CHAPTER 5: GENERAL DISCUSSION

The general discussion chapter initially elucidates the methodology of the research project followed by observations regarding the incidence and survival of *Bacillus sporothermodurans* during UHT processing. The observations regarding the effects of wet heat treatment on the structural and chemical components of *B. sporothermodurans* spores will also be elucidated.

5.1 METHODOLOGICAL CONSIDERATIONS

SPCs were conducted for all retail milk samples (raw, pasteurised and UHT treated) in order to determine their bacterial quality. Similarly, milk samples at various points of the processing line at processor D were also collected and the bacterial quality determined. It is essential to use SPC which is a conventional technique employed by most quality assurance laboratories. An alternative method that could have been used to enumerate bacteria in milk samples is the flow cytometry. However, flow cytometry requires the clearing of milk and the staining of bacteria with a fluorescent stain thereby making sample handling cumbersome. Despite the rapid nature of flow cytometry, it has a particularly low detection limit with up to 10^4 total bacteria per ml in some cases. However, it can detect non-culturable, dead and injured cells (Gunasekera, Attfield & Veal, 2000). It would have been interesting to enumerate the bacteria using both methods.

The pure cultures of all the samples were then analysed using the *B. sporothermodurans* specific PCR to determine whether or not isolates were *B. sporothermodurans*. The choice of 4 or 5 colonies taken per plate was to ensure that tested isolates were representative of the total bacterial flora in the different milk packages. It would have been useful to profile all the bacteria isolated from each milk sample using extragenic palindromic polymerase chain reaction (REP-PCR) and sequencing in order to determine their identity and genetic relationship considering that REP-PCR has been used successfully to differentiate *Bacillus sporothermodurans* species (Herman & Heyndrickx, 2000). This technique would have enabled us to

alternatively confirm the absence *Bacillus sporothermodurans* in raw and pasteurised milk as established in this research.

The determination of viable spore counts of UHT milk samples was conducted by plating serial dilutions of milk or broth samples that have been heat treated at 95 °C for 30 min followed by SPC. The direct epifluorescent filter technique (DEFT), which is a rapid technique for viable spore enumeration, could have been very useful in this research. This technique includes heat activation followed by germination with l-alanine and the subsequent counting of green and orange fluorescing cells (Moran, Rowe & Gilmour, 1991). This method can be used alongside the SPC method considering both methods have been found to have a good correlation factor (r= 0.98)

B. sporothermodurans spores to be used for thermokinetic analysis were obtained from BHI agar plate growth that had been incubated for 15 days at 37 °C. The reason why plates were incubated for 15 days at 37 °C was to ensure complete sporulation. Spores at this stage must have attained complete maturity. In this study, spores were washed and thermokinetics studies were conducted without delay to ensure that spores did not undergo any physiological changes prior to analysis.

In this study, the thermokinetic characteristics of spores were obtained by heating spores suspension (10^8 spores/ml) in sterile pressure tubes. The use of a UHT steriliser would have been ideal to monitor the inactivation and the effect of wet heat treatment on spores. However, this was not case because of lack of equipment as well as knowing that local milk processors would not wish to have their UHT sterilisers inoculated with spores.

In order to observe the mechanisms of destruction of spores at different times, TEM analysis of resin-polymerised spores was conducted. The TEM analysis can only show structural differences in spores across the different heating times. It would have been interesting to do scanning electron as well as phase contrast microscopy in order to see morphological changes across the different heating times.

The analysis of leaked protein and DPA was carried out in order to determine the amount released at different times. Even though the amount of DPA release is not a reflection of heat resistance, it gives an indication of the mechanism of spore destruction as well as an indication of the extent of destruction during which death occurs.

Two methods of extraction were used to extract the genomic DNA for molecular analysis in this study. The isolation of genomic DNA for PCR with agarose gel detection was conducted as described by Scheldeman *et al.* (2002) with some modifications. The modifications introduced were the centrifugation of the boiled bacteria and the quantification of the DNA in the supernatant. This enables the determination of the right amount of genomic DNA required for the PCR reactions. However, the quantification of DNA from crude cell lysate was problematic due to the presence of impurities but this did not influence the PCR reaction as DNA was diluted in the PCR reaction volumes. On the other hand, DNA for real time PCR with SYBR Green was extracted and purified from pure cultures using the ZR Fungal/Bactrial DNA Kit™ (Zymo Research Corporation, USA) because real time PCR will not give accurate results if the genomic DNA utilised is not pure.

The molecular identification of *B. sporothermodurans* was conducted using *B. sporothermodurans* specific primers described by Schelderman *et al.* (2002). An alternative PCR primer developed by Herman *et al.* (1997) can also be used to detect *B. sporothermodurans*. The problem with this PCR is that it can detect only heat resistant spore forming *B. sporothermodurans* while the method described by Schelderman *et al.* (2002) can detect both heat resistant spore formers as well as non-heat resistant spore formers. Other reputable identification techniques like the REP-PCR with gel separation (Klijn *et al.*, 1997; Herman *et al.*, 1997) and ARDRA (ribotyping) (Guillaume-Gentile *et al.*, 2002) were not considered because they are more complex than the normal PCR identification.

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

The SPC values of raw milk from processor D were higher than the 300 000 (log 5.48) cfu/ml maximum acceptable SPC level for raw milk prior to pasteurisation as stipulated by the European Council Directives 85/397/EEC, 1985. The same limit is also endorsed by the Dairy Standard Agency of South Africa. SPC values of this magnitude reflect poor fresh milk handling sanitation and herd health conditions (Muir, 2007). Export of UHT milk with this quality is prohibited particularly to EU member countries and the USA. Ineffective cooling, poor maintenance of milking equipment and poor water quality are often the cause of a high bacterial load in fresh milk (Muir, 2007). Similarly, the pasteurised milk packages had SPC higher than 50 000 (log 4.70) cfu/ml which is the maximum SPC level stipulated by the European Council Directives 85/397/EEC, 1985 and the Dairy Standard Agency of South Africa. This level is also a reflection of the poor quality of the raw milk that was pasteurised. It should be noted that the quality of processed milk is reflected by the quality of the raw fresh milk.

The fact that high *B. sporothermodurans* counts were determined in UHT treated milk to be used for re-processing is of great concern because of the inevitable re-introduction of spores and vegetative cells originating from spores that survived the previous UHT treatment in the UHT processing line. This practice has grave implications, as it is likely to result in the introduction of a high quantity of bacterial protein from vegetative cells into UHT milk destined for a long period of storage. Furthermore, spores of *B. stearothermophilus* that survived a sub-lethal heat treatment have been found to exhibit a heat-induced resistance to subsequent heat treatment (Etoa & Michiels, 1988). Therefore, spores of *B. sporothermodurans* that may have survived a previous UHT treatment and have not germinated could acquire a heat-induced resistance thereby increasing their survival during subsequent UHT reprocessing. However, results from this research did not indicate any heat-induced resistance and proliferation of *B. sporothermodurans*.

In a similar experiment, pre-heating of broth culture before UHT treatment did not influence the survival of *B. sporothermodurans*, contrary to the findings of Teofila *et al.* (1998), where pre-heating was found to induce heat resistance in spores of *Bacillus* spp. This may be attributed to the fact that spores had not attained the required level of heat resistance induction during pre-heating considering that heat-induced resistance is relative to the duration of exposure to sub-lethal temperature (Teofila *et al.*, 1998). Heat-induced resistance only becomes effective at the point where there is an expansion of the cortical peptidoglycan against an intact coat resulting in more water in the cortex and less water in the protoplast. The idea behind the pre-heating experiment was to determine the effect of pre-heating on the survival of *B. sporothermodurans* following UHT treatment as this may have some implication for UHT processes in dairy industries with *B. sporothermodurans* contamination. However, looking at the results of this research, pre-heating did not affect the survival of *B. sporothermodurans* following UHT treatment.

The SPC of contaminated UHT milk in this study were less than 10^5 cfu/ml that was obtained from 37% of contaminated Italian UHT milk packs analysed over a period of two years (Montanari *et al.*, 2004). It should be mentioned that 10^5 cfu/ml is the maximum *B. sporothermodurans* load that has been reported so far for contaminated UHT milk packs after incubation for 15 days at 30 °C. Even though this research has confirmed the presence of *B. sporothermodurans* in the South African dairy industry, the exact route of contamination remains unknown. This is exacerbated by the fact that the many attempts to isolate *B. sporothermodurans* from raw and pasteurised milk have been futile.

The results of the PCR reactions indicated that isolates from contaminated UHT milk packages were *B. sporothermodurans*. Given the fact that at least one isolate from each of the UHT milk packs tested positive for *B. sporothermodurans*, confirmed the incidence of this highly heat-resistant spore-producing bacteria in the South African dairy. The adoption of the PCR methodology of Schelderman *et al.* (2002) to the RT PCR methodology used in this research to detect *B. sporothermodurans* was successful. This is the first time in which *B. sporothermodurans* has been detected

using RT PCR with SYBR Green using the primers; BSPO-F2 (5'-ACG GCT CAA CCG AG-3') and BSPO-R2 (5'-GTA ACC TCG CGG TCT A-3').

The adopted RT PCR methodology was efficient considering that 32 out of 33 isolates tested positive after a single run. This result could be due to the introduction of the DNA extraction kit that enabled extraction of pure DNA, unlike the case of crude DNA extraction when the method described by Schelderman *et al.* (2002) was used. The high number of false negative results after a single run using the PCR detection agarose gel electrophoresis was a result of the crude DNA extracts which contained impurities that might have interfered with the PCR reaction. Impurities in the crude DNA might also have hampered the accurate quantification of the crude DNA. The presence of an inappropriate amount of genomic DNA in the reaction mixture can result in the replication of the wrong segment or abort the PCR reaction (Roux, 1995).

In addition, the detection method of the RT PCR with SYBR Green is internalised and is more robust and less labour intensive than the agarose gel electrophoresis detection method. The fluctuation of the Tm between 86–87 °C could be attributed to minor variations in the nucleotide sequences within the amplified segment of the 16S rRNA gene. A similar fluctuation was observed in a study involving the melting curve analysis for the identification of *Plum poxvirus* strains (Varga & James, 2005).

Herman *et al.* (2000) used REP-PCR combined with non-denaturing separation in polyacrylamide gel to analyse 37 *B. sporothermodurans* strains. This technique offers a very powerful tool to discriminate between species and strain of *B. sporothermodurans* and other *Bacillus* spp. The PCR detection technique used in this research can be used in combination with the REP-PCR molecular typing to study the contamination route of *B. sporothermodurans* in raw and heated milk as well as on farms. When comparing the different PCR methods utilised for the identification of *B. sporothermodurans*, it can be concluded that RT PCR with SYBR Green was quicker and less labour intensive than other methods.

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

UHT treatment at 140 °C for 4s in this research was severe enough to completely inactivate broth cultures of *B. sporothermodurans*. This finding is contrary to the finding of Huemer *et al*. (1998) where spores of *B. sporothermodurans* were found to be more resistant at temperatures above 130 °C. However, Huemer's finding was on *B. sporothermodurans* spores that were harvested from a 7-day-old ONA^{+} agar plate culture. The 24h *B. sporothermodurans* culture used in this study could only possibly have contained spores that were older than 24h and mature enough to display high heat resistance (Knott *et al*., 1995). It should be recalled that the aim of this research was to find out if the spores present in broth cultures for not more than 24h could acquire heat-induced resistance after reprocessing.

In an experiment conducted to determine the thermokinetics of *B. sporothermodurans* spores, Huemer *et al*. (1998) found that *B. sporothermodurans* spores have heat resistance comparable to that of *B. stearothermophilus* and *B. subtilis* within the 110–125 °C temperature range. On the other hand, they also discovered that *B. sporothermodurans* exhibited an extremely high heat resistance within the 130–145 °C temperature range. The spores used in that thermokinetic study were from *B. sporothermodurans* cultures that were cultured on ONA^{+} agar plate for seven days at 37 °C or 55 °C.

Chilling rendered *B. sporothermodurans* spores susceptible to UHT treatment when compared to spores that were not subjected to chilling prior to UHT treatment. This result is in agreement with research conducted by Movahedi and Waites (2002) in which *Bacillus subtilis* spores were found to be susceptible to temperatures above

100 °C, but not to an increase in heat resistance observed at temperatures between 85–90 °C. Movahedi and Waites (2002) did not elucidate on the mechanism through which chilling followed by heating affects the components of *Bacillus subtilis* spores to make them more susceptible to high temperatures. Until now, no work has been done to determine the effects of chilling at 85–90 °C on the heat resistance of spores of *B. sporothermodurans* and what effect this has on the UHT processing of milk contaminated with *B. sporothermodurans* spores. Such findings could be used to modify processing technology so that spores may be inactivated by a relatively less severe heat treatment.

It is logical to determine the effect of H_2O_2 on spores of *B. sporothermodurans* considering that it is one of the most widely used disinfectants for sanitising UHT milk packaging materials. The significant differences in the survival counts of the spores observed during the different exposure time intervals was due to the effectiveness of the sporicidal activity of 30% H_2O_2 which increases with increasing incubation times (Toledo *et al.*, 1973). The inactivation of *B. sporothermodurans* spores followed a first order kinetic with a $D_{30\%}$ value of 3.33 min indicating that 30% H_2O_2 concentration can be used as a sterilising agent to disinfect packages for UHT treated milk contaminated with *B. sporothermodurans* spores. For this to be effective, treatment with 30% H_2O_2 should be followed by the application of hot air (Khadre & Yousef, 2001). The sporicidal properties of H_2O_2 have been well documented by many authors. Conversely, the exact mechanism of spore inactivation has not been determined (Melly *et al*., 2002).

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

In this research, spores of *B. sporothermodurans* were found to have compact cores and a relatively large cortex, which is in agreement with previous findings (Scheldeman *et al.*, 2006). The release of protein (µg/ml) from heated spores was significantly ($p \leq 0.05$) different between the two isolates. This could be attributed to

structural difference in protein composition within the spores of the different *B. sporothermodurans* isolates. The composition of the exosporium and coat structures of *Bacillus* spores has been found to be species and strain specific (Henriques & Moran, 2007). On the other hand, the release of DPA across the heating times was not significantly different between the two strains. This is in agreement with similarities observed in the inactivation pattern and structural damage of both strains at different heating times.

From the TEM, spores began losing their structural components after 4 min of heating. The cortical membrane was the area first affected by wet heat and had the first visible signs of structural damage. The reason for this early damage is due to the washing away of peptidoglycan materials that are the main constituent of the cortex (Setlow, 2006). At this time, heat must have penetrated the spore layers into the spore core resulting in the release of more protein than DPA. This finding is in agreement with previous findings in which the DPA release from heated *Bacillus subtilis* spores was accompanied by a large amount of protein release (Coleman *et al.*, 2007).

The fact that a small proportion of spores did not show visible structural damage after 4 min of heating could be that damage is not acquired at the same rate by all spores. This is in agreement with findings of a similar study in which *B. stearothermophilus* spores had been inactivated at different rates following heating at UHT temperatures (Feeherry *et al.*, 1987).

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

By 12 min there was complete destruction of spore components with the cortex almost entirely washed off leaving behind a reduced and less dense spore core surrounded by loosely attached coat and surface layers. This is due to the total destruction of spores after prolonged heating which results in the flushing out of a considerable amount of soluble protein from spores. This is explained by the levelling off of protein release between the 8th and the 12th min of heating. Contrary to soluble protein release, the amount DPA released at this stage did not level off. DPA is located exclusively in the spore core, the last area in which visible structural damage was detected during the entire heating time as seen on the TEM.

By integrating the TEM, protein and DPA analyses, the mechanism of *B. sporothermodurans* spore inactivation over time can be summarised as follows: heat penetrates into the spore core; hot moisture follows and rehydrates the spore; spore structures are destroyed; massive inactivation of spores occur (small amounts of DPA escape); finally, total inactivation of spores occurs (large amounts of DPA escape). The release of soluble protein levels off while the DPA release rises until there is complete spore destruction.

Bacteria spores following heat treatment often die as a result of injury acquired during heating. These injuries often lead to the inability of spores to germinate in the vegetative cells. However, bacterial spores can recover from their heat injury. The recovery time differs among individual spores with some taking longer than others (Speck & Busta, 1968). This phenomenon is of major concern considering that quality control testing procedures do not take into account any injured spores with a longer recovery duration. In this research, injured spores that took long to germinate were accounted for by incubating BHI agar plates for up to one week.

5.4 PROPOSALS FOR FUTURE RESEARCH

For rapid identification and quantification more research needs to be conducted on *B. sporothermodurans*. Such techniques should be able to detect and quantify *B. sporothermodurans* in milk samples without culturing. Such techniques should be

simple in order to be incorporated easily into an automated system. It should be mentioned that current detection techniques involve culturing and isolation of suspected colonies before identification.

More research needs to be done to determine the effect of recontamination during processing using large volumes of milk. This research used broth cultures. Similarly, it would be interesting to determine the response of *B. sporothermodurans* to UHT processing with larger percentage recontamination. Only 10% recontamination was used in this study.

Based on findings in this research, spore death occurs after hot moisture has breached the structural barrier of spores and gained entry to the spore's core. In this regard, more research should be conducted to identify various non-thermal methods to compromise the structural barrier of spore prior to UHT treatment. If this is achieved, technological processes could be modified in such a way that the inactivation of *B. sporothermodurans* would be achieved with a less severe heat treatment. In addition, further research could determine the possibility of activating and germinating spores in milk prior to UHT treatment, as this will also enable the inactivation of spores using a less severe heat treatment.

In this research, chilling was found to render *B. sporothermodurans* spores susceptible to UHT treatment at 130 °C. Further research should be conducted with spores in a large volume of milk using a pilot plant in order to ensure industrial application and also to ascertain how chilling prior to UHT treatment would affect the quality of the end product.

Further research needs to be conducted to determine the production of stress response protein within spores of *B. sporothermodurans* after having subjected the growing cell and spores to various types of stress considering that the production of spore protein is driven by morphogenetic factors in the mother cell (Henriques & Moran, 2007). Studies on the production of heat-shock protein will be of great interest considering the unusual thermokinetic properties of *B. sporothermodurans*.

The prospect of modifying processing parameters in order to enhance the inactivation of *B. sporothermodurans* spores with the application of a comparatively less severe heating should also be studied. The coupling of non-thermal processing techniques such as high-pressure and high-voltage pulse electric field processes that can cause some structural defect on spores prior to the application of heat treatment could be a solution. This idea is feasible considering that spore death is due to the movement of heat into the spore. Any method that accomplishes this compromises the structural integrity of spores and increases the severity of inactivation.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

B. sporothermodurans is present in UHT milk but the incidence is not extensive or widespread and the PCR detection protocol adopted for the RT PCR is effective in confirming *B. sporothermodurans*. Chilling renders *B. sporothermodurans* spores more susceptible to UHT treatment while the onset of DPA released during wet heat treatment coincides with visible signs of structural damage and significant inactivation of spores. Visible signs of spore structural damage emanate at different rates while the amount of protein release seems to be strain specific.

Further research should be conducted to explore the mechanism of spore destruction during wet heat treatment in order to provide a less severe heat treatment that will ensure the inactivation of spores without high-level thermally induced changes in UHT milk. Non-thermal techniques such as High Voltage Pulse Electric technology and High Pressure technology could be explored in this regard.