate did not have the HRS clone except for MB 1505, which was isolated from silage (Herman et al., 1998).

REP-PCR is a method whereby sequences are highly conserved in transcribed sequences (Herman & Heyndrickx, 2000) and is an established method that is recognised for bacterial
typing (Versalovic & Lups, 2002). The use of REP-PCR allows for the identification of *B. sporothermodurans* and differentiating between colony related isolates from UHT and raw milk as well as from feed concentrate and feed silage. Scheldeman et al. (2006) used REP-PCR and ribotyping for milk and farm isolates from different countries. Their results showed a compact cluster mostly made up of the UHT isolates suggesting that there is a clonal origin called the HRS clone, whereas the farm isolates showed a more distributed group (Pettersson et al., 1996). Results from Herman et al. (1998) showed that the grouping of different isolates indicated a common contamination source within or between production entities because of their high genetic homogeneity.

(GTG)$_5$ is also a genotypic technique used to differentiate and characterize bacterial organisms such as lactobacilli and enterococci. (GTG)$_5$ fingerprinting is known to be a promising genotypic tool that allows for more rapid and reliable results (De Vuyst et al., 2008) and has not yet been used in strain identification of *B. sporothermodurans* (Herman & Heyndrickx, 2000). This method may be potentially beneficial as these primers are easily obtained internationally therefore becoming a more simple tool for strain identification between laboratories (Wiid et al., 1994).

2.5. Contribution of environmental stresses to Bacillus spores

2.5.1. Introduction

Stress response of *Bacillus* species are of interest in the dairy industry as it influences their survival during dairy processing. *Bacillus* species may adapt to certain stresses when subjected to the same stress continuously (Henriques & Moran, 2007).

The spore surface layers such as the exosporium and spore coat contribute to the resistance of the spores against extreme physical and chemical stresses. Even though spores are in their dormant state, metabolically inactive, they still interact with the environment. These interactions mainly occur with the exosporium and coat layers. The spore surface layers are flexible thereby expanding or retracting in response to environmental conditions such as humidity (Driks, 2003). The exosporium and coat layer also contain enzymes that increase spore protection, control (modulate) germination and most likely affect the environment and the behaviours of neighbouring organisms. Spore heat resistance depends on a number of
factors such as the number of heat-labile macromolecules important for germination and outgrowth, the stabilization by the spore itself and the intrinsic stability of these macromolecules as well as the environment in which the spores are formed (Lindsay et al., 1990).

Past research showed the spores of *B. subtilis* and *B. coagulans*, produced at different temperatures, had an altered spore heat resistance. Reasons for this may be due to a correlation between the calcium and DPA content (Lechowich & Ordal, 1962). Recently however, direct correlations between sporulation temperature and spore heat resistance in a number of *Bacillus* spp. was ascribed to the involvement of membranes and heat shock proteins (Khoury et al., 1987; Palop et al., 1999). This differed for *B. cereus* whereby the sporulation temperature (Palop et al., 1999) did not significantly influence the heat resistance. However, Palop et al. (1999) demonstrated that the relationship between heat resistance and sporulation temperature is not always linear. Studies suggest that some bacterial species are more sensitive to changes in sporulation temperature at the lower investigated temperatures and some species are more sensitive to changes closer to higher investigated temperatures (Palop et al., 1999).

It is not surprising that spores have obtained a number of different mechanisms to protect themselves during dormancy. These mechanisms protect the spores from damage by various treatments (Setlow, 2006). The opinion of Palop et al. (1999) states that “microorganisms would inherit their capability to sporulate at a given range of temperatures and their final heat resistance would be determined by their sporulation temperature”.

### 2.5.2. Heat Treatment

#### 2.5.2.1. Heat Treatment of *B. sporothermodurans*

There are two types of heat treatments namely dry heat and wet heat. Wet heat uses temperatures above 100°C for short periods. With wet heat, the proteins of the spores are found to be damaged due to denaturation because of the increased water content in the spore core resulting in inactivation of the spore. There is also a large amount of DPA released from the spore (Coleman et al., 2007).

Effective heat treatment against the spores of *B. sporothermodurans* would require longer times (i.e. 8 minutes) at 135-140°C (Tabit & Buys, 2010). When spores such as those from *B.*
*sporothermodurans*, are subjected to extreme heat treatments, even the spores will become inactive (Scheldeman et al., 2006). Due to noticeable changes in sensory and nutritional quality with high temperature and long time, this treatment would not be viable in the dairy industry. For one to determine the safety of a product, one needs to determine the heat resistance of spores in the product in question (Palop et al., 1999).

Using the imaging from the transmission electron microscope (TEM), Tabit & Buys (2010) looked at the structural damage of the spores from heat. Structural damage occurred mainly in the cortical region after 4 minutes. After 8 minutes, the damage of the cortical membrane widened with a loss of the cortical material. At this time, the core was also losing some of its material and the exosporium was more compressed. After 12 minutes, the spores were severely damaged with the exosporium and cortex losing most of their components. The density of the core was also reduced (Tabit & Buys, 2010).

One way to determine at what temperature the spore coat layers are affected and how much energy is needed to denature the proteins, is the use of differential scanning calorimetry (DSC). The DSC measures heat absorbed or lost from matters at specific temperatures. When a vegetative bacterial cell is placed in a DSC, a number of disturbances occur to the specific heat capacity of the cell. This is recorded as an exothermic or endothermic transition (Belliveau et al., 1992). Belliveau et al. (1992) conducted research on *B. magaterium* spores. The DSC scan profile of dormant *B. magaterium* spores showed endothermic peaks at 56, 100, 114°C and a large exothermic peak at 119°C. Belliveau et al. (1992) concluded that because the spore was made up of a number of cellular components, it was difficult to determine which component caused the peak. However, endothermic peaks are often related to protein denaturation as well as to the DNA and RNA unfolding, depolymerization of complex cell structures and denaturation-induced aggregation of macromolecules. Depolymerization and aggregation reactions are more complex and can either be endothermic or exothermic (Lepock et al., 1988). The large exothermic peak is often associated with spore death. The transitions were irreversible which was most likely due to the unfolding and the denaturation of the protein (Belliveau et al., 1992). The peaks also play a role in the DSC scan profiles. The higher the peak, the more energy is needed to denature the proteins. Therefore, at each peak, there was either a high protein concentration or a number of different proteins with peaks at the same temperature. When looking at the DSC profile of *B. magaterium*, the first endothermic (56°C) reaction is associated with spore activation (i.e. germination) and the third endothermic peak
was associated with the outer coat membrane complex (114°C) (Albett et al., 1999; Maeda et al., 1978). However, it is important that water is present for this heat activation to occur. With lower water content, the heat activation peak does not even occur (Maeda et al., 1978). Albett et al. (1999) explained two reasons for endothermic events from occurring. The first, the enthalpy of the peak increase with an increase in moisture content, the second, the temperature peaks increase by carefully annealing the temperature of the sample. In comparison to B. cereus, the first endothermic peak also occurred at 56°C. Another two endothermic peaks occurred at 95°C and 103°C and an exothermic peak at 105°C (Maeda et al., 1978). Their degree of protein denaturation differs at different temperatures. Even though the activation temperatures were, the same, B. megaterium seemed to show protein denaturation of the spore coat at a higher level thereby concluding that B. megaterium spores are able to withstand higher temperatures than B. cereus spores. Maeda et al. (1978) also concluded that the thermodynamic process mainly occurred at the spore coat since this coat is made up of proteins and disulfide bonds that are mainly denatured at 56 °C. The slower these spores germinate the more difficult it is to kill these spores as a result of heat resistance (Maeda et al., 1978). One can observe from these results that spores from different species seem to be unique in terms of their ability to withstand high temperatures.

2.5.2.2. Heat Resistance of B. sporothermodurans

There are three main factors contributing toward the heat resistance of spores namely protoplast dehydration, mineralization (i.e. increased concentration of Ca²⁺) and thermal adaptation (Beaman & Gerhardt, 1986).

Core dehydration is extremely important in the ability of bacterial spores to withstand high temperatures. This dehydration is mainly due to the spore cortex peptidoglycan (Atrih et al., 1996). Heat resistance may be increased in two ways namely increase in sporulation temperature or the inclusion of heat shock proteins (HSPs). HSPs are proteins synthesised by induced heat shock or by growth at a higher temperature (Sedlak et al., 1993). It was found in Bacillus stearothermophilus that partly activated spores became more resistant to heat due to the heat shock (Sedlak et al., 1993).

2.5.3. Mineralization
It is thought that mineralization as well as other factors will have an indirect effect on the spores’ core water content. The reduction in the spores’ water content will decrease the association of water with the proteins, thereby stabilizing the proteins during denaturation (Beaman & Gerhardt, 1986). The core of *B. sporothermodurans* spores is very compact and the surrounding cortex is relatively large. As the core becomes more compact, it results in greater dehydration, which is an important factor in heat resistance of the bacterial spores (Beaman & Gerhardt, 1986). Calcium may also accumulate in the spores’ core and chelate with DPA. As milk contains minerals such as calcium, it may stimulate the formation of a Bacillus spore in dairy processes (Burgess *et al.*, 2010).

### 2.5.4. Chemical Resistance

Bacterial spores are resistant to a number of chemicals, however there are only certain chemicals which are effective in killing spores (e.g. formaldehyde, nitrous acid, alkylating agents) (Russell, 1990; Setlow, 2006). Spore killing by chemicals is mainly by DNA damage as a result of survivors accumulating mutations that in turn will sensitize the spores to these chemical agents. However, some oxidising agents are found to kill spores by damaging the spores external layers and as a result, when germination occurs, the damaged membranes rupture resulting in spore death. The precise mechanism of the damage to the inner membrane however is not known (Setlow, 2006). The spore layers play a protective role by detoxifying the chemicals before they penetrate further into the spores. An enzyme called superoxide dismutase may have the potential to detoxify damaging chemicals and has been associated with exosporium or spore coats of certain species (Setlow, 2006).

Packaging material is often sterilized by a number of different methods such as heat, hydrogen peroxide, irradiation, infrared light, etc (Ansari & Datta, 2003). A combination of these may also be used depending on ones’ preferences. With the use for example of hydrogen peroxide (H₂O₂), the chemical is seen as an indirect sterilant of food or milk. Regulations specify that a maximum concentration of 35% H₂O₂ may be used for sterilizing food contact surfaces. The final product is also not allowed to contain more than 0.5 ppm H₂O₂ (Ansari & Datta, 2003). It has been reported that 0.25 ppm of H₂O₂ present in the food, has little harmful effects on consumers. Many aseptic packaging systems use around 30-35% H₂O₂ as a sterilant for aseptic packaging in conjunction with hot air (60-125°C) to enhance the sterilization effect as well as to remove any residual H₂O₂ after sterilization (Ansari & Datta, 2003; Russell, 1990).
of H₂O₂ is most effective in combination with heat, as at ambient temperatures its functionality is reduced. It however becomes a problem when spores such as B. sporothermodurans are highly heat resistant (Ansari & Datta, 2003). H₂O₂ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivates the proteins. The main target for spore killing by H₂O₂ is at the exterior of the spores’ inner membrane (Melly et al., 2002). Tabit & Buys (2010) experimented with H₂O₂ whereby the spores were incubated in a 30% H₂O₂ solution for 0, 3, 6, 9 or 15 minutes at room temperature. They found that as the exposure time increased, the B. sporothermodurans levels decreased however; there was still a presence of viable B. sporothermodurans spores after 15 minutes (Tabit & Buys, 2010).

2.5.5. pH

A number of studies have shown that maximum heat resistance of spores are at pH values close to neutrality and that the resistance decreases closer to acidification (Gould & Dring, 1975). The mechanism by which low pH decreases spore heat resistance is not well known. Gould and Dring (1975) suggested that closer to acidification, the protonization of the carboxyl groups occurs causing the collapse of the whole structure allowing for rehydration of the protoplast thereby decreasing the heat resistance of the spore. Another hypothesis is that the decrease in heat resistance at a low pH may be due to the acid wash of minerals resulting in the spores being more sensitive to denaturation at high temperatures (Palop et al., 1999).

2.5.6. Relative Humidity

A dormant spore has low water content. Often when in a humid environment, the spore swells in two phases. The first phase takes 1 minute and the second takes 8 minutes. From these two phases one can determine the rate of entry of water into different areas in the spore (Driks, 2003). Resistance mainly occurs by three structures mainly the core, the cortex and the spore coat. The cortex consists of a multilayered shell coat that resembles woven fabric, is a tight conformation and allows the core to remain dry (Westphal et al., 2003). This design allows for small molecules such as water to pass through. Even though water passes through, the core still remains dry due to the squeezing action of the cortex on the core. Low moisture allows the DNA to be preserved during dormancy (Driks, 2003). When spores are dormant, they are metabolically inactive however; they are able to sense the environment around them. Westphal
et al. (2003) found that when responding to one environmental parameter such as humidity, it prepares the spore for a change in another parameter such as nutrition to allow for germination to occur. They also found that the coat and the cortex are permeable to water and contain free water whereas the core contains bound water.

When swelling occurs, the diameter of the spore increases allowing inactivating gases to pass through more easily (Westphal et al., 2003). This contraction of water resulting in the spores having low moisture content may also be important for the increase in heat resistance. The uneven distribution of water is mainly due to osmosis. The cortex consists of electronegative peptidoglycan and positively charged counterions associated with it forming an osmotic potential. Because of this, the cortex must be in equilibrium with the core to allow water to pass from the core to the cortex to equalize the osmotic pressure. Even in a high moisture environment, the core will still have a low moisture content to balance the osmotic equilibriums with the cortex. When hydration occurs, the calcium in the core neutralizes electronegativity of the cortex to reduce the osmotic pressure allowing the hydration of the core (Gould, 1977).

Research on B. sporothermodurans and their spores has increased over the years and has focused more on PCR methods as well as looking at the effects of heat, pH and hydrostatic pressure. Esteban et al. (2013) looked at the effect of heating medium characteristic on the thermal inactivation of B. sporothermodurans spores when exposed to heating and cooling treatments using models (i.e. Weibull and Geeraerd) to predict the survival of the spores after treatment. Results showed that a lower pH lead to a decrease in heat resistance and that non linear models can predict the thermal resistance of spore forming bacteria in different media (Esteban et al., 2013). Research by Aouadhi et al. (2013) looked at the behaviour of heat resistant spores under pressure so as to select the optimal condition to maximize germination. Results showed germination was greater in milk than in water. Milk allows for a higher germination rate due to the free amino acid which acts as strong germinated (Aouadhi et al., 2012).

2.6. Quorum sensing

Bacteria often send out signalling molecules into their environment to control the behaviour of individuals of populations so that they may respond the same way to the surrounding environment. Therefore, not only does nutrient availability bring about sporulation, but the
density of the cell population as well. This is called quorum sensing. Quorum sensing by 
*Bacillus* spp. is brought about by the release of small peptides from the *phr* gene (Miller & 
Bassler, 2001). Gram positive bacteria secrete peptides as autoinducers. The peptides are 
secreted by an ATP binding cassette (ABC) transporter. They also use a signalling mechanism 
called the phosphorylation/dephosphorylation signalling cascade. Figure 3 is a general quorum 
sensing model for Gram positive bacteria (Miller & Bassler, 2001). The diagram shows peptide 
autoinducers being secreted in concentrations according to the cell population density. The two 
component sensor kinases detect the secreted peptide signals. These detections result in 
interactions with the peptide ligand bringing about a phosphoryl event that ends with the 
phosphorylation of cognate response regular proteins. The phosphorylation of the response 
regulator results in the activation allowing it to bind to the DNA and alter the transcription of 
the controlled target gene resulting in quorum sensing (Miller & Bassler, 2001).

Cell to cell communication is an important aspect in survival and the interaction with the 
natural habitat (Miller & Bassler, 2001). Studies have shown that communications between 
spores exist. The closer the spores are together the better is their germination synchronisation 
(Zhang et al., 2011).

![Figure 3: A general model for peptide-mediated quorum sensing in Gram-positive 
bacteria](image)

**Figure 3: A general model for peptide-mediated quorum sensing in Gram-positive 
bacteria** (Miller & Bassler, 2001)

2.7. Speciation
Evolutionary biology looks at two concepts, adaptation to their environment and organism diversity. Often populations diverge when mutations occur in a population for them to adapt to different environmental conditions (Curie, 2012). In environments where temperate conditions are more variable and unpredictable, the environmental stress may have an impact on evolutionary and ecological processes. These impacts can affect and shape the genetic structure and evolution of the population in these environmental stresses. This concept may apply to bacterial strains such as *B. sporothermodurans*. A concept to look at is that organisms and populations should be least stressed in their environment and become more stressed in an unknown environment (Bijlsma & Loeschcket, 2005). This explains how organisms and populations should be better adapted to their daily and seasonal changes in temperature compared to extreme temperature changes sporadically. Because of these extreme changes, the organisms and populations can respond phenotypically or genotypically and evolve to adapt to the stresses around them. Adaptation is defined by Bijlsma & Loeschcket (2005) as, “The process of change of an organism to conform better to the new environmental conditions, whereby the organism acquires characteristics, involving changes in morphology, physiology or behaviours that improve their survival and reproductive success in the particular environment.” Adaptation may occur through changes in allele frequencies due to selection pressure from the environment, namely evolutionary adaptation (Bijlsma & Loeschcket, 2005). The occurrence of evolutionary adaptation and the rate at which it occurs is dependent on the presence and the fitness of the mutations involved (de Visser & Rozen, 2005). Bacteria have evolved to help face the changes in environmental conditions allowing for a greater chance of survival. Environmental stresses include UV radiation, heat and hydrogen peroxide (Abee & Wouters, 1999).

2.8. Conclusion

Past research has used various fingerprinting techniques (Herman & Heyndrickx, 2002; Scheldeman *et al.*, 2002) such as BOX PCR and REP-PCR to characterize *B. sporothermodurans* and differentiate between strains, however, (GTG)$_3$ is a quick and easy technique that has not yet been used to differentiate *B. sporothermodurans* strains. Research has been conducted to determine the effects of heat on the structure of *B. sporothermodurans* spores (Tabit & Buys, 2010); however exactly at what temperature the spores are affected the most is unknown. *B. sporothermodurans* has been placed in hydrogen peroxide to determine
the rate of survival of the spores (Tabit & Buys, 2010). The extent of damage on the spore structure has not yet been microscopically characterised.

2.9. Hypotheses and Objectives

2.9.1. Hypotheses

The main difference between *Bacillus sporothermodurans* strains present in UHT milk (i.e. heat resistant strains) and those from other sources (*B. sporothermodurans* strains isolated from raw milk, feed concentrate and silage: non-heat resistant strains) is genotypically (i.e. their genes). Factors such as where the spores were isolated (i.e. UHT milk, raw milk, silage or feed concentrate), in which country and whether the strains have the HRS clone or not may differentiate the strain genotypically (Scheldeman *et al.*, 2005). Because of the diverse environmental conditions that the *B. sporothermodurans* strains are subjected to, the strains isolated from UHT milk and other farm sources may differ phenotypically as well because of their environmental adaptations to their environments (Dale & Park, 2004).

The spore structure of the *B. sporothermodurans* spores will contribute to the heat resistance at temperatures of between 120-140°C. The exosporium layer is mainly made up of proteins, specifically glycoproteins and may just be an expansion of the outermost coat layer. The exosporium and spore coat layers will not contribute to heat resistance as much as the cortex layer will (Burgess *et al.*, 2010). The spore cortex is made up of peptidoglycan which is important for the reduction of water content in the spore and as a result is important in determining the heat resistance of the spore. The type of coat proteins and their degree of cross linking plays a role in heat resistance. The inner spore membrane is particularly important, as it is highly resistant toward heat so as to prevent any DNA damage present in the core (Setlow, 2006).

The presence of H$_2$O$_2$ will damage the layers of the spore. H$_2$O$_2$ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivate the proteins. The main target for spore killing by H$_2$O$_2$ is at the exterior of the spores’ inner membrane (Melly *et al.*, 2002).
2.9.2. Objective

To determine whether there is a genotypic difference in spore structure between strains isolated from UHT milk (i.e. heat resistant strains) and those isolated from farm sources (*B. sporothermodurans* strains isolated from raw milk, feed concentrate and silage: non-heat resistant strains).

To determine the effect of heat on the spore coat structures of *Bacillus sporothermodurans* spores.

To determine the effects of H$_2$O$_2$ on the structure of *B. sporothermodurans* spores.
Chapter 3: Research

3.1. Molecular characterisation of *Bacillus sporothermodurans* using (GTG)$_5$ fingerprinting.

Abstract

*Bacillus sporothermodurans* is a Gram positive, sporeformer which has the potential to survive sterilization or UHT treatments. Survival of UHT treatments is due to *B. sporothermodurans* producing highly heat resistant spores (HRS). Detection of *B. sporothermodurans* from various sources can be identified by PCR based methods, HRS-PCR and the more general *B. sporothermodurans* (*BSPO*) PCR. (GTG)$_5$ PCR is a repetitive element based DNA fingerprinting technique that has been used to differentiate and characterize bacteria organisms such as lactobacilli and enterococci. (GTG)$_5$ fingerprinting is known to be a promising genotypic tool that allows for more rapid and reliable results in comparison with other PCR methods such as REP-PCR. This method has not yet been used in strain differentiation of *B. sporothermodurans*. The aim of this study was to genotypically compare *B. sporothermodurans* isolates from UHT milk and those from the dairy farm sources using (GTG)$_5$ PCR. Nine *B. sporothermodurans* strains were previously isolated from UHT milk or farm sources from different countries. Two recently isolated strains, QA1 and F3, from Belgium and SA, respectively, were identified using the general *B. sporothermodurans* (*BSPO*) method and the more specific HRS-PCR method identifying the HRS clone. REP-PCR and (GTG)$_5$ PCR fingerprinting was performed on all 9 isolates. From (GTG)$_5$ PCR, all *B. sporothermodurans* strains were shown to have a number of identical bands at ca. 400, 550, 798-800, 1090, 1200, 1400 and 1500 bp. The two recently isolated UHT strains, QA1 and F3, were shown to be closely associated by the (GTG)$_5$ PCR patterns and were positive for the HRS clone. Results go on to show that the *B. sporothermodurans* strains of the HRS clone are mainly responsible for the production of heat resistant spores. One can conclude that the two recently isolated strains F3 and QA1 isolated from UHT milk belong to the HRS clone. (GTG)$_5$ PCR has been used to produce DNA fingerprints for a number of species and in this present study we showed that this method could be used to evaluate the genotypic differentiation of *B. sporothermodurans* strains.
3.1.1. Introduction

Ultra high temperature processing (UHT) is a process whereby milk is treated at high temperatures of between 135ºC- 140ºC for 3 to 4 seconds (Pettersson et al., 1996). *Bacillus sporothermodurans* is a Gram positive, mesophilic sporeformer that has the potential to survive sterilization or UHT treatments (Pettersson et al., 1996). Survival of UHT treatments is due to *B. sporothermodurans* producing highly heat resistant spores, hence its original designation as highly heat resistant spore former or HRS. These highly heat resistant spores will most likely begin to germinate and grow in the processed milk during storage due to little or no competition from the faster growing vegetative cells of other (non-spore forming) microorganisms (Pettersson et al., 1996; Scheldeman et al., 2006). Heat resistant spores were first detected in UHT treated milk from southern Europe in 1985 and thereafter from Germany in 1990. Up until now, the presence of HRS in UHT treated milk has become more widespread in a number of European countries such France, Spain, Belgium as well as in Brazil and South Africa (Pettersson et al., 1996; Tabit & Buys, 2010). Contamination of milk by *B. sporothermodurans* may occur on the farm. A contamination source is cattle feed whereby spores are able to survive in the digestive tract of cattle and as a result be shed in the faeces (Varewijck et al., 2001).

The ability of *B. sporothermodurans* to survive UHT treatment is dependent on the strain to produce highly heat resistant spores (Guillaume-Gentil et al., 2002). Detection of *B. sporothermodurans* from various sources can be identified by PCR based methods, HRS-PCR and the more general *B. sporothermodurans* (*BSPO*) PCR (Herman et al., 1997). A more general *B. sporothermodurans* method is needed since not all *B. sporothermodurans* strains belong to the HRS clone (Scheldeman et al., 2002). The HRS-PCR method determines the allocation of a strain to this HRS clone.

A number of PCR methods have been used to characterize *B. sporothermodurans* at strain level, such as random amplified polymorphic DNA analysis and repetitive extragenic palindromic (REP)-PCR (Guillaume-Gentil et al., 2002; Herman et al., 1998; Scheldeman et al., 2002). The use of REP-PCR in combination with high resolution separation by non-denaturing polyacrylamide gel electrophoresis and silver staining allows for the differentiation between *B. sporothermodurans* by clonally related isolates from UHT milk. \((\text{GTG})_3\) is a repetitive element based DNA fingerprinting technique that has been used to
differentiate and characterize bacterial organisms such as lactobacilli and enterococci (De Vuyst et al., 2008). (GTG)$_5$ fingerprinting is a technique using (GTG)$_5$ primer, which is a sequence of 5x GTG. (GTG)$_5$ fingerprinting the is known to be a promising genotypic tool that allows for more rapid and reliable results in comparison with other PCR methods such as REP-PCR (De Vuyst et al., 2008). This method has not yet been used in strain differentiation of B. sporothermodurans.

The aim of this study was to genotypically compare B. sporothermodurans isolates from UHT milk and from dairy farm sources using (GTG)$_5$ PCR.

### 3.1.2. Materials and methods

#### 3.1.2.1 Bacterial strains and methods used

Nine B. sporothermodurans strains were obtained from UHT milk or farm sources (e.g. feed concentrate, silage and raw milk) from different countries, two of them (F3 and QA1) were isolated more recently (Table 2). DNA was extracted by the method of Flamm et al. (1984). Two other species from milk were included namely Paenibacillus lactis and Bacillus fordii. The 2 recent strains, F3 and QA1, were identified using the general B. sporothermodurans PCR as well as the more specific HRS-PCR method identifying the HRS-clone. REP-PCR and (GTG)$_5$ PCR fingerprinting were performed on all 9 isolates and thereafter the fingerprints were analysed in a dendrogram (Guillaume-Gentil et al., 2002).
Table 1: *B. sporothermodurans* and other test species and their original sources

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Strain</th>
<th>Source</th>
<th>Country of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>F3</td>
<td>UHT milk</td>
<td>South Africa*</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>QA1</td>
<td>UHT milk</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>DSMZ 10599</td>
<td>UHT milk</td>
<td>Germany</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 372</td>
<td>UHT milk</td>
<td>Germany</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 385</td>
<td>Raw milk</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 1505</td>
<td>Silage</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 1317</td>
<td>Feed concentrate</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 1499</td>
<td>Feed concentrate</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>MB 1668</td>
<td>Feed concentrate</td>
<td>Belgium</td>
</tr>
<tr>
<td><em>Paenibacillum lactis</em> (negative control)</td>
<td>MB 1871</td>
<td>Raw milk</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus fordii</em> (negative control)</td>
<td>MB 1878</td>
<td>Raw milk</td>
<td></td>
</tr>
</tbody>
</table>

*Strain obtained from the department of Food Science, University of Pretoria. DSMZ 10599 obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. All other isolates were obtained from the Institute for Agricultural and Fisheries Research (ILVO) in Belgium.

### 3.1.2.2. Growth conditions

The *B. sporothermodurans* isolates were grown on Brain Heart Infusion agar (Oxoid, Hampshire, UK) in combination with bacteriological agar (15 g.L⁻¹) and vitamin B₁₂ (1mg.L⁻¹) (Sigma, St. Louis, MO), for 24h at 37°C. *Bacillus fordii* MB 1878 and *Paenibacillus lactis* MB 1871 were grown on brain heart infusion agar (Oxoid) for 24h at 37°C.

### 3.1.2.3. *Bacillus sporothermodurans* PCR (BSPO PCR)

BSPO PCR was performed according to the methods of Scheldeman *et al.* (2002) with one modification: the first cycle started with an initial denaturation of 1 min at 95°C and with an annealing of 15s at 57°C. The primers used for *B. sporothermodurans* identification were BSPO-F2 (5’ ACG GCT CAA CCG TGG AG 3’) and BSPO-R2 (5’ GTA ACC TCG CGG TCT A 3’).
3.1.2.4. Heat Resistant-PCR (HRS-PCR)

HRS-PCR was performed according to the methods of Herman et al. (1997) with one modification: the denaturation step was at a temperature of 95 °C for 15s. The primers used were SH2-F1 (5’ GAT TCA GGC AGA ATG TAG CA 3’) and SH2-R1 (5’ TTT CGC CAC TTG ATG GTA 3’) (Herman et al., 1997).

3.1.2.5. Repetitive Element Palindromic-PCR (REP-PCR)

REP-PCR was performed using the primers REP 1R-I (5’ III ICG ICG ICA TCI GGC 3’) and REP 2-I (5’ ICG ICT TAT CIG GCC TAC 3’) (Isogen, Bioscience) as described by Herman et al. (1998) with one modification: after LE (Low electro endo osmosis) agarose gel (1.5%) gel (Seakem) electrophoresis, the gels were stained using ethidium bromide solution instead of silver staining. The amplifications were performed on a GeneAmp 9700 (Applied Biosystems). The scanned gel images were analysed using Bionumerics version 6.6 (Applied maths, Kortrijk, Belgium). The REP PCR patterns were grouped according to the Pearson similarities using a UPGMA (unweighted pair group method using arithmetic averages) dendrogram (Guillaume-Gentil et al., 2002).

3.1.2.6. (GTG)$_5$ PCR

(GTG)$_5$ PCR was performed using 1μl of DNA (25 ng/μl) using the (GTG)$_5$ primer, which is a sequence of 5x GTG. The amplification reaction was performed on a final volume of 25 μl containing 0.6μg of primer, 2.5 μl 10x PCR buffer II (100mM Tris HCl pH 8.3; 500mM KCl), 25 mmol$^{-1}$ MgCl$_2$ (Applied biosystems), 2 mmol$^{-1}$ dNTP (GE Healthcare), 0.3 IU Goldstar DNA polymerase (Eurogentec). The amplifications were performed on a GeneAmp 9700 (Applied Biosystems) thermocycler using a temperature profile of: 7 min at 95ºC, 30 cycles for 1 min at 94ºC, 1 min at 40ºC, 8 min at 65ºC, thereafter to a final extension of 16 min at 65ºC. (GTG)$_5$ PCR profiles were separated using LE agarose gel (1.5%) (Seakem). Two molecular weight markers namely the 100 bp (Invitrogen) and the 500 bp (Bio-Rad) were combined. The gels were run for 4 hours at 130 V. After electrophoresis the gels were stained using ethidium bromide solution. The stained gel was examined visually and scanned
using a G Box scanner (Westburg). The scanned gel images were analysed using Bionumerics version 6.6 (Applied maths, Kortrijk, Belgium). The (GTG)$_5$ patterns were grouped according to the Pearson similarities using a UPGMA dendrogram.

3.1.3. Results

3.1.3.1. Identification of recent strains

\textit{B. sporothermodurans} PCR (\textit{BSPO}) confirmed that the 9 isolated strains were \textit{B. sporothermodurans} (Table 3). The 2 recently isolated \textit{B. sporothermodurans} strains were the most recent strains isolated from UHT milk, QA1 and F3, tested positively for the HRS clone (Table 3). As previously indicated by Scheldeman \textit{et al.} (2002), the 4 isolates from UHT milk and feed concentrate tested negative for the HRS clone and MB 1505 sourced from silage was the only farm isolate that was positive for the HRS clone.

\textbf{Table 2: Determination of heat resistant and non-heat resistant Bacillus sporothermodurans isolates obtained from various geographic origins and isolated from dairy and farm sources using the polymerase chain reaction (PCR)}

<table>
<thead>
<tr>
<th>\textit{B. sporothermodurans} strains</th>
<th>Product</th>
<th>Country of Origin</th>
<th>\textit{B. sporothermodurans} PCR</th>
<th>HRS PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>UHT milk</td>
<td>South Africa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>QA1</td>
<td>UHT milk</td>
<td>Belgium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DSMZ 10599</td>
<td>UHT milk</td>
<td>Italy</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB 372</td>
<td>UHT milk</td>
<td>Germany</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB 385</td>
<td>Raw milk</td>
<td>Belgium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MB 1505</td>
<td>Silage</td>
<td>Belgium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB 1317</td>
<td>Feed concentrate</td>
<td>Belgium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MB 1499</td>
<td>Feed concentrate</td>
<td>Belgium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MB 1668</td>
<td>Feed concentrate</td>
<td>Belgium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Paenibacillus lactis}</td>
<td>Raw milk</td>
<td>Belgium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Bacillus fordii}</td>
<td>Raw milk</td>
<td>Belgium</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.1.3.2. (GTG)₅ –PCR

Figure 1: Dendrogram of *B. sporothermodurans* strains and other milk species (MB 1871 and MB 1878), obtained by (GTG)₅ PCR. The scale bar represents the percentage similarity.

Cluster analysis (Fig. 4) of (GTG)₅ PCR showed that *B. fordii* and *Paenibacillus lactis* had very little similarities with any of the *B. sporothermodurans* strains. The *B. sporothermodurans* strains showed an overall similarity of 40%. All *B. sporothermodurans* strains were shown to have a number of identical bands at ca. 400, 550, 798-800, 1090, 1200, 1400 and 1500 bp.

Two clusters were observed. In Cluster C1, strains QA1, F3 and MB 372 belonging to the UHT milk strains were closely similar (80%) with a difference in the presence or absence of two bands between ca.700-900 bp. Strains QA1 and F3 showed the greatest similarity of 95%. MB 1505 from silage had a 72% similarity to the UHT milk strains. This differentiation was mainly due to a strong band present at ca. 2000 bp in the silage strain. MB 1505 was the only non UHT milk isolate in cluster 1 showing a greater similarity to the UHT milk isolates.

From cluster C2, type strain DSMZ 10599 from UHT milk had a similarity of 80% with MB 1499 isolated from feed concentrate. Similarity was due to the band intensity at ca. 800 and ca. 1200 bp. Differentiation between the two strains was due to the presence of a strong band of MB 581 at ca.1700 bp. Feed concentrate isolates MB 1317 and MB 1499 showed a 75% similarity with two strong major bands at ca. 600 and 700 bp. Farm isolates MB 385 (raw milk) and MB 1668 (feed concentrate) showed the lowest similarity to the other strains with a 64% and 41% similarity respectively. MB 385 showed more unique strong bands between ca. 1800- 2100 bp. The feed concentrate strain MB 1668 showed a very low similarity with the
other feed concentrate strains due to the presence of a unique strong band present at ca. 200 bp.

3.1.3.3. REP-PCR

Figure 2: Dendrogram of 9 strains of *B. sporothermodurans* and other milk species (MB 1871 and MB 1878), obtained by REP-PCR. The scale bar represents the percentage of similarity.

In general, less bands were generated with REP-PCR compared to (GTG)$_5$ PCR. Cluster analysis (Fig. 5) from the REP-PCR patterns showed that *B. fordii* and *P. lactis* had very little similarities with any of the *B. sporothermodurans* strains. The *B. sporothermodurans* strains showed an overall similarity of 50%. The most prominent common bands from all *B. sporothermodurans* strains were at ca. 980 and 950 bp.

Two clusters could clearly be observed (i.e. C1 and C2) with C1 being the larger cluster. From the first cluster DSMZ 10599 and MB 1317 had a similarity of 90% even though these two strains were isolated from different sources. When observing the bands of the two strains however, the presence of a single band at ca. 950 bp was observed. The two UHT milk isolates DSMZ 10599 and MB 372 showed an 80% similarity with differentiation due to the absence of a strong band at ca. 1900 bp in one of the strains. Even though there was a 78% similarity between MB 385 (raw milk), MB 372 (UHT milk) and F3 and QA1, MB 385 showed a significant difference due to the presence of a unique strong band at ca. 380 bp. QA1 and F3 had a high similarity (90%) with similar strong bands present in each strain. The main difference in similarity may be due to the difference in intensities of the bands.
In cluster C2, the two feed concentrate isolates MB 1499 and MB 1668 showed an 88% similarity with only one prominent band at \( ca. \) 980 bp. MB 1505 had a 75% similarity to the two feed concentrate strains. MB 1505 showed greater differences with the presence of more than one major band at \( ca. \) 600, 1900 and 1700 bp.

**3.1.4. Discussion**

*B. sporothermodurans* strains from this study had previously been compared using the REP-PCR method by Guillaume-Gentil *et al.* (2002) but using another gel electrophoresis and staining technique. REP-PCR patterns obtained in this study using a conventional agarose gel electrophoresis and ethidium bromide staining were difficult to compare to that of Guillaume-Gentil *et al.* (2002), showing only one common band at \( ca. \) 1000 bp. For this reason, to compare the percentage similarity was more difficult. However, even with relatively less bands, REP-PCR patterns from this paper compared to that of Guillaume-Gentil *et al.* (2002), the percentage between the strains were similar in relation to the percentages generated from dendrogram. One would postulate that one band is vital in the genetic makeup of all *B. sporothermodurans* strains.

The two new strains, F3 (isolated in 2007) and QA1 (isolated in 2007) were confirmed as *B. sporothermodurans* strains and belonged to the HRS clone. Even though the QA1 and F3 strains were genetically very similar, they were isolated from two different countries and continents namely Belgium (QA1) and South Africa (F3).

The two feed concentrates MB 1499 and MB 1668 showed a high degree of similarity in REP PCR with MB 1317 showing more similarity to the UHT milk strains. In \((GTG)\_5\) PCR MB 1499 showed a greater similarity towards MB 1317 than MB 1668. When looking at the bands only, the three strains were extremely similar other than the intensity of certain bands. This was expected, as they were all isolated from feed concentrate in Belgium.

The sporadic occurrence of contamination of UHT milk may be caused by the circulation of the HRS *B. sporothermodurans* within and between the UHT production plants (Guillaume-Gentil *et al.*, 2002). One would assume that initial contamination occurs via raw milk from the farm environment. However, the \((GTG)\_5\) patterns show that most of the farm isolates do not have any similarities with the UHT isolates. MB 1505 was the only farm isolate that showed a significant similarity to the UHT strains. Similarities may be due to the acquisition
of the HRS clone by both MB 1505 and the UHT strains. Farm isolates such a MB 1505 from silage, have been found to be highly heat resistant however whether these spores are able to withstand UHT treatment is not known (Guillaume-Gentil et al., 2002). The latter may explain if farm isolates may contribute to UHT milk contamination as certain farm isolates can also be HRS PCR positive. However, if contamination occurred from farm isolates then the REP and (GTG)$_5$ patterns would be expected to be similar whether the strains were isolated from UHT milk or the farm environment. This was a similar finding to that of Guillaume-Gentil et al. (2002). Even though most of the farm isolates do not have the HRS clone, their spores are still heat resistant; however whether they will be able to withstand the UHT treatment still needs to be determined. To date no studies have used (GTG)$_5$ PCR for characterization of B. sporothermodurans and is a relatively new method mainly used for characterisation of lactobacilli spp. For this reason, comparison of the (GTG)$_5$ PCR results of B. sporothermodurans strains to that of previous studies on B. sporothermodurans, is difficult..

Research has looked at possible sources of cross contamination between countries for UHT treated milk. Milk powder is a potential source since certain countries prepare UHT treated milk with imported milk powder (Hammer et al., 1995). This may explain the spread of the same B. sporothermodurans HRS clone over different continents (Guillaume-Gentil et al., 2002). More research would need to be done on the B. sporothermodurans strains and the spread during processing.

3.1.5. Conclusion

One can conclude that the two more recent strains, F3 and QA1, isolated from UHT milk belong to the HRS clone which was already isolated for the first time in the eighties of previous century. Results go on to show that the B. sporothermodurans strains of the HRS clone are mainly responsible for the production of heat resistant spores. As of yet, no studies have used (GTG)$_5$ PCR to characterize B. sporothermodurans. (GTG)$_5$ PCR has been used to produce DNA fingerprints for a number of species and in this present study we showed that this method could be used to evaluate the genotypic differentiation of B. sporothermodurans strains without the need for a more laborious and expensive polyacrylamide gel electrophoresis technique and silver staining as described previously for REP-PCR. To determine whether the B. sporothermodurans strains have the HRS clone is important in the
dairy industry as the quality and safety of dairy products is vital in the industry. The genetic variability may play a large role in the evolutionary adaptation of *B. sporothermodurans* strains however more research would need to be done on the extent of adaptation. This may give an understanding on how adaptable *B. sporothermodurans* will be if changes occur in dairy processing of UHT milk and whether anything can be done to prevent this.
3.2. Morphological characterisation and the effects of hydrogen peroxide and heat on *Bacillus sporothermodurans* spores

Abstract

*Bacillus sporothermodurans* is a facultative thermophilic sporeformer that may occur in UHT milk. Bacterial spores have the same basic structure such as the spore coat, inner and outer membrane, the cortex as well as the core. Structures such as appendages are found more often in *B. clostridium* and *B. cereus* spores. A number of factors such as chemicals and heat may affect the structure of the spore. In this study the morphological characteristics of spores of heat resistant and non-heat resistant *B. sporothermodurans* strains were compared. The effect of the chemical H$_2$O$_2$ on the structure of *B. sporothermodurans* spores was also determined as well as at what temperature the effect of heat was greatest on the *B. sporothermodurans* spores. The spores of reference strains of *Bacillus sporothermodurans* DSMZ 10599, Leibnitz and *B. sporothermodurans* F3, Pretoria, SA, were isolated from UHT milk. Reference strains isolated from feed concentrate obtained from the ILVO institute in Belgium (MB 1499 & MB 1668) were also used. The *B. sporothermodurans* spores were similar in structure. All the spores had an exosporial layer, spore coat, cortex and core. Appendages were not observed in the *B. sporothermodurans* spores. Even though a typical appendage-like structure could not be observed, a node-like structure was clearly visible in the spore coat of the heat resistant strains. The nod-like structures were absent in the spore coat of MB 1499. From the DSC, a thermogram with heat values versus temperature is illustrated. The initial scan of DSMZ showed 3 transitions, one at 79°C, the second at 87°C and the third at 113°C. From the study the chemical disinfectant hydrogen peroxide clearly affected the spore layers. At different times, different layers were affected. As a result, the longer the spores were in contact with H$_2$O$_2$, the more H$_2$O$_2$ was able to inactivate the proteins and penetrate the spore layers. From the DSC and H$_2$O$_2$ results, it was found that *B. sporothermodurans* spore structure contributes to the resistance of heat and H$_2$O$_2$. The structure says a lot about the environment they are in and therefore much more work would need to be done on the proteins present in the layers of the *B. sporothermodurans* spore and whether there is a difference between UHT and farm isolated strains of *B. sporothermodurans*. *B. sporothermodurans* has now been found to have short node-like appendages present which
may explain attachment to steel pipe surface resulting in post and cross contamination in UHT milk.
3.2.1 Introduction

*Bacillus sporothermodurans* is a facultative thermophilic sporeformer that may occur in UHT milk (Klijn *et al.*, 1997). *B. sporothermodurans* has not only been found in UHT milk but also in farm sources such as feed concentrate, silage and raw milk (Scheldeman *et al.*, 2002). The main observed difference between the strains found in UHT milk and the farm source strains is genotypic. *B. sporothermodurans* strains present in UHT milk have the HRS clone which produces heat resistant spores (Scheldeman *et al.*, 2002). Although genotypic characteristics of the *B. sporothermodurans* spores play a role in heat resistance, phenotypic characteristics may also play a role.

Bacterial spores from different species have the same basic structure such as the spore coat, inner and outer membrane, the cortex as well as the core. A less common layer, an outer loosely fitted exosporium layer is present in only certain species such as *Bacillus cereus* and *B. sporothermodurans*. Other structures such as appendages are more often present in *B. clostridium* and *B. cereus* spores (Wrath, 1987). The spore surface layers such as the exosporium and spore coat contribute to the resistance of the spores against extreme physical and chemical stresses. Even though spores are in their dormant state, meaning that they are metabolically inactive, they still interact with the environment. These interactions mainly occur with the exosporium and coat layers. The spore surface layers are flexible thereby expanding or retracting in response to environmental conditions (Driks, 1990). Microscopes such as atomic force microscopy (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are powerful tools for studying natural surfaces of biological material. Several studies of spore surface morphology suggest that closely related species may be differentiated by their surface structures (Zolock *et al.*, 2006). A number of factors such as chemicals and heat may affect the structure of the spore.

Bacterial spores are resistant to a number of chemicals however there are only a few chemicals which are effective in spore killing (e.g. formaldehyde, nitrous acid, alkylating agents) (Russell, 1990; Setlow, 2006). Spore killing by chemicals is mainly by DNA damage as a result of survivors accumulating mutations which in turn will sensitize the spores to these chemical agents. The precise damage to the inner membrane however is not known (Setlow, 2006). Packaging material is often sterilized by a number of different methods such as heat, hydrogen peroxide, irradiation, infrared light, etc. With the use for example of hydrogen...
peroxide (H$_2$O$_2$), the chemical is seen as an indirect sterilant of food or milk (Ansari & Datta, 2003; Russell, 1990). H$_2$O$_2$ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivates the proteins (Melly et al., 2002).

The level of spore heat resistance can depend on a number of factors such as the amount of various heat-labile macromolecules important for germination and outgrowth, the stabilization by the spore itself and the intrinsic stability of these macromolecules as well as the environment in which the spores are formed (Lindsay et al., 1990). The effect of heat treatment has been determined on a number of bacterial spores such as Bacillus subtilis and Bacillus megaterium using differential scanning calorimetry (DSC).

In this study the morphological characteristics of heat resistant and non-heat resistant B. sporothermodurans spore strains were compared. While investigations have been done on the spore surface morphology of Bacillus species such as B. cereus (Appendix 1), B. thuringiensis, B. anthracis as well as B. globigii, there is little information on the surface morphology of B. sporothermodurans spores isolated from UHT milk and farm sources. The effect of H$_2$O$_2$ and heat on the structure of B. sporothermodurans spores was determined.

### 3.2.2. Materials and Methods

#### 3.2.2.1. B. sporothermodurans strains

Reference strains of Bacillus sporothermodurans spores isolated from UHT milk were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ 10599- originally isolated from Italy) and the University of Pretoria (Department of Food Science) in South Africa (F3). A reference strain isolated from feed concentrate obtained from the Institute for Agricultural and Fisheries (ILVO) in Belgium (MB 1499 & MB 1668) was compared to the UHT strains. Strain DSMZ 10599 was positive for the HRS clone whereas MB 1499 and MB 1668 were not (Guillaume-Gentil et al., 2002). The spores of three B. sporothermodurans strains namely F3, DSMZ 10599 and MB 1499 were compared using transmission electron microscopy and atomic force microscopy.
3.2.2.2. Spore Preparation

*B. sporothermodurans* spores were prepared as described by Belliveau *et al.* (1992) and Tabit & Buys (2010). From an overnight culture of each strain, 1 ml was collected and spread on BHI agar plates and incubated for 14 days at 37 °C until 100% sporulation was attained. Sporulation was checked using a light microscope. The growth on the surface of the agar plates was scraped off and placed in a 1.5 ml Eppendorf tube with sterile saline. The spores were washed with double distilled water and centrifuged at 10,000 x g for 3 minutes. The pellets were diluted to about $10^8$ spores per ml.

3.2.2.3. Transmission electron microscopy (TEM) analysis of spores

Transmission electron microscopy was conducted with the cleaned spores as described by Van der Merwe and Coetzee (1992) with modifications. The spores were centrifuged at 3000 x g for 3 min. The pellets were suspended in 2.5% glutaraldehyde, 0.075M of phosphate buffer for 1h. The pellet suspension was centrifuged at 3000 x g for 5 min and rinsed in 0.075M phosphate buffer. The centrifugation and rinse procedure was repeated three times. After washing, the pellets were suspended in 0.5% OsO₄ for 1h and centrifuged afterwards. After centrifuging, the pellets were washed three times with distilled water. The pellets were dehydrated in at 30%, 50%, 90 and 100% ethanol for 10 min. Between each dehydration step, the spores were centrifuged at 3000 x g for 3 min. The pellets were also washed at 100% ethanol 3 times. After dehydration the pellets were infiltrated with 50% quetol in ethanol for 1h. Thereafter the pellets were infiltrated with pure quetol for 4 hours. Pellets were polymerised and cut into sections and placed on grids and stained with uranyl acetate and lead acetate. The micrograph was taken using the Jeole JEM-2100F FEG TEM (Tokyo, Japan).
3.2.2.4. Atomic force microscopy (AFM) preparation

AFM preparation and analysis was conducted according to the methods of Plomp et al. (2005) with some modifications. After spore preparation the samples were placed on a glass slide instead of in liquid. The washed spores were spread on a glass slide and left to dry. AFM images were taken using the Veeco Zeiss AFM (California, USA).

3.2.2.5. High Resolution Scanning Electron Microscopy (High Res SEM) preparation

High resolution SEM preparation and analysis was conducted according to the methods of Van der Merwe and Coetzee (1992) with some modifications. The High Res SEM method was similar to that of the TEM method up until the 100% ethanol washing. After dehydration, the samples were dipped in HMDS (Hexamethyldisilazane), placed, and dried on a glass slide. The glass slide was then taped unto an aluminium stub and coated with carbon. Analyses of the spores were conducted using a Zeiss ultra plus 55 high resolution SEM (Germany, Munich).

3.2.2.6 Differential Scanning calorimetry

Differential scanning calorimetry was conducted on the B. sporothermodurans strain DSMZ 10599. Sample preparation and analysis was conducted according to the methods of Belliveau et al. (1992). After spore preparation, the spores were sonicated to remove the exosporial layer. Thereafter the spores were centrifuged at 5000 x g and left overnight at 4°C. Spore samples in an aqueous suspension were taken with a dry weight of 30 mg. The samples were crimped in a stainless steel pan and then heated in a differential scanning calorimeter (model DSC 827; Mettler Toledo, Johannesburg, South Africa) at a rate of 10°C/min from 10 to 140°C and cooled rapidly afterwards. The graph was analysed on STARe Thermal Analysis Software (Johannesburg, South Africa).
3.2.2.7 Hydrogen peroxide experiment

The strains F3, DSMZ 10599 and MB 1668 were compared in the hydrogen peroxide experiment. Sample preparation and methods were conducted according to the methods of Tabit & Buys (2010). A 30% H₂O₂ (Sigma Aldrich, Midrand, South Africa) concentration was mixed with *B. sporothermodurans* spores. The suspensions were incubated for 0, 3, 6, 9 and 15 min. After each interval, 1 ml of the suspension was added to 9 ml of catalase solution and left to stand for 1 minute to end the H₂O₂ reaction. The spores then underwent the TEM preparation as described by Van der Merwe and Coetzee (1992). Micrographs were taken using the Jeole JEM-2100F FEG TEM (Tokyo, Japan).
3.2.3. Results

3.2.3.1. Morphological characterisation of *B. sporothermodurans* spores

![Figure 3: Transmission electron microscopy images of *B. sporothermodurans* spores. A & B: strain F3; C: strain DSMZ 10599; D & E: strain MB 1499. a, exosporial layer; b, cortex; c, spore core; d, spore coat; e, folds of the spore coat.](image)

All three strains were found to have similar TEM profiles. As shown from Figure 6 (a-e), *B. sporothermodurans* spores consist of four main layers, namely, cortex, spore coat, exosporium and the core. The core (Fig. 6 & 7) is surrounded by the cortex and is dense in structure. DSMZ 10599 and F3 (i.e. both isolated from UHT milk) had a smaller core (Fig. 6.)
a-c) with a significantly larger cortex layer (Fig. 7). MB 1499 had a less compact core (i.e. larger core) with a thin cortex layer compared to that of the UHT strains (Fig. 6 & 7). The cortex layer is surrounded by the spore coat and the exosporium layer surrounds the spore coat. The exosporium is a loosely fitted outermost layer that comes into contact with the environment (Fig. 6 & 7). The coat, has multiple layers (Fig. 7a-e). The first layer is the thick outer coat, next comes the middle coat made up of 4-5 lamellae layers consisting of a large number of proteins and the last layer is the inner spore coat. The coat layer appears to show folds (Fig. 6 & 7) allowing for observable small spaces to be present within the coat layer. It had to be noted that DSMZ 10599 images were difficult to attain during TEM imaging due the impermeability of the layers.
Figure 4: Transmission electron microscopy images of the spore layers of *B. sporothermodurans* spores. A & B: strain F3; C & D: strain MB 1499; E: DSMZ 10599. a, exosporium; b, outer coat; c, middle coat; d, inner coat; e, cortex layer; f, core
Figure 5: High resolution SEM images of *B. sporothermodurans* spores. A: F 3; B: DSMZ 10599; C: MB 1499.

High resolution SEM images (Fig. 8) showed a similarity in shape of the spores of all three strains which were between round and ovular. The exosporial layer of DSMZ 10599 and F3 looked to have been removed during preparation with the exosporial layer of MB 1499 spores only partially removed for high resolution SEM imaging (Fig 8). The outer coat layer was not smooth compared to the exosporium layers due to a number of indentations (Fig 8). A node-like structure was observed in F3 (Fig. 6b & Fig. 8 a-b.) developing from the middle coat layer. The node is short and thick and more than one node developed per spore. Even though the TEM figures did not show a node like structure on DSMZ 10599, the high resolution SEM images showed DSMZ 10599 to have similar node-like structures to F3 (Fig 8). The node-like structures were absent on the spore coat of MB 1499 therefore it would be difficult to confirm whether the non HRS strains do not have these node-like appendages. Further SEM and TEM work would need to be done on different non HRS strains to confirm whether these strain do not in fact have these node-like appendages.
AFM images showed DSMZ 10599 and F3 with the exosporial layer present. The exosporial layer was extremely sensitive as tip scratching (cantilever tip causing marks on the exosporium) was visible on the exosporium. The exosporium of F3 was significantly loose compared to that of DSMZ 10599. From the AFM images F3 and MB 10599 were round in shape compared to MB 1499 which showed a more ovular shape. DSMZ 10599 spores had an average width ±1 μm and a length of ±1.33 μm whereas F3 had an average width of 500 nm and length of 800 nm this including the exosporial layer. MB 1499 had an average width of 730 nm and length of 1.39 μm. Figure 9 D showed DSMZ 10599 without an exosporial layer. From the coat layer one could observe more than one appendage like structure attaching to the surface.
Figure 6: AFM images of *B. sporothermodurans* spores. A, B: F3, C, D, E: MB 10599, F: MB 1499
3.2.3.2. The effect of hydrogen peroxide on *B. sporothermodurans* spores

The effect of H$_2$O$_2$ on the spores of three *B. sporothermodurans* strains namely F3 (isolated from UHT milk in South Africa), DSMZ 10599 (one of the first strains isolated, from UHT milk in Italy) and MB 1668 (isolated from feed concentrate in Belgium) was compared using TEM.

![Transmission electron microscopy images on the effect of hydrogen peroxide on *B. sporothermodurans* spores of strains F3 (1), DSMZ 10599 (2), MB 1668 (3)](image)

Figure 7: Transmission electron microscopy images on the effect of hydrogen peroxide on *B. sporothermodurans* spores of strains F3 (1), DSMZ 10599 (2), MB 1668 (3)

Figure 10 shows that, the three *B. sporothermodurans* strains were affected similarly by H$_2$O$_2$. After 3 minutes, the exosporial and coat layer were completely degraded with significant swelling of the spore core. The cortex layer was still however visible. Strain MB 1668 (isolated from feed concentrate) was the only strain to show rupture of the core after 3 minutes. Rupture of the core of strains DSMZ 10599 and F3 occurred after 6 minutes. After 9 minutes, the core outer layer of F3 and MB 1668 was completely degraded however not fully for DSMZ 10599. At 15 minutes the core was exposed to the environment however even though DSMZ 10599 core layer was still not completely degraded, the core was still exposed to the environment.
3.2.3.3. The effect of heat on *B. sporothermodurans* spores

![Graph showing DSC profile](image)

**Figure 8: DSC profile of *B. sporothermodurans* spores strain DSMZ 10599**

Figure 11 shows a DSC thermogram with heat values versus temperature. The initial scan of DSMZ 10599 showed 3 transitions, one at 79°C, the second at 87°C and the third at 113°C. The first peak was associated with the activation (increase in reproduction) of the spores due to the heat. The heat values were found to increase from the first to the last transition.

3.2.4. Discussion

*B. sporothermodurans* spores are made up of a number of layers, each one playing a role in the spores survival/durability. The three isolates, even though one strain was isolated from non UHT source, showed similarities with each other. All strains had an exosporial layer, spore coat, cortex and core. Very little is known about the role of the exosporium, however since the exosporium layer is the first layer to come into contact with the environment it may have the potential role of sensing the environment. The important aspect of germination is rehydration. There were a number of folds present due to the dehydration of the spores. The more dehydrated the spores, the better the chance of survival during dormancy. When
germination is most likely to occur after dehydration, the folds of the coat allow for expansion. Due to the looseness of the exosporium and the folds of the coat, it allowed for expansion and contraction in response to parameters such as humidity (Driks, 1990). One could observe that the less folds there were, the more expanded the cortex layer was. The cortex layer showed quite a great deal of space where the water could fill in when germination occurred. The cortex layer is made up of crosslinking peptidoglycan layers and varies along the spores radius (Driks, 2003). Bacillus spores in general are made up of a number of enzymes that protect the spore from chemical stresses and bring about germination. The tough coat also allows for smaller molecules such as nutrient molecules to access the coat for when germination occurs (Driks, 2002). B. sporothermodurans would most likely have these enzymes present as well. The composition of the spore layers would therefore give a good reflection of the environment they are in. Since not much is known about the composition of B. sporothermodurans layers, further research would need to be conducted on the composition of the B. sporothermodurans spore layers.

Appendages have been observed in species such as Bacillus cereus and Clostridium taeniosporum, however appendages have yet to be observed in B. sporothermodurans spores. Even though a typical appendage-like structure could not be observed, a node like structure was clearly visible on the spore coat. This node-like structure may be a shorter version of an appendage. The appendages observed seemed to remain short when in contact with the surrounding spores, however it lengthened slightly when attached to the glass surface. This ease of adaptability may explain their close attachment to each other and surfaces which can clearly be seen from the AFM images (Fig. 9). The short length of these appendages could have allowed for close attachment to the surrounding spores and to the steel pipes. The potential of B. sporothermodurans spores to attach to steel pipes in UHT processing and to each other is high. This may prove difficult to remove with heat and chemicals as little penetration occurs to the steel surface. Past research has shown that adherence is more involved with the number of appendages and the degree of hydrophobicity (Faille et al., 2010). One would need to determine the degree of hydrophobicity of B. sporothermodurans spores.

Research has shown that the spore coat plays an important role in protecting the spore against extreme physical stresses such a chemical stress (Henriques & Moran, 2007). From the
figures, the chemical hydrogen peroxide clearly affected the spores’ layers. At different times of exposure, different layers were affected. As a result, the longer the spores were in contact with H₂O₂, the more H₂O₂ was able to inactivate the proteins and penetrate the spore layers. The only difference between the three strains was that the outer core membrane of MB 1668 was degraded at 3 minutes instead of at 6 minutes. Therefore, the stability of the spores would most likely occur more quickly H₂O₂ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivate the proteins (Melly et al., 2002). Even though the core was exposed to the environment, little DNA damage occurred. These results could be correlated with those of Tabit & Buys (2010), whereby after H₂O₂ treatment, the spore concentration decreased significantly after an increase in time however spores were still viable after treatment. Therefore damage to the spore was not fully effective where DNA damage is minimal or the damage of the spore proteins was reversible.

Three endothermic peaks were observed for the DSC profile of DSMZ 10599. The first endothermic peak at 79°C was associated with the heat activation of the spore meaning that the increase in temperature brought about germination. This germination occurred because some water was present. The drier the environment the less the peak that is present. The endothermic peak of B. sporothermodurans was relatively high compared to that of B. cereus (56°C) and B. megaterium (56°C). This may explain why B. sporothermodurans are low in number when present in UHT milk since B. sporothermodurans germinate at higher temperatures, the other sporeforming bacteria have already utilised the nutrients in their ideal environment making it difficult for B. sporothermodurans to compete. The endothermic peaks are most likely from the coat and cortex, core layer. The coat is made up of a number of proteins and endothermic peaks are often related to protein denaturation as well as the DNA and RNA unfolding (Belliveau et al., 1992). One peak may not always mean one transition occurring at that temperature. Bacterial spores are made up of a number of components; therefore there could be thousands of potential endothermic transitions at a specific temperature. Therefore one transition may be made up of a number of small transitions or it is a transition whereby there is a high concentration of a one component in the spore (Belliveau et al., 1992). The concentration of the spore layers plays a role in the amount of heat needed to denature the proteins. From the thermogram, the third peak had the highest peak area, therefore the concentration of the proteins was high or there was a combination of proteins with a number of peaks at the same temperature (Belliveau et al., 1992).
The third endotherm was most likely due to the high protein concentration in the spore coat layer. Often killing of a spore is observed by large exothermic transitions. For example *B. megaterium* was found to have a large exothermic transition at 119°C. However no exothermic transition occurred in the thermogram of *B. sporothermodurans*. This correlated with the general information that *B. sporothermodurans* is able to survive UHT temperatures of up to 140°C. Further studies on *B. sporothermodurans* using a more sensitive DSC to determine whether the protein damage is reversible would be interesting to observe as well. A combination of heat and hydrogen peroxide is said to have a greater affect in a shorter time span. This would most likely be true since H₂O₂ would degrade the spore layers and heat would further denature the protein and DNA of the spore.

### 3.2.5. Conclusion

From the above research *B. sporothermodurans* spore structure was found to contribute to the resistance to heat and chemicals. The structure may be affected by the environment they are in and therefore a much more work would need to be done on the proteins present in the layers of the *B. sporothermodurans* spore and whether there is a difference between UHT resistant and non UHT resistant isolates of *B. sporothermodurans*. *B. sporothermodurans* has now been found to have short node-like appendages present on its spores which may explain attachment to steel pipe surfaces resulting in post and cross contamination in UHT milk. A further investigation would be interesting to determine whether this species is able to produce a biofilm. Furthermore, it would be interesting to determine whether the damage done to the spore by heat is reversible.
Chapter 4: General Discussion

*Bacillus sporothermodurans* are the only known strains whose spores are able to survive the UHT treatment. With companies having the disadvantage of not being able to increase the temperatures during milk processing as a result of negative sensory properties, a better understanding on the structure of *B. sporothermodurans* spores and how exactly *B. sporothermodurans* are able to survive such high temperatures has become of great importance in the dairy industry. The main objective of this study was to determine whether there was a genotypic difference between *B. sporothermodurans* strains present in UHT milk and the strains present in other farm sources. The morphological structure of *B. sporothermodurans* spores and the effect of heat and hydrogen peroxide on the spore structure was also determined.

During growth of *B. sporothermodurans* strains on brain heart infusion agar, it was found that the strains struggled to grow, the older the plated out isolates were. Additional nutrients in the agar plates such as vitamin B12 would have helped with the growth of *B. sporothermodurans*, strain F3 and DSMZ, as few colonies were formed under optimal conditions.

A comparison of the molecular methods, namely REP-PCR and (GTG)$_5$ PCR, was investigated to determine which method was most efficient in characterising *B. sporothermodurans* strains. For the the method of preparation, (GTG)$_5$ PCR was easier and quicker than the REP-PCR method with the (GTG)$_5$ PCR programme not having a temperature “ramp” (temperature profile in the PCR programme) stage. The two new *B. sporothermodurans* strains, F3 (isolated in 2007) and QA1 (isolated in 2007) were confirmed as *B. sporothermodurans* and belonged to the HRS clone confirming these strains produce highly heat resistant spores. It was of interest to observe that strains F3 and QA1 were isolated in the same year and in two different countries. It was looked into whether there was import or export of milk from South Africa to Belgium resulting in cross contamination between the two countries however it was unlikely and difficult to determine due to the lack of information, for example, batch of milk, if ever, sent to Belgium.

It was observed that these two recently isolated heat resistant strains (isolated from UHT milk) were the closest in similarity compared to the heat resistant strains such as MB 10599 and MB 372. A reason could be due to environmental conditions differing between Belgium and South Africa. It can be hypothesized that two populations of the same species being separated
geographically will differ as they adapt to their respective environment (Dale & Park, 2004). Due to changes in environmental factors, the organisms will often adapt phenotypically and genetically to reduce the impact of stress on them. (Bijlsma & Loeschke, 2005). When comparing the more recent strains, F3 and QA1, isolated in 2007, to the strains isolated from UHT milk previously, the strains showed an 80% association with MB 372 and an even lower association with DSMZ 10599. One thought could be that because the less recent UHT isolates have had time to evolve to their different conditions, the close association is less whereas with the two recent strains, they have had less time to evolve, resulting in a close association. When looking at the differentiation of the strains isolated in different years and environments, bacterial adaptation has been found to play a role in the bacterial genome. The gene loss, gain or rearrangement is involved with the evolutionary processes of bacteria resulting in a high genetic variation due to the differences in environmental circumstances (Lawrence & Hendrickson, 2005). The greater the degree of genetic variation, the more successful evolutionary adaptation is. One could deduce that evolutionary adaptation and environmental adaptation may have a played a role in genetic differentiation of B. sporothermodurans strains however this would need to be looked into more detail.

Feed concentrate had the highest contamination of B. sporothermodurans in farm sources (i.e. feed concentrate silage, raw milk) (Scheldeman et al., 2002; Varewijck et al., 2001). It was observed that even though the feed concentrate strains originated in Belgium, the similarity was not more than 80% in both molecular methods. Even though the farm isolates were isolated from the same country, the strains seemed to have adapted to different stresses (i.e. environmental conditions) in different ways (Varewijck et al., 2001).

When comparing heat resistant to non-heat resistant B. sporothermodurans strains, there seems to be no similarity between the two. MB 1505 was the only farm isolate that showed a significant similarity to the UHT strains. Similarities may be due the acquisition of the HRS clone by both MB 1505 and the UHT strains. Results go on to show that the B. sporothermodurans strains of the HRS clone still seem mainly responsible for the production of heat resistant spores. To determine whether the B. sporothermodurans strains have the HRS clone is important in the dairy industry as the quality and safety of dairy products is vital. The genetic variability may play a large role in the evolutionary adaptation of B. sporothermodurans strains however more research would need to be done on the prominence of adaptation. This may provide an understanding of how adaptable B. sporothermodurans will
be if changes occur in dairy processing of UHT milk and if anything can be done to prevent this.

It was also determined if there was a difference in the structure of spores of the heat resistant (isolated from UHT milk) and non-heat resistant (isolated from farm sources). The present study found the spores to be similar in structure with the only difference being genotypic as discussed above. What was interesting was the possibility of appendages on the *B. sporothermodurans* spores. Appendages have been observed in species such as *B. cereus* and *Clostridium taeniosporum*, however appendages were previously not observed in *B. sporothermodurans* spores. During the present study, appendage structures were confirmed to be present on *B. sporothermodurans* spores protruding from the coat layer. It may be that these structures could be associated with the ability of the spores to attach to surfaces such as steel pipe surfaces and to each other. This could potentially cause layer upon layer of spores on the surfaces making it difficult for heat penetration during cleaning of the pipes. Appendages attach themselves to a surface allowing for germination to occur leading to the growth of vegetative cells (Panessa-Wareen, 2007). Studies have shown that spores of *B. cereus* adhere better to hydrophobic than hydrophilic surfaces. It has been suggested that spores adhere to surfaces by increasing the hydrophobicity of the spore surface (i.e. adheres to stainless steel surfaces found in processing equipment) (Husmark & Ronner, 1990). The degree of hydrophobicity and the presence of appendages have not yet been determined for *B. sporothermodurans* spores. Such attached cells may also produce biofilms, thereby becoming more resistant to sanitizers. Biofilms are defined by Costerton *et al.* (1987) as “a functional consortium of microorganisms attached to a surface and is embedded in the extracellular polymeric substances (EPS) produced by microorganisms”. These biofilms are a menace in the dairy industry because they can reduce the heat flow across the surface, increase the fluid frictional resistance at the surfaces and increase the corrosion rate at the surface resulting in product loss (Ganesh Kumar & Anand, 1998). The potential for *B. sporothermodurans* to form biofilms has not yet been determined but may be a potential future study as this information would be of great importance in the food industry.

With the use of the Atomic Force Microscope (AFM), it was extremely difficult to zoom in on the spore coat surface to observe the arrangement of the bond. This finding would have been very interesting to determine whether the arrangement differed to that of, for example, *B. cereus* spores as it may contribute to the heat resistance of the spore. Although the spores were observed using a dry preparation method, examining the spores in liquid under the AFM may
be the preferred method as the spores began to dehydrate and lose shape after a while making it difficult to get a substantial number of photos.

The differential scanning calorimetry (DSC) was used to determine how much energy was needed to break down the spore layers and at what temperature this occurred. The endothermic peaks were most likely from the coat and cortex, core layer. The third endothermic peak was most likely due to the high protein concentration (i.e. more energy needed to degrade the large protein concentration and bonds) of the spore coat layer. No exothermic transition (i.e. spore death) occurred for the B. sporothermodurans spores. This correlated with the general information that B. sporothermodurans are able to survive UHT temperatures of up to 140°C. However the lack of spore death detection may be due to the less sensitive DSC machine. The main aspect I would change in this experiment would be to use a more sensitive DSC. Because such small amounts of sample were used, the results should have shown more peaks at different temperatures thereby relating better specificity at what temperature each different spore layer would be affected.

From the transmission electron microscope (TEM) images, it was found that H₂O₂ had damaged the spore surface layers but not the spore core containing the DNA. As a result, growth of the strains still occurred. Results showed that the longer the spores were in contact with H₂O₂, the more the H₂O₂ was able to inactivate the proteins and penetrate the spore layers. Past work has shown that H₂O₂ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivate the proteins (Tabit & Buys, 2010). Using H₂O₂ may be an advantage as it helps to reduce the bacterial count and prolong the shelf life of the milk. Bacterial spores are resistant to a number of chemicals although, there are some chemicals which are effective (e.g. formaldehyde, nitrous acid, alkylating agents) (Russell, 1990; Setlow, 2006). Spore killing by chemicals is mainly by DNA damage as a result of survivors accumulating mutations that in turn will sensitize the spores to these chemical agents. However, some oxidising agents have been found to kill spores by damaging the external layers of the spore and as a result, when germination occurs, the damaged membranes rupture resulting in spore death. The precise damage to the inner membrane however is not known (Setlow, 2006). The main role of the spore layers is to exert a protective role by detoxifying the chemicals before they penetrate further into the spores. An enzyme called superoxide dismutase may have the potential to detoxify damaging chemicals and has been associated with exosporium or spore coats of certain species (Setlow, 2006).
Packaging material is often sterilized by a number of different methods such as heat, hydrogen peroxide, irradiation, infrared light, etc (Ansari & Datta, 2003). A combination of these may also be used depending on one's preferences. With the use for example of hydrogen peroxide (H₂O₂), the chemical is seen as an indirect sterilant of food or milk. It has been reported that with the use of H₂O₂, 0.25 ppm is present in the food as residual that has no real harmful effect on consumers. Regulations specify that a maximum of 35% H₂O₂ may be used for sterilizing food contact surfaces. The final product is also not allowed to contain more than 0.5 ppm residual H₂O₂. Many aseptic packaging systems use around 30-35% H₂O₂ as a sterilant for aseptic packaging in conjunction with hot air (60-125°C) to enhance the sterilization effect as well as to remove any residual H₂O₂ resin after sterilization (Ansari & Datta, 2003; Russell, 1990). The use of H₂O₂ is most effective with heat, as at ambient temperatures its affectivity decreases. It however becomes a problem when spores such as *B. sporothermodurans* are highly heat resistant (Ansari & Datta, 2003). H₂O₂ does not kill spores by DNA damage; however, it does penetrate the spore core and inactivate the proteins. The main target for spore killing by H₂O₂ is at the exterior of the spores inner membrane (Melly *et al*., 2002).
Chapter 5: Conclusions & Recommendations

5.1. Conclusions

The present study has shown that *B. sporothermodurans* spore structure contributes to the resistance of heat and chemicals. It can be confirmed that even though the spores do need to have the HRS clone to be highly heat resistant, the spore layers such as the spore coat and spore cortex do contribute to the heat resistance of the spore. The structure reveals information about the environment they were isolated from and therefore much more work would need to be done on the proteins present in the layers of the *B. sporothermodurans* spore and whether there is a difference between UHT and non UHT isolated strains of *B. sporothermodurans*. Prevention would be to make sure any and post UHT contamination decreases in factories. *B. sporothermodurans* has now been found to have short appendages present which may be involved in attachment to steel pipe surfaces resulting in post and cross contamination in UHT milk. With the potential of the appendages resulting in attachment to surfaces, it may be the main reason why it is so difficult to clean and remove the spores from pipes resulting in cross contamination. A better understanding of the degree of attachment to the surfaces will provide improved cleaning methods and prevention of cross contamination.

5.2. Recommendations

- Since not much is known about the composition of *B. sporothermodurans* spore layers, further research would need to be conducted on the composition of the *B. sporothermodurans* spore layers such as determining the protein content of each spore layer and the crosslinking of the proteins in the spore coat. This finding may explain whether the composition will make a greater contribution to heat resistance of the *B. sporothermodurans* spores.

- Since \( \text{H}_2\text{O}_2 \) does not completely kill the spore, a recommended experiment would be to use \( \text{H}_2\text{O}_2 \) and heat together and determine the extent of damage using TEM. A combination of heat and hydrogen peroxide is said to have a greater affect in a shorter
time span than H₂O₂ alone. This would most likely be true since H₂O₂ would degrade the spore layers and heat would further denature the protein and DNA of the spore.

- A future experiment would also be recommended to determine if the damage of spores is reversible using the DSC since no spore death was indicated at 140°C.

- Further studies would need to be performed to determine the degree of hydrophobicity to surfaces and most likely the potential for *B. sporothermodurans* strains to develop biofilms.
Chapter 6: References


• Herman, L., Heyndrickx, M. & Waes, G., 1998. Typing of *Bacillus sporothermodurans* and other Bacillus species isolated from milk by repetitive element sequence based PCR. *Letters in Applied Microbiology* **26**, 183-188.


Appendix 1

High resolution SEM imaging of *B. cereus* spores strain 10876

TEM imaging of *B. cereus* spores strain 10876