Bacillus and *Paenibacillus* spp. associated with extended shelf life milk

By

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DECLARATION

I, Desmond Tichaona Mugadza declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

November, 2017

ABSTRACT

***Bacillus* and *Paenibacillus* spp. associated with extended shelf life milk**

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Degree: PhD Food Science

Extended shelf life (ESL) milk combines longer shelf life and better organoleptic characteristics; a combination that is absent in both pasteurised and ultra-heat treated (UHT) milk. Bacterial spoilage remains the main cause of food loss worldwide, which also includes milk and dairy products, despite advances in food preservation technology. The objectives of this study were to identify and characterise the spore-forming population associated with ESL milk during processing and chilled storage as well as characterising *Bacillus cereus* isolates obtained from ESL milk processing and during storage.

Characterisation of spore-formers associated with ESL milk was done by analysing bacteriological quality of milk samples collected at various processing stages and during storage. Isolates were identified with MALDI-TOF-MS. *B. cereus* strains obtained from ESL milk and filler nozzles were characterised using $(GTG)_5$ Rep PCR fingerprinting; the presence of virulence genes; cytotoxin K (*cytK*), nonhemolytic enterotoxin A (*nheA*), emetic toxin cereulide (*cer*) and enterotoxin hemolysin BL (*hblA*). The isolates were further discriminated as psychrotrophic and

mesophilic strains using 16S rDNA. Furthermore, *B. cereus* isolates were selected for 16S partial sequencing. Some of the *B. cereus* strains obtained from ESL milk and filler nozzles were further characterised using *rpoB* partial sequencing and multilocus sequence typing (MLST).

Milk had spore counts < 2 $\log_{10}$ cfu/ml and 4 $\log_{10}$ cfu/ml during processing and storage, respectively. *Bacillus pumilus* dominated the bacterial population. In addition *B. subtilis*, *B. cereus*, *B. sonorensis*, *B. licheniformis* and *Paenibacillus* spp. were among the main spore-formers identified in the study. Bacterial species were inoculated in sterile milk for a shelf life study and population change observed over 42 days at 7 ^{o}C. Despite high prevalence of *cer*, *hblA* and *nheA*; *cytK* was not widely distributed. There was 100% and 8% prevalence of mesophilic and psychrotrophic signatures, respectively in *B. cereus* isolates. Although ESL milk process was effective in the reduction of bacterial counts and species diversity, the presence of *B. cereus* shows a potential safety problem in ESL milk. Despite the large diversity of the *B. cereus* strains in this study, there is evidence that biofilms associated with filler nozzles and raw milk are a source of contamination of *B. cereus* in ESL milk. Furthermore, the study has also shown that *rpoB* partial sequencing and MLST can be used as a tool for source tracking in ESL milk processing.

DEDICATION

I dedicate this work to my late grandparents Mr and Mrs S.H Chiware. It is through the firm foundation you laid in my life that I fought to see this dream turn into reality. May your souls rest in eternal peace.

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CHAPTER ONE

GENERAL INTRODUCTION

Extended shelf life (ESL) milk bridges the gap between high temperature short time (HTST) pasteurised milk, with a shelf life of around 10 days under refrigeration, and ultra-heat treated (UHT) milk with a shelf life of at least 3 months at ambient storage temperatures (Fitzgerald 2012). Currently, there are two methods used in the production of ESL milk, ever since its existence (about 6 years) in the South African market. In the first method, milk is subjected to bactofugation, pasteurised and finally packaged aseptically, while, in the second method milk is subjected to UHT temperatures (135 ^{o}C) for about 0.5 s and packaged. The objective of the product is to combine longer shelf life and better organoleptic characteristics, a combination that is absent in both pasteurised and UHT milk (Rysstad and Kolstad 2006). Bacterial spoilage remains the main cause of food loss worldwide, including milk and dairy products, despite these and other advances in food preservation technology (Gram *et al.* 2002; Ranieri *et al.* 2012). Previous studies have also indicated that most customer complaints emanate from microbial spoilage compared to other factors in processed fluid milk (Hayes *et al.* 2002; Fromm and Boor 2004; Ranieri *et al.* 2012). The production of thermostable proteases and lipases that can remain active even after the elimination of the vegetative microorganisms by heat treatments applied has been reported as one of the biggest hurdle in extending the shelf life of milk (Júnior *et al*. 2017). The ability of *Bacillus* and *Paenibacillus* spp. among other bacteria to form endospores has also emerged as another great obstacle in extending the shelf life of milk. Most endospores are heat resistant and upon germination the organisms are able to grow under a wide range of temperatures and pH, (Huck *et al.* 2007). Psychrotrophic bacteria have been recognised as a pertinent problem in the dairy industry and they contribute to about 25% of shelf life problems in conventionally pasteurised milk and greatly limit shelf life extension of fluid milk and related

products (Francis *et al.* 1998; Stenfors and Granum 2001; Huck *et al.* 2007; Huck *et al.* 2008). Research has documented that *Bacillus* spp. dominate the endospore forming population in milk (Coorevits *et al.* 2008; De Jonghe *et al.* 2010; Schmidit *et al.* 2012; Aoudhi *et al.* 2014), while *Paenibacillus* spp. increase in population during storage of pasteurised milk under refrigeration to outnumber the previously dominating *Bacillus* spp. at the beginning of the shelf life of pasteurised milk (Ranieri and Boor 2009; Ranieri *et al.* 2012).

Despite the high diversity of *Bacillus* spp. in milk (Fromm and Boor 2004; Aouadhi *et al.* 2014), *Bacillus cereus* attracts great attention in food processing. In addition to causing spoilage problems in milk, *B. cereus* has also been reported to be an opportunistic human pathogen (Bartoszewicz *et al*. 2008) that causes two principal types of food poisoning, which are, the emetic and diarrhea (Hansen and Hendriksen 2000). Although it is regarded as a mesophile, some researchers have reported the existence of psychrotolerant strains of *B. cereus* (Stenfors and Granum 2001). These strains have been reported to have the ability to germinate at temperatures between 4-6 ^{o}C and grow well at temperatures below 10 ^{o}C (Larsen and Jørgensen 1997).

Although raw milk has been implicated as an important source of endospores in milk products (Miller *et al*. 2015), other studies have shown that a different population of endospore forming microorganisms exists in raw milk and other milk products and has been attributed to a number of reasons including post heat treatment contamination by processing equipment. (Scott *et al.* 2007; Burgess *et al.* 2010; Hill and Smythe 2012). *B. cereus* contamination has been linked to

processing equipment such as milk fillers (Khoza 2016) since its spores are highly adhesive (Anersen 2007).

Although several studies on ESL milk have been reported, nothing has been documented on the bactofugation based ESL milk product. The objective of this study was to characterise the *Bacillus* and *Paenibacillus* spp. associated with ESL milk spoilage, during processing and chilled storage, with the aim of validating the effectiveness of heat and bactofugation based ESL milk process on the spore-formers as well as understanding the route of ESL milk contamination in a processing plant.

CHAPTER TWO

LITERATURE REVIEW

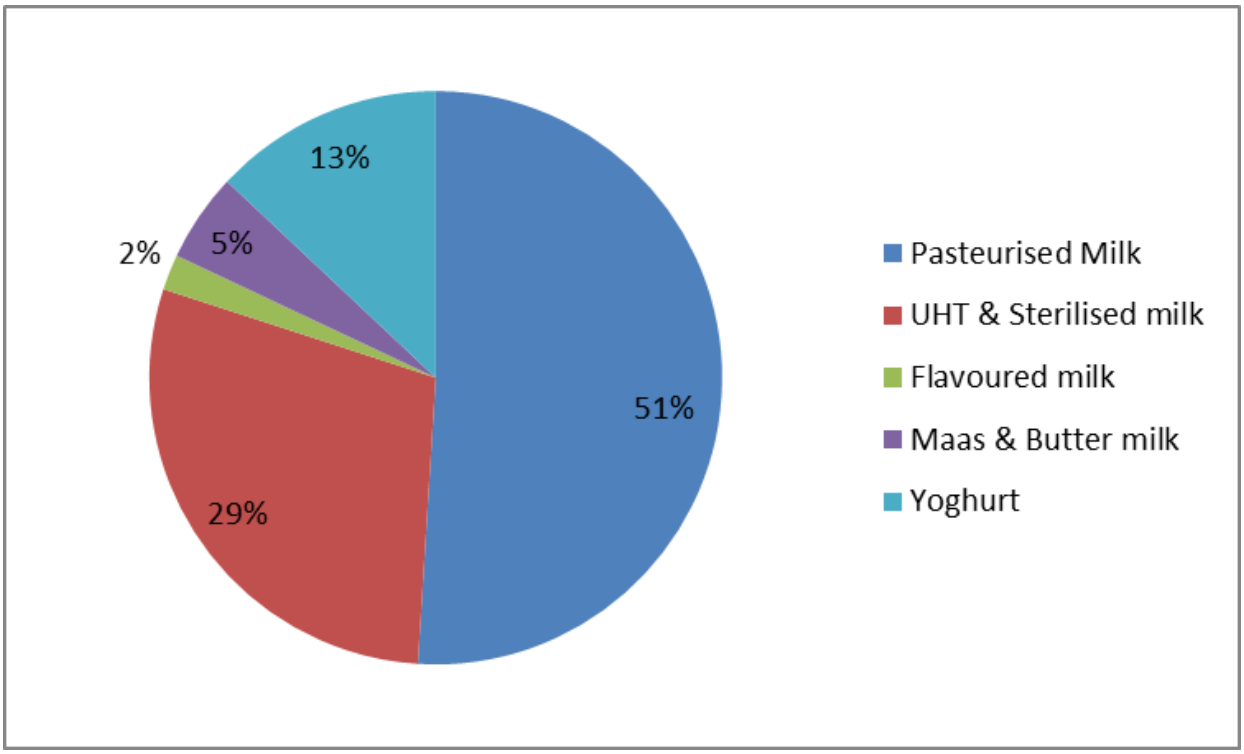
2.1 Milk production and consumption trends

Global milk production has increased by 50% in the past 3 decades from 482 million tonnes in 1982 to 754 million tonnes in 2012 (FAO 2016). While the greatest expansion in milk production has been in South Asia since 1971 and other countries such as USA and New Zealand, little growth has been observed in Africa due to poverty and adverse climatic conditions in other countries (Hemme *et al*. 2010). World milk production declined by 9% in 2005 indicating that the world milk production has not kept pace with increase in world population, despite the increase in global milk production the per capita (Knips 2005).

Asia is the highest consuming region with 42% of total dairy demand, followed by Europe (26%). Asia still has large growth potential as its per capita consumption (75 kg per person per year) is still low compared to other areas, with the exception of Africa (49.2 kg). While UN estimates a 16% global population increase by 2030, the OECD/FAO agricultural outlook, projects that the global average per capita dairy consumption should increase by 13.7% between 2013 and 2023 (FAO 2016). The main drivers remain the growth in the global population, income levels and urbanization. Faster growth is expected in developing countries with current low per capita consumption.

While the average global cost of milk production is US$ 46/100 kg, the average cost of production of milk in South Africa lies slightly above US$ 35 per 100 kg of milk, which is at par with the New Zealand cost level but lower than most other dairy countries. Similar to global trends, the South African dairy industry has also seen growth in production with a 22% increase of milk in between 2009 and 2016 (Lacto data 2016). South African dairy market is divided into

58% liquid and 42% concentrated products (Lacto data 2016). Pasteurised liquid milk and UHT milk are the major liquid products, while hard cheese is the major concentrated product. Pasteurised milk has increased by 2% while UHT dropped by 3% in the period between 2009 and 2015 (Lacto data, 2015). Liquid milk distribution in South Africa is shown in figure 2.1



*Maas refers to a fermented milk product with no preservatives that is mainly consumed in Southern Africa.

Figure 2.1: Percentage composition of liquid milk in South Africa (Lacto data 2015)

Although UHT milk and sterilised milk have better microbial and keeping qualities, as well as long shelf life under ambient temperature, their usage is still lower than that of pasteurised milk due to the favourable sensory characteristics possessed by the pasteurised milk. (Wolf *et al.*

2013). The classification of pasteurised milk includes recently introduced extended shelf life (ESL) milk which comprises of pasteurisation coupled with bactofugation as a non-thermal hurdle.

2.2 Extended shelf life milk

ESL milk bridges the gap between high temperature short time (HTST) pasteurised milk, with a shelf life of around 10 days under refrigeration, and ultra-heat treated (UHT) milk with a shelf life of at least 3 months at ambient temperature storage (Fitzgerald 2012). Although a generally accepted definition of ESL does not exist, the term has been used to refer to fresh milk with an extended shelf life regardless of the process used(Buckenhüskes 2014). The objective of the product is to combine longer shelf life and better organoleptic characteristics, a combination which lacks in both pasteurised and UHT milk (Rysstad and Kolstad 2006). The milk undergoes treatment in a manner that reduces the microbial count beyond normal pasteurisation, is packaged under extreme hygiene conditions, and has a defined prolonged shelf life under refrigeration conditions (Rysstad and Kolstad 2006; Lorenzen *et al*. 2011). Since the same conditions may be achieved by different temperature/time profiles, 'ESL' is an umbrella term for many different types of milk which also vary with regard to composition and flavor (Grabowski *et al*. 2013). Various processing schemes of ESL milk have been described by several authors ranging from high heat treatment for a few seconds to coupling pasteurisation with a non-thermal process. Buckenhüskes (2014), listed five available methods of ESL milk processing, while other researchers have only classified them as two methods (Rysstad and Kolstad 2006; Lorenzen *et al*. 2011; Grabowski *et al*. 2013). Apart from bacterial count reduction due to various techniques

applied in ESL milk, the longer shelf life of ESL milk is also a result of reduced post process contamination due to the use of aseptic packaging as illustrated in Figure 2.2.

Literature has indicated that the high heat treatment method of ESL milk is based on 123-127 ^{o}C for 1-5 s or 135 ^{o}C for 0.5 s (Mayr *et al.* 2004a; Britz and Robinson 2008; Lorenzen *et al*. 2011). In South Africa, milk is subjected to 135 ^{o}C for about 0.5 s and packaged in the conventional manner. Although nothing has been published on the South African ESL milk produced using this method, it has been reported that generally this method causes sensory characteristics problems in the final product (Shmidt *et al.* 2012). Apart from high heat treatment another commonly used method is a combination of HTST pasteurisation and a non-thermal process such as microfiltration (Hoffman 2006) and bactofugation (Fox and McSweeney 1998; Fox *et al*. 2015) coupled with aseptic packaging. The main steps in ESL processing using pasteurisation and a non-thermal step for bacteria removal start with the chilling of raw milk, followed by heat treatment, homogenisation, bactofugation or microfiltration and lastly aseptic packaging. In South Africa the dairy industry use the bactofugation based process.

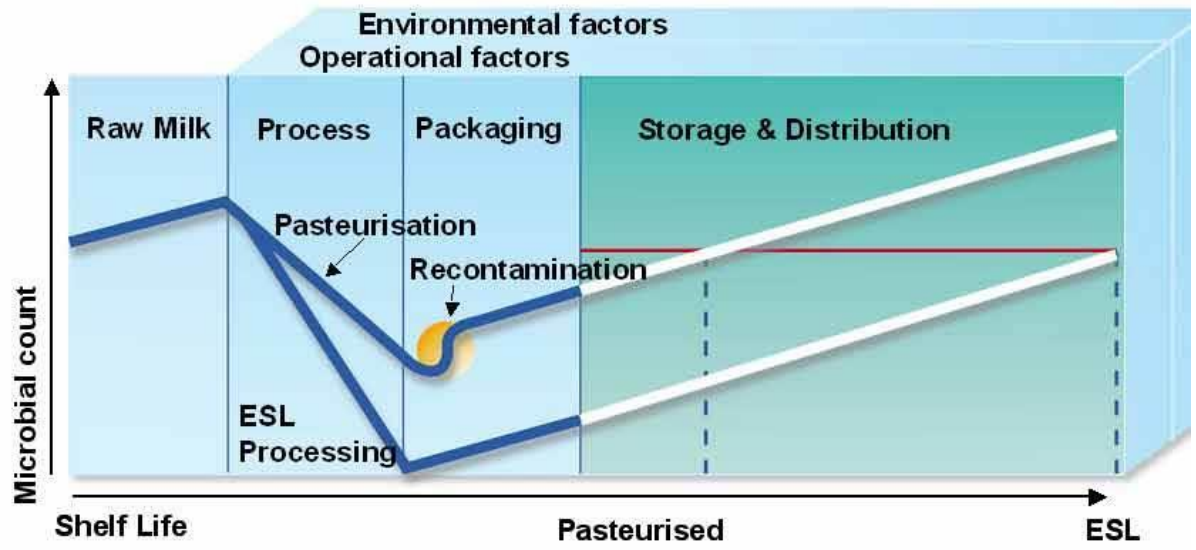


Figure 2.2: Process design for the production of ESL milk and conventionally pasteurised milk http://www.drgailbarnes.com/2013/01/extended-shelf-life-future-for-chilled.html (Available online. Accessed 10 March 2016)

2.2.1 Milk chilling

Chilling is a quick process of cooling milk to a temperature range of 2-7 ^{o}C. The purpose of chilling is to inactivate microbial activity and minimise micro-induced changes thereby elongating shelf life of milk, although it neither kills microorganisms nor render the milk safe for human consumption (FAO 2016). Chilling is done at different stages of milk processing but the first stage of chilling is just after milking or as soon as milk is received at a processing plant (FAO 2016). Along the processing line, chilling is applied after a heating process as well as during product storage for products such as pasteurised milk. It prevents and / retards the multiplication of thermophilic and mesophilic bacteria including most known pathogens. The extent of control of growth of microorganisms depends on the type of organisms as some

organisms like *Staphylococcus* spp. do not grow below 10 ºC. Growth stops for most type of bacteria such as *Escherechia coli, Bacillus proteus* and *Micrococcus* spp. between 0 and 5 ºC. However, chilling is ineffective on psychrotrophs like *Paenibacillus* spp. which continue to grow at temperatures below 8 ºC (Ranieri *et al*. 2012), hence milk stored at low temperature for too long can be undesirable due to increased psychrotrophic organisms which may produce extremely heat resistant lipases and proteases that will subsequently have an effect on the product quality.

2.2.2 Heat treatment of milk

Depending on the specifications of different organisations some processes may have a preheating which usually ranges from thermisation temperatures to pasteurisation temperatures, followed by final heating which ranges from pasteurisation to UHT temperatures. Thermisation is a mild heat treatment of milk at a temperature range of 57-68 ºC for 15 s with the ultimate goal of shelf life extension by reduction of psychotrophic microorganisms followed by refrigeration in raw milk that is to be stored for some time before use (FAO/WHO 2000; Smit 2003; McSweeney 2007). Thermisation inactivates psychrotrophic bacteria in milk, preventing the growth of heat-resistant enzymes and allowing the milk to be stored below 8 ºC for three days (Lewis 2006) or stored at 0-1 ºC for seven days (McSweeney 2007). Many experts are of the opinion that thermisation has a favourable effect on certain spore-forming bacteria. The heat treatment causes many spores to revert to the vegetative state, which means that they are destroyed when the milk is subsequently pasteurised (Lewis 2006). However, some manufactures prefer tyndallisation, a process that inactivates spores by sequential heat treatments (Smit 2003; Tammine 2009). Tyndallisation is

heat sterilisation by steaming the food or medium for a few minutes at atmospheric pressure on three or four successive occasions, separated by 12-18 h intervals of incubation at a temperature favorable for bacterial growth (Gould 2006). The process is based on inactivating spore-formers using high heat then incubating the milk at temperatures that allow germination of spores so they can be inactivated in their vegetative form at the second or third heating stage. Such milk is mostly used for UHT milk because of the altered sensory characteristics.

2.2.2.1 Pasteurisation of milk

Named after Louis Pasteur a French chemist and microbiologist after doing a follow up on Nicolas Apert's discoveries. Pasteurisation has been defined by IDF as, "a process applied to a milk product with the objective of minimising possible health hazards arising from pathogenic microorganisms associated with milk by heat treatment which is consistent with minimal chemical, physical and organoleptic change of the product," (Staal 1986). Pasteurisation aims to reduce the number of pathogens to a level where they do not constitute a significant health hazard, reduce the level of undesirable enzymes and spoilage bacteria, thereby increasing the keeping quality and achieving the preceding two goals without destroying the original characteristics of the product. The original type of heat treatment was a batch process in which milk was heated to 63 ^{o}C in open vats for 30 min. This method is called the holder method or low temperature, long time (LTLT) method (Tammine 2009). Currently, milk is heat treated in continuous processes known as high temperature short time (HTST) pasteurisation. HTST process involves heating milk to 72-75 ^{o}C with a hold of 15-20 s before it is cooled (FAO/WHO

2000; Tammine 2009). The phosphatase enzyme is destroyed by this time/temperature combination hence the phosphatase test is used to check that milk has been properly pasteurised. Without pasteurising, food poisoning through milk is a possibility, with diseases such as tuberculosis, salmonellosis sand listeriosis dominating. However, pasteurisation cannot destroy spores and some of these spore-forming microorganisms such as *Bacillus* spp. are able to grow at temperatures below 8 ^{o}C (Tammine, 2009; Ranieri *et al*. 2012). Pasteurisation can affect the nutrient composition and flavor of milk. HTST causes less damage to the nutrient composition and sensory characteristics of foods as compared to LTLT. The mandate of the manufacturer is to choose the time/temperature combination that will be effective on microorganisms while preserving heat sensitive nutrients and sensory characteristics. Figure 2.3 shows the relationship of the pathogen destruction, nutrient loss and pasteurisation temperature.

2.2.2.2 UHT treatment and sterilisation of milk

To destroy most or all heat resistant microbes UHT is applied, where milk is pumped through a plate exchanger for 2-5 s at 135-140 ^{o}C and rapidly cooled to prevent the Maillard reaction (Smit 2003). The processing of milk at high temperatures is aimed at destroying vegetative cells as well as endospores present in raw milk so that it can be stored for prolonged periods, generally several months, without refrigeration (Tabit 2010). Another high heat treatment is sterilisation, which is a high-temperature/long-time heat treatment aimed at producing a commercially sterile product which can be stored at room temperature. Sterilisation destroys all microorganisms and any residual microorganisms are unlikely to cause spoilage under normal storage condition. The temperatures for sterilisation should be 110 to 125 ^{o}C in combination with appropriate holding

times such as 121 ºC for 3 min or 115 ºC for 13 min (FAO/WHO 2000). The purpose of both sterilisation and UHT is to produce a commercially sterile product which can be stored at room temperature.

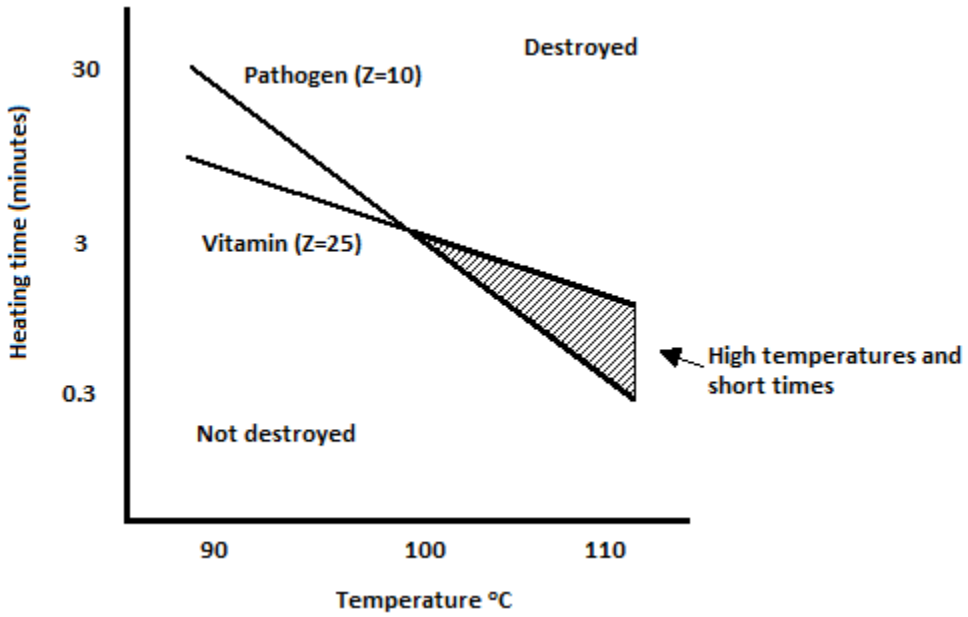


Figure 2.3: Relative changes in time temperature profiles for the destruction of microorganisms and vitamins in milk during pasteurisation. https://www.uoguelph.ca/foodscience/book-page/thermal-destruction-microorganisms. (Available online. Accessed 13 March 2016)

2.2.3 Microfiltration of milk

Microfiltration is a type of physical filtration process where a contaminated fluid is passed through a special pore-sized membrane to separate microorganisms and suspended particles from

process liquid. The filters used in the microfiltration process are specially designed to prevent particles such as bacteria from passing through. The process was first implemented in the milk and cheese production in the late 80s (Hoffman *et al*. 2006; Schmidt *et al*. 2012). The principle of this technique during milk processing is to remove bacterial cells and spores from milk mechanically using ceramic membrane with pore diameter of 0.8-1.4 mm (Rysstad and Kolstad 2006). Most experiences with microfiltered ESL milk are based on the patented Bactocatch® system (Holm *et al*. 1986). This process and its variants comprise microfiltration of separated skim milk resulting in a permeate. The permeate is added with or without subsequent HTST pasteurisation to the highly heated (115-130 ºC, 4-6 s) mixture of microfiltration retentate and required an amount of cream. Finally, the recombined and fat-adjusted milk is filled aseptically (Hoffman *et al*. 2006).

2.2.4 Bactofugation of milk

It is a process used to eliminate the bacteria contained in the milk by means of centrifugal force. Its effectiveness increases with increase in temperature. Bactofugation compliments pasteurisation and does not replace it (Fondation de technologie laitière du Québec 1985; Lund *et al*. 2000). Effectiveness of bactofugation varies according to size and type of bacteria because sedimentation by centrifugal force is greater for larger and denser bacterial cells. The process is believed to reduce 90-99% of bacterial cells and clostridal spores which cause late blowing of Swiss cheese (Fox and McSweeney 1998; Faccia *et al*. 2013). Bactofugation has proved to be an efficient way of reducing the number of spores in milk. This method is claimed to be effective at

removing bacterial spores but it can be plagued by problems with recontamination (Lund *et al.* 2000a; Faccia *et al*. 2013).

2.2.5 Aseptic packaging

Aseptic filling is important to control contamination during packaging in order to achieve the goal of extended shelf life milk processing. It is the process by which a sterile product is packaged in a sterile container in a way that maintains sterility. Burton (1988), points out that in order to achieve aseptic packaging the process must satisfy the following conditions;

- Container and method of closure must not allow passage for microorganisms.
- The part of the container that is in contact with milk must be sterilised when formed and before filling.
- Container must be filled without contamination from equipment and surrounding atmosphere.
- If closure is needed it must be sterilised before application.
- Closure must be applied and sealed in place to avoid or prevent the passage of contaminants.

Saturated steam has been used for container sterilisation although it has economic challenges in setting up and energy costs. Dry heat in form of hot gas or hot non aqueous liquid such as glycol is also used although it desiccates microorganisms and makes them more resistant. The heating processes normally achieve temperatures between 91-146 ^{o}C. Apart from heat treatments,

hydrogen peroxide (H_2O_2) has been successfully used for aseptic packaging of UHT milk (Ansari and Datta 2003), while UV light at a wavelength of 250 nm has also proved to be effective (Burton 1988). UV light, however, has practicality difficulties of ensuring uniformity during application. Ionising radiation such as gamma rays has been used to sterilise the interior of sealed but empty containers, particularly those made of materials which cannot withstand temperatures needed for thermal sterilisation. The most common packaging for aseptically packed milk is laminates of paperboard cartons coated internally and externally with polyethylene. An oxygen barrier like aluminum foil is a common inclusion to the laminate. Other common packaging materials include plastic pouches and blow moulded bottles (Burton 1988).

2.3 Bacteria associated with milk

2.3.1 Raw milk

Bovine milk as it is secreted by the cow is sterile. However, microorganisms associated with the teat move up the teat canal into the interior of the udder, indicating that even aseptically drawn milk will have a certain number of microorganisms (Ryser *et al.* 1998; Ozer and Akdemir-Evrendilek 2014). Fresh, aseptically drawn milk from a healthy cow may contain < 100 cfu/ml (Walstra *et al*. 2005). Other scholars have indicated that a practical range is between < 1000 and 20 000 cfu/ml (Chambers 2005). While the interior of the udder contributes a few microorganisms to raw milk, most microorganisms in raw milk are contaminants from outside the udder such as milking equipment and human handlers. Developments of closed milking systems, use of bulk tanker for transport and improvements in refrigeration systems has resulted in a change of the micro flora in raw milk from predominantly Gram positive, acid producing to

Gram negative psychrotrophic microorganisms primarily the *Pseudomonas* spp. (Ryser *et al.* 1998; Chambers 2005).

Psychrotrophs that have been defined as bacteria that grow at 7 ^{o}C or less, irrespective of their optimal temperature, have become of great importance in the dairy industry from both a spoilage and safety stand point (Ryser *et al.* 1998). The most common Gram negative bacteria of primary importance include *Pseudomonas, Achronobacter*, *Aeromonas*, *Alcaligenes*, *Chromobacterium* and *Flavobacterium* spp. These bacteria produce some heat stable enzymes that may participate in product spoilage during refrigeration. While *Yersinia enterocolitica* and *Escherecia coli* are Gram negative pathogens, *B. cereus* and *L. monocytogenes* are Gram positives that are of safety concern in raw milk. *B. cereus* has been extensively reported in milk and its products (Ryser *et al.* 1998; Chambers 2005; Ozer and Akdemir-Evrendilek 2014). Research has shown that its existence in milk depends on the season among other factors, with winter exhibiting the highest prevalence (Ryser *et al*. 1998). Enterobacteriaceae is another dominant group of microorganisms in raw milk. This includes *Lactobacillus, Acinetobacter, Staphylococcus, Falvobacterium* and *Micrococcus* spp. The most common spoilers of raw milk are the acid producing ones (Ozer and Akdemir-Evrendilek 2014). Although they come in low numbers, raw milk also contains spore-forming bacteria with the ubiquitous *Bacillus* spp. dominating the spore-forming group (Ryser *et al*. 1998; Ozer and Akdemir-Evrendilek 2014).

2.3.2 Heat treated milk

2.3.2.1 Pasteurised milk

Although most pathogenic bacteria are destroyed, pasteurised milk has been reported to contain both spoilage and some pathogenic organisms (Ntuli *et al*. 2016). There are two types of microorganisms in pasteurised milk, which are, post process contaminants that enter after heating and heat resistant bacteria which survive heating. Post process contaminants are usually Gram negative psychrotrophic bacteria that include members of the Enterobacteriaceae, such as *Serratia*, *Enterobacter*, *Citrobacter* spp. among others (Varnam and Sutherland 2001). Nevertheless, the ultimate spoilage microflora usually consists of Gram negative rods such as *Pseudomonas*, *Alcaligens* and to a lesser extent *Flavobacterim*. It is the competitive nature of these organisms that make them out grow the Enterobacteriaceae during storage at 8 ^{o}C (Touch and Deeth 2009; Tammine 2009).

While other studies have shown that *Pseudomonas* spp. was the only bacteria causing defects in milk stored at 4-7 ^{o}C (Craven and Macauley 1992 in Tammine 2009), some have shown that the endospore forming bacteria *Paenibacillus* dominates in pasteurised milk as it ages (Ranieri *et al*. 2012). The presence of spore-formers is inevitable in pasteurised milk, however, some scholars point out that, although pasteurisation virtually kills all vegetative thermophilic bacteria, post pasteurisation contamination by *Psuedomonas* spp. at levels of 10^3 cfu/ml, frequently occurs. Other post pasteurisation contaminants include *Lactobacillus* and *Lactococcus* spp. (Varnam and Sutherland 2001; Deeth *et al*. 2006).

The most common heat resistant organisms in pasteurised milk are those that attach to the plates and grow during the regeneration stage usually at 45-60 ^{o}C, which is their optimum temperature, resulting in recontamination before milk leaves the pasteuriser (Lund *et al*. 2002; Tammine 2009). These bacteria will dominate in milk stored at temperatures above 10 ^{o}C. *B. licheniformis* as well *S. thermophilus* have also been implicated in post pasteurisation contamination. *Bacillus* spp. are the most significant heat resistant organisms because of their ability to adapt to various conditions through formation of endospores as well as ability to grow at refrigeration storage. *Bacillus* spp. usually becomes the dominant spoilage organisms at storage temperature below 5 ^{o}C when competitive Gram negative bacteria are low in numbers (Ryser *et al.* 1998). This usually occurs when milk is manufactured under conditions of good hygiene, for which a long storage period is expected. *B. cereus*, *B. licheniformis*, *B. mycoides*, *B. circulans* and *B. coagulans* have been frequently isolated in pasteurised milk at levels $\leq 10^2$ cfu/ml (Tammine 2009).

2.3.2.2 ESL milk

The most common microflora in ESL milk are spore-formers and post process contaminants. Myar *et al*. (2004b) reported that a level of 13-130 spores/L has been observed in ESL milk. While some studies reported that *B. circulans* was the dominating organism in milk pasteurised at 72-88 ^{o}C for 15 s (Cromie *et al*. 1989 in Tammine 2009), other studies revealed that *B. licheniformis* (73%) was the dominating organism followed by *B. subtilis*, *B. cereus*, *Brevibacillus* spp. and *B. pumilus* in milk heat treated at 127 ^{o}C for 5 s (Mayr *et al*. 2004b). Commercial milk directly heated at 120-132 ^{o}C for 4 s was observed to harbor only *B.*

licheniformis, *B. coagulans* and *B. cereus.* (Ozer and Akdemir-Evrendilek 2014). Ranieri *et al*. (2012), reported *Paenibacillus* spp. as the dominating microbe in pasteurised based ESL milk and Schmidit *et al*. (2012) observed *Microbacterium* spp. (40%) followed by *Microbacterium lacticum* (34%), spore-formers (20%) in ESL. *B. subtilis* was the dominating spore-former followed by *B. licheniformis*, *B. cereus* and *B. pumilus* among others. Mayr *et al*. (2004a) also reported non spore-forming organisms in commercial ESL milk that includes *Rhodococcus, Anquinibacter, Arthrobacter, Microbacterium, Enterococcus, Staphylococcus* and *Micrococcus* among others and these were attributed to recontamination.

2.4 Contamination routes in milk processing

2.4.1 Milk at the farm

It is generally accepted that milk drawn from a healthy cow under hygienic conditions contains relatively few organisms. However, during milking the milk can be subjected to a number of sources of microbial contamination such as the udder, equipment and the atmosphere (Ryser *et al*. 1998).

2.4.1.1 Interior of the udder

The most common bacteria in the udder are *Micrococci* and *Streptococci*. These are also present on the skin of the teats. However, when the cow has mastitis, high numbers of environmental bacteria such as *E. coli*, coliforms and *Pseudomonas* spp. may also be present on the teats especially when the udders are exposed to mud and manure (Ryser *et al*. 1998). The counts from these sources can be as high as 10^5-10^7 cfu/ml under certain circumstances. Apart from mastitis

related bacteria, lactic acid bacteria are other usual inhabitants of the skin and streak canal of the teats hence their presence in milk is inevitable, though in low numbers. (Tammine 2009)

2.4.1.2 Exterior of the udder

Cow udders are contaminated by the enviriomeent in which the animal stays. Animal feed may contain from 10^5-10^8 cfu/g of psychrotrophs and lactic acid bacteria are associated with silage and animal feeds (Bramely and Mckinnon 1990 in Tammine 2009). Urine and faeces also add microorganisms on the bedding material. The bedding and feeding material consequently contaminate the exterior of the udder. The most common groups on the teats that later contaminate milk are micrococci and aerobic spore-formers such as *Bacillus* spp. Spore counts of *Bacillus* spp. range from 10^2-10^5 per teat depending on the environmental conditions. Although water and silage play a role in spore contamination of raw milk the major sources of contamination are soil and faeces on the teats (Cook and Sandeman 2000).

2.4.1.3 Water

It has been reported that water used for dairy farm contain psychrotrophic bacteria even when it is chlorinated (Tammine 2009). Hence, its use for cleaning and rinsing milking equipment provides direct means of milk contamination. These psychotrophic bacteria are often very active producers of extracellular enzymes and grow rapidly at refrigeration temperature (Hantsis-Zacharov and Halpern 2007). *Pseudomonas* spp. dominates the psychrotrophic flora in water while *Bacillus* spp. and coliforms are in lower numbers. Furthermore, heat resistant spore-

formers have been isolated from farm water supplies, including hot water used for washing milking equipment (Depiazzi *et al*. 1997).

2.4.1.4 Milk handling equipment

Despite the use of stainless steel on much dairy equipment, some microorganisms are still able to attach to equipment and are often difficult to inactivate by chemical sanitisation. Milk handling equipment and utensils are the major sources of Gram negative psychrotrophic bacteria (Tammine 2009). Studies have shown the presence of *B. cereus*, micrococci and thermophilic strains of *Enterococcus faecalis* on milk handling equipment (Touch and Deeth 2009). Contributing factors include poorly designed and constructed pipeline systems (Varnam and Sutherland 2001). Previous studies have shown that counts of psychrotrophic bacteria in bulk tanks may be up to 10^3 cfu/cm^2 (Hayes 1985), hence these can be another major source of psychotropic bacteria in raw milk.

2.4.2 Milk at the processing plant

Pipelines, tanks, valves, and filling machines have been cited as the major sources of contamination after pasteurisation or any heat treatment (Fredsted *et al.* 1996).

2.4.2.1 Processing equipment

Filling equipment is a common source of psychrotrophs in packaged milk. Even when filling equipment is effectively cleaned and sanitised, it can still become a source of contamination due to psychrotrophic microorganisms which accumulate during continuous use (Eneroth *et al*. 2000;

2001). Holding tanks can also participate in contamination, by protecting microorganisms in microscopic fissures (ICMSF 1998). Some microorganisms such as *Pseudomonas* spp. are able to adhere to surfaces of milk processing equipment. Furthermore, *Bacillus* spp. produces highly hydrophobic spores and adheres firmly to stainless steel. After adhesion, there is colonisation if environmental conditions allow spore germination. This leads to biofilm formation which has proved to be a menace for the dairy industry for a long time (Faille *et al*. 2002; Simmonds *et al*. 2003).

2.4.2.2 Air in the processing plant

Microbial population of air in a dairy processing plant has been reported to be 85% bacteria, 10% moulds and 5% yeasts (Fredsted *et al.* 1996). However, most of the bacteria are Gram positives that do not grow well at low temperatures. Many sources of air contamination are usually ventilation systems, flow drains and personnel. Products can therefore, be easily exposed to contaminated air during packaging. The influence of microorganisms in the air on the microbiological spoilage of milk is of minor importance if premises are well designed; maintained and internal hygiene measures are taken. (Ozer and Akdemir-Evrendilek 2014). In modern dairy processing the effect of air has further been reduced by use of a closed system and aseptic packaging.

2.4.2.3 Packaging materials

It is very important that packaging material used should not cause contamination as it will be in direct contact with the product during its entire shelf life. Although a previous study revealed

that interval counts on surfaces of packaging material was < 10^3 cfu/cm^2 (Frested *et al*. 1996), another study has implicated packaging in contamination of ESL milk with Gram positive bacteria (Mayr *et al*. 2004a). Various milk contamination routes may collectively or singly result in a shortened shelf life of the ESL milk product.

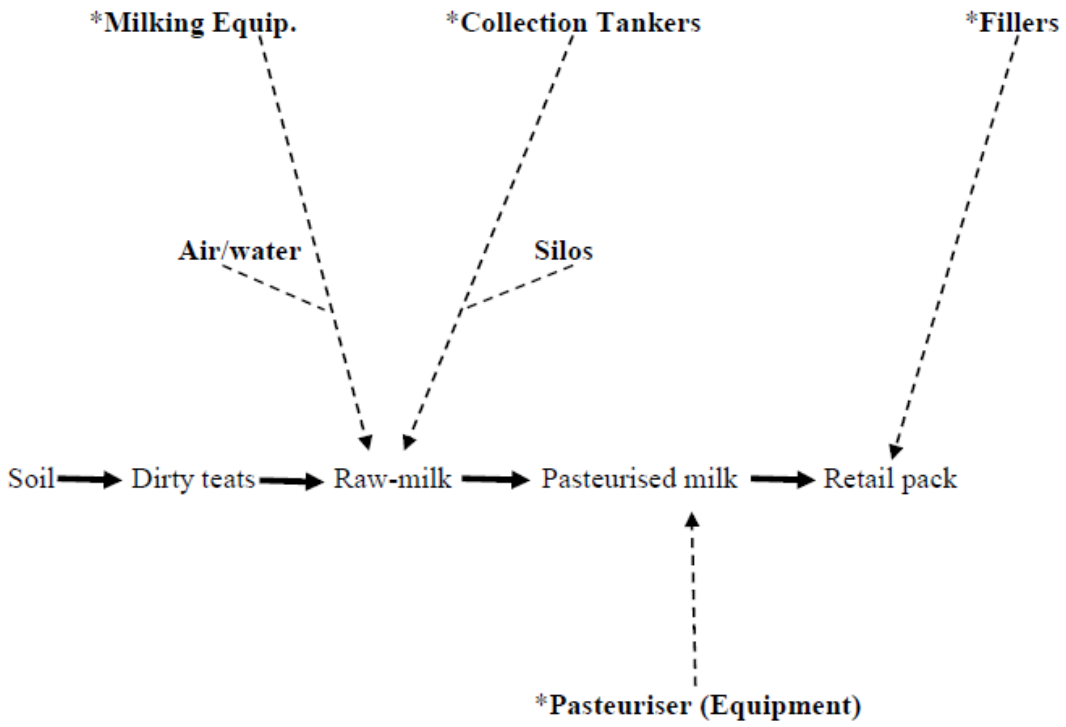


Figure 2.4: Contamination route of *Bacillus cereus* in a production chain (Heyndrickx 2011)

*Possible sources or routes of contamination is indicated by dotted arrow

2.5 Adaptation mechanisms of bacteria during milk processing

Microorganisms develop mechanisms that help them to adapt and survive various processing conditions. Among other adaptation mechanisms are; formation of endospores that will assist them in surviving heat treatments, biofilm formation, which assists microorganism to survive

cleaning and sanitisation and other organisms have managed to develop adaptation to cold stress helping them to grow in milk during refrigeration (Drenkard 2003; Abel-Santos 2012).

2.5.1 Bacterial endospores

Bacterial endospores are resistant dormant structures produced by stressed vegetative cells (Driks 2002). Usually sporulation is induced when microorganisms are challenged by nutritional stress (Abel-Santos 2012). This special type of cell can withstand a wide range of assaults that would otherwise destroy its vegetative cell form. Endospores of the genus *Bacillus* are several times more resistant to heat, desiccation, UV light, oxidizing chemicals and other genotoxic drugs. Exposing actively growing *Bacillus* cells to limited nutrients leads to the commencement of the stationery phase (Driks 2002; de Hoon *et al.* 2010). During the transition to stationery phase, bacterial cells initiate some adaptive processes which allow adaptation of cells to adverse environmental conditions (Pedraza-Reyes and Yasbin 2004). The sporulation process is genetically controlled by transcriptional regulatory network (Piggot and Hilbert 2004). The extraordinary spore resistance properties are due to a well-structured multilayer morphology. The layer comprises of the exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and the central core. Apart from the protein based multilayer, the low water content in endospores accounts for the enzymatic dormancy and heat resistance (Cowan *et al.* 2003). The resistance mechanism of endospores makes them difficult to handle when they contaminate milk at any point in the process chain. The stages of endospore formation are summarised in Figure 2.5.

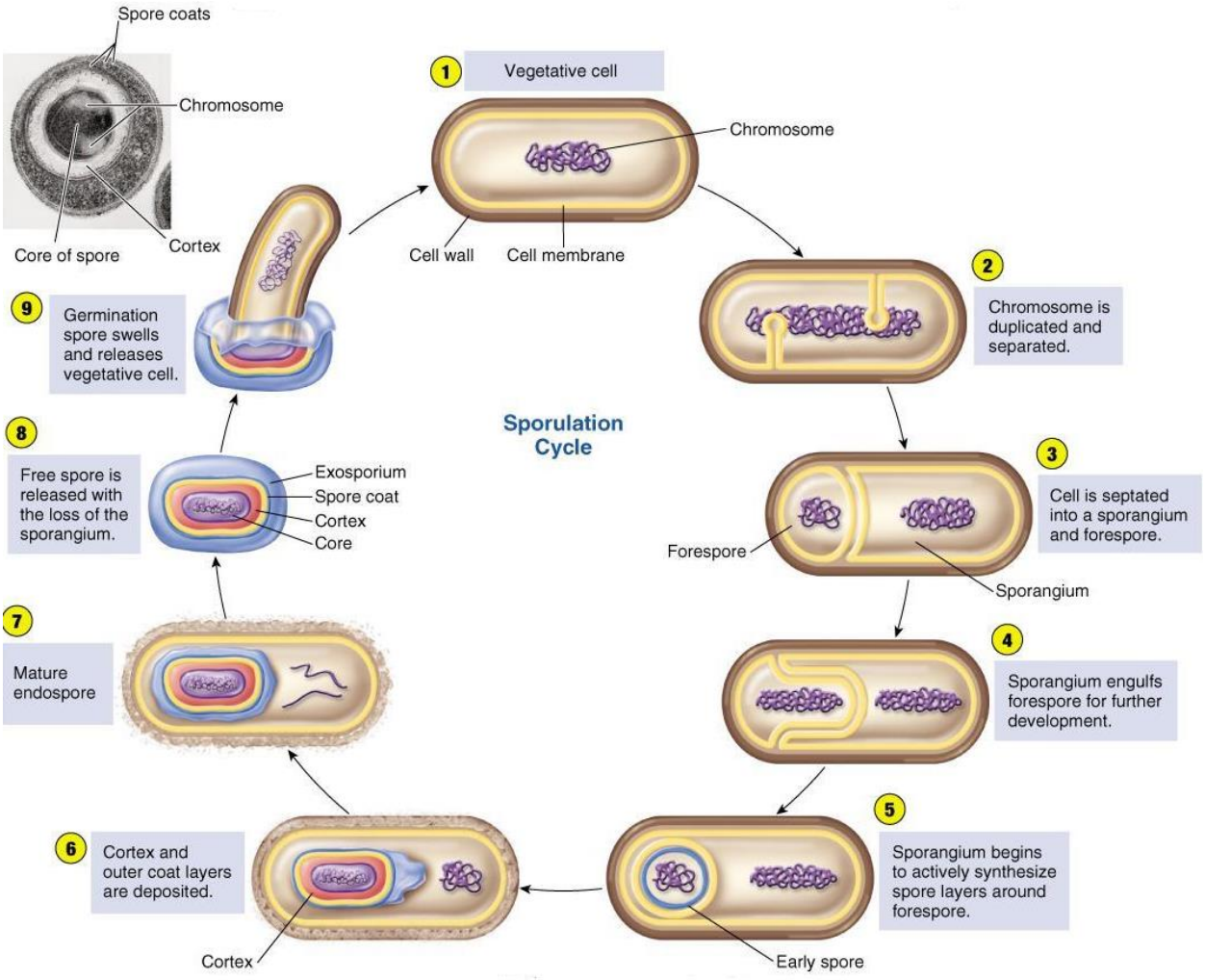


Figure 2.5: Stages that a bacterial cell goes through in a sporulation cycle http://zf2t.dromibi.top/c/spore-formation/ (Available online. Accessed 27/03/2016)

2.5.2 Biofilms

A biofilm can be defined as the sessile community of microbes characterised by cells that are irreversibly associated with a surface embedded in a matrix of extracellular polymeric substances

and display an altered phenotype with respect to gene expression, protein production and growth (Dhillon 2012). Microorganisms in a biofilm display some particular features that are not shared with the same microorganisms in suspension form. Microorganisms in a biofilm can be up to a 1000 times more resistant to antimicrobials than their planktonic counterparts (Drenkard 2003). Biofilm formation begins with attachment of a single cell to the surface. Studies have shown that *B. cereus* among others spore-formers can attach to stainless steel (Khoza 2016). Adhesion of bacterial cells to the conditioning layer then follows (Dhillon 2012). Irreversibly attached cells utilise nutrients present in the conditioning film to multiply (Khoza 2016). Continuous attachment of cells over time results in formation of a biofilm. Mixed species biofilms are more common as they are more stable than single cells (Dhillon 2012). This allows microorganisms to protect one another during the application of cleaning chemical agents. As the biofilm matures the attached bacteria in order to survive, detach and disperse to colonise a new niche (Myszka and Czaczyk 2011). It is at this detachment stage that the milk is contaminated by organisms from the biofilm.

2.5.3 Cold shock response

Some microorganisms have developed mechanisms to grow well under cold temperatures and amongst them are some members of *Bacillus* and *Paenibacillus* spp. The ability to produce cold shock proteins (CSP) allows microorganisms to continue with metabolic processes at low temperatures. Psychrotrophic strains are said to display a particular sequence in their *cspA* gene that was proposed to be a psychrotolerant-associated signature (Francis *et al*. 1998). Another psychrotolerant-associated signature was also proposed based on the rrs sequence, while a 16S

based signature has also been described (von Stetten *et al*. 1998). In a classification proposed by Guinebretierre *et al*. (2008; 2010) it was reported that group 2 comprises of both mesophilic and psychrotrophic strains with 50% of the stains being intermediate. It is important, however, to note that this group does not have a *cspA* signature, owing their psychrotrophic nature to a different signature.

When bacterial cells are subjected to a temperature downshift they elaborate an adaptive response known as cold shock response (Requena 2012). The first notable aspect under cold stress is that growth significantly slows down or stops, representing the lag phase and this correspond to a cold acclimation phase. This is followed by a massive and transient synthesis of CSP and a significant decrease of non-CSP synthesis including housekeeping proteins. The amount of cold acclimation proteins (CAP) already present also increases moderately (Requena 2012). The second phase of cold adaptation begins with a decrease in synthesis of CSP and CAPs, concomitantly to a non-CSP protein neo-synthesis CSPs and CAPs proteins are involved in several processes such as DNA and RNA metabolism, cell metabolism, protein folding and degradation among other functions (Phadtare 2004).

Compatible solute acquisition also plays a role in cold adaptation as was shown that glycine betaine is an effective protectant of *B. subtilis* during cold adaptation (Hoffman and Bremer 2011). Membrane fluidity is also modified when temperature drops (Zhang and Rock 2008, 2009). Bacterial cell membrane switches from a liquid fluid crystalline to a rigid state under low temperatures (Requena 2012). A study on *B. subtilis* also suggests a great need for DNA excision repair during growth at low temperature (Budde *et al.* 2006). During adaptation to cold stress,

protein folding and misfolding is also observed (Requena 2012). Metabolic pathways modification is also noticed during cold adaptation (Budde *et al.* 2006). While other studies have shown that low temperature influences spore production in *B. weihenstephanensis* (Garcia *et al.* 2010), it has also been established that *B. cereus* spores can germinate at temperatures below 6 ^{o}C. It is this complex adaptive mechanism of *Bacillus* spp. that makes it an important organism in extending the shelf life of milk.

2.6 Spore-forming bacteria associated with ESL milk

2.6.1 *Paenibacillus* spp.

Formerly known as *Bacillus polyxma* that was reclassified in 1993, *Paenibacillus* spp. emerged from early phylogenetic dissection of *Bacillus sensu lato* based on the 16S rDNA gene sequences (Ash *et al.* 1993). Similar to the *Bacillus* spp., *Paenibacillus* spp. are rod shaped Gram positive microorganisms that form endospores. However, they regularly appear gram-negative under the microscope (Bergey 2009). All *Paenibacillus* spp. produce endospores that are usually of greater diameter than the mother cell and under suitable conditions some produce capsules. *Paenibacillus* differs with the *Bacillus* spp. in the DNA encoding their 16S RNA (Ouyang 2008). It was once believed that *Paenibacillus* was not pathogenic however, research has shown that *P. thiaminolyticus* was the cause of a renal failure case in the USA (Ouyang 2008). *Paenibacillus* is a thermoduric (Huck *et al*. 2007), aerobic endospore forming microorganism that is characterised by a ubiquitous nature and to aid to their survival skills are the ability to secrete peptide antibiotics, signal molecules as well as enzymes though variations exist (Gardener 2004). Some members of the *Paenibacillus* such as *P. polymyxa* are able to fix nitrogen in the soil

(Yegorenkova 2008). In milk *Paenibacillus* and *Bacillus* are the predominant spore formers that spoil milk after pasteurisation (Ranieri 2012). *Bacillus* represents the bacteria that dominate in the early stages of shelf life in pasteurised milk, however, *Paenibacillus* increases with time at refrigeration temperatures to contribute about 95% of the dominating microbes in the pasteurised milk (Ranieri 2012). This clearly shows the ability of *Paenibacillus* to grow as a psychrotroph in milk. *Paenibacillus* is able to degrade proteins (Ash *et al.* 1993), polyaromatic hydrocarbons (Daane *et al*. 2002) and polysaccharides (Scheldeman *et al.* 2004). *Paenibacillus* usually produces small translucent, light brown/white sometimes pink/yellowish colonies on agar plates (Bergey 2009). Methods of isolation, characterisation and identification for *Paenibacillus* vary but most are almost the same as those used for the *Bacillus* hence the PCR is used to differentiate the two.

2.6.2 *Bacillus* spp.

These are aerobic endorespore forming gram-positive rods. The genus was created in 1872 by F. Cohn who changed the name of Ehrenberg's 1935 *Vibrio subtilis* to *Bacillus subtilis* (Harwood 1989). Representatives of this genus are widely distributed in the air, soil and water. Some *Bacillus* strains are able to tolerate extreme conditions such as high and low pH as well as high and low temperatures. In the soils, they have been isolated from extreme desserts as well as Antarctic samples. Soils of low organic matter are dominated by *B. subtilis, B. licheniformis* and *B. cereus* but the range increases with increase in fertility of the soil. *Bacillus*, contribute to 20% of heterotrophic flora in the seawater (Harwood 1989). In fresh water most *Bacillus* bacteria represent the microflora of the soil. Few species have been isolated in plant leaves. *B. macerans,*

is often isolated from the roots while cereals such as rice and pulses have a varied *Bacillus* flora including *B. cereus*. *Bacillus* flora of food is related to the distribution of those bacteria in soil water and plants. The ubiquitous distribution of *Bacillus* spp. makes them prominent milk spoilage organisms. *B. sporothermodurans* has been identified as a cause of spoilage in UHT milk (Tabit and Buys 2011). Lecithinase-positive strain of *B. cereus* is responsible for the broken flavour or bitty cream condition (Jan·Tová *et al*. 2006), although others occur in raw milk and pasteurised milk e.g. *B. sphaecicus, B. megatarium* and *B. subtilis* (Scheldeman *et al*. 2004).

2.6.2.1 *B. pumilus*

B. pumilus is widely distributed in clinical, veterinary, food ingredients, leather, paper samples and has also been isolated from the interior of Sonoran desert basalt (Benardini *et al*. 2003), while its spores usually reside in soils and some colonise the root area of some plants where it has some antibacterial and antifungal activity. *B. pumilus* has also been reported to be the second most predominant *Bacillus* species in space crafts (La Duc *et al*. 2003). In addition, it has been isolated in both milk and milk processing environments (Schmidit *et al*. 2012; Aoudhi *et al*. 2014; Khoza 2016). Although *Bacillus* spp. spores are notoriously resistant to unfavorable conditions such as low or no nutrient availability, extreme desiccation, H_2O_2, UV, gamma-radiation, or chemical disinfection (Nicholson *et al*. 2000). *B. pumilus* has shown elevated resistance to these factors when compared to the spores of other *Bacillus* members (Nicholson *et al*. 2000; Kempf *et al*. 2005). *B. pumilus* has also been used at industrial level. Some purposes of *B. pumilus* that are being researched are its involvement in bacterial hay preservation and the use of *B. pumilus* plasmids in gene transfer systems. The proteases from *B. pumilus* are used in

various industries such as food, chemical, detergent, and leather industries (Pan *et al*. 2004). Although *B. pumilus* is regarded as non-pathogenic, previous studies have shown toxigenic potential in some of its strains through production of pumilacidins (Suominem *et al*. 2001; From *et al*. 2007; Nieminem *et al.* 2007).

2.6.2.2 *B. subtilis*

Originally named *Vibrio subtilis* in 1835, this organism was renamed *B. subtilis* in 1872. *B. subtilis* is a Gram positive, catalase-positive bacterium. Although *B. subtilis* has been isolated in milk and dairy environment (Schmidit *et al*. 2012; Aoudhi *et al.* 2014), it is usually found in soil and grass hence the name grass bacillus. *B. subtilis* is considered an obligate aerobe, but can also function anaerobically in the presence of nitrates or glucose (Heyndrickx and Scheldeman 2002). Along with enzymes, *B. subtilis* also produces a toxin called subtilisin (Ryan and Ray 2004). Subtilisin can cause allergic reactions if there is repeated exposure in high concentrations. Industrially *B. subtilis* can be used to produce proteases and amylase enzymes (van Dijl and Hecker 2013). Previously it was widely used as a broad spectrum antibiotic, however, this was lost after the ability to produce cheaper, large-scale antibiotics although it is still used in Western Europe and the Middle East in alternative medicine. Other commercial applications of *B. subtilis* include cleaning agents in detergents, de-haring and batting in the leather industry, production of special Japanese and Korean food, starch modification, de-sizing of textiles and other specialized chemicals. *B. subtilis* has become the model agent in laboratory research because of its easy genetic manipulation (Earl *et al*. 2008).

2.6.2.3 *B. cereus*

B. cereus is a ubiquitous, Gram positive, spore forming, and motile aerobic bacteria with some strains that have shown ability to grow well anaerobically. Its habitats ranges from milking parlours to cattle pans, decaying organic matter, fresh and marine waters, vegetables and fomites, and the intestinal tract of invertebrates among others (Jensen *et al*. 2003; Arnesen *et al.* 2008; Bottone 2010). *B. cereus* contamination can be mediated by various substrates including raw milk and processing equipment such as milk fillers (Khoza 2016) as its spores are highly adhesive (Arnesen *et al.* 2007). Spores germinate when they come into contact with organic matter or within an insect or animal host (Arnesen *et al.* 2008).

Although it is non-competitive, *B. cereus* has managed to adapt to different and diverse habitats ranging from cold to hot environments and from refrigerated to dehydrated foods (Larsen and Jogernsen 1997; Guinebretiere *et al.* 2008). *B. cereus* has previously been reported as mesophilic bacteria, however, emerging research has shown a number of strains that are psychrotrophic (Stenfors *et al.* 2001*). B. cereus* spores can germinate at 4-6 ^{o}C and they grow well at temperatures below 10 ^{o}C (Larsen and Jogernsen 1997). The ability to adapt to various conditions has raised the concern of *B. cereus* as it is said to account for 25% of shelf life related problems in pasteurised milk (Francis *et al*. 1998). *B. cereus* is also known to cause sweet curdling in milk due to its ability to hydrolyse casein and studies have also shown that some strains are able to utilise lactose (Kotiranta *et al.* 2000) while others can hydrolyse lipids (De Jonghe *et al.* 2010). In addition, *B. cereus* is also involved in gelatin hydrolysis, DNase, lecithinase, protease, amylase as well as xylanase production (Molva *et al*. 2008).

2.6.2.3.1 *B. cereus* pathogenicity and outbreaks

Apart from causing spoilage problems in milk, *B. cereus* is an opportunistic human pathogen (Bartoszewicz *et al*. 2008) that causes two principal types of food poisoning; emetic and diarrheal (Hansen and Hendriksen 2001). The emetic poisoning is caused by a small cyclic heat stable peptide which induces vomiting a few hours after ingestion while the diarrheal types are attributed to enterotoxins that induce abdominal pain and diarrhoea after incubation for 8-16 h and vegetative growth of the bacteria in the intestine (Hansen and Hendriksen 2001). Three main toxins that have been commonly implicated in the diarrhoeal disease are cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (*cytK*) (Arnesen *et al.* 2008; Ehling-Schulz *et al.* 2004; Lund *et al.* 2000b). *B. cereus* enterotoxins are heat-labile proteins and can be inactivated by trypsin, pepsin and pronase as well as low pH (pH < 4) (Wang *et al*. 2014). Haemolysin is a three component toxin consisting of the proteins B, L_1 and L_2 (Beecher and Wong 1997) encoded by the genes *hblA*, *hblD* and *hblC* respectively (Ryan *et al*. 1997). Similar to the Hbl is the Nhe complex that is also a three component toxin consisting of the proteins NheA, NheB and NheC encoded by the *nheABC* operon (Granum *et al*. 1999). Both proteins of Nhe and Hbl have shown homology that suggests that they originate from a similar gene. Furthermore, they have all been termed to belong to the tripartite family of toxins (Arnesen *et al*. 2008). Despite the similarities that exist between these toxins, cooperation between them is very limited (Lund and Granum 1997). Several studies have described the effects of *B. cereus* toxins and are summarised in Table 2.1.

Apart from the tripartite family toxins another diarrhoeal toxin is *cytK* that falls under the β-barrel pore-forming toxins (Lund *et al*. 2000b). *CytK* is a 34 kDa water-soluble protein with dermonecrotic, cytotoxic and haemolytic activities, and shows similar cytotoxic potency towards cell cultures as Hbl and Nhe (Lund *et al*. 2000b). Originally isolated from *B. cereus* strain NVH 391/98 that was responsible for a severe foodborne outbreak of diarrhoeal disease in a French nursing home 1998 which claimed three elderly people and several suffering bloody diarrhoea (Lund *et al*. 2000b), *cytK* virulence was later shown to be depended on expression level of the gene not mere presence of the gene (Brillard and Lereclus 2004; Fagerland *et al*. 2007; Arnesen *et al*. 2008).

The emetic syndrome that has a rapid onset of 0.5-6 h after consumption is caused by the toxin cereulide, a cyclic dodecadepsipeptide with a molecular mass of 1.2 kDa (Arnesen *et al*. 2008). The toxin is produced by a peptide synthetase encoded by the 24 kb cereulide synthetase (ces) gene cluster (Ehling-Schulz *et al*. 2005) and it consists of a ring structure with three repeats of four amino and/or oxy acids; [d-*O*-Leu-d-Ala-d-*O*-Val-d-Val]$_3$ (Ehling-Schulz *et al*. 2005). Although cereulide that is also said to be closely related to potassium ionophore valinomycin is not antigenic, it is resistant to heat, pH and proteolysis (Arnesen *et al*. 2008). Its production commences at the end of the logarithmic phase during vegetative growth of *B. cereus* and reaches its highest level during the early stages of the stationary phase (Arnesen *et al*. 2008). Its synthesis takes place between 12-22 ^{o}C, although a previous study reported two isolates belonging to *B. weihenstephanensis* that showed ability to produce cereulide at 8 ^{o}C (Thorsen *et al*. 2006). Cereulide inhibits mitochondrial activity by inhibition of fatty acid oxidation as it acts

as a cation ionophore like valinomycin (Mikkola *et al*. 1999). Previous studies have also shown that cereulide causes cellular damage and inhibit human natural killer cells of the immune system among other biological effects (Yokoyama *et al*. 1999; Paananem *et al*. 2002). The production of cereulide varies depending on a number of factors such as bacterial strain, type of food, temperature, oxygen levels, pH and the presence of specific amino acids (Finlay *et al*. 2000; Agata *et al*. 2002; Rajkovic *et al*. 2006; Shaheen *et al*. 2006).

Table 2.1: Effects of the tripartite cytotoxins on different cells

Toxin	Effects	Reference
Nhe	Fluid accumulation in rabbit ileal loops	Beecher *et al.* 1995b
	Dermonocrotic activity, vascular permeability	Beecher and Wong 1994
	Cytotoxic activity towards Vero cells and retinal tissue	Beecher *et al*. 1995a
	Haemolytic activity towards erythrocytes from several species	Beecher and MacMillan 1990; Beecher and Wong 2000
Hbl	Haemolytic activity towards erythrocytes from several mammalian species	Fagerlund *et al*. 2008
	Cytotoxic activity towards Vero cells	Lindback *et al*. 2004
	Rapid disruption of plasma membrane of epithelial cells and formation of pores in planar lipid bilayers	Fagerlund *et al*. 2008
	Cell death through colloid osmotic lysis by forming transmembrane pores	Fagerlund *et al*. 2008

B. cereus diarrheal food poisoning was first identified after an investigation of a hospital outbreak in Norway, while *B. cereus* emetic syndrome was originally identified in the early 1970s as a result of consumption of cooked rice in Chinese restaurants in the United Kingdom

(Wang *et al.* 2014). While CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) get sick, 128 000 are hospitalized, and 3 000 die of foodborne diseases. It is reported that *Bacillus* spp. food borne outbreaks in Europe were 1.4% of the total outbreaks in 2005 (Arnesen *et al.* 2008). Naranjo *et al*. (2011), also reported of a *B. cereus* food poisoning case that claimed a life in America while Al-Abri *et al*. (2011), reported of an outbreak implicating hospital kitchen food affecting 58 people and Dierick *et al*. (2005), reported of 5 children from the same family who were seriously ill in a *B. cereus* poisoning incident that claimed the life of the youngest girl. In another incident in July 2014 Public Health England reported of *B. cereus* outbreak implicated in intravenous liquid (Total Parenteral Nutrition, TPN). In this out break 23 cases were reported, 19 were confirmed while 3 children died and the whole batch of the product recalled (https://www.gov.uk/government/news/bacillus-cereus-infections). In Argentina the Health Ministry reported 5783 cases of food-borne disease between 2008 and 2013, of which 26 were positive for *B. cereus.* Despite the fact that *B. cereus* outbreaks are believed to be under reported on official lists (Al-Abri *et al*. 2011), food poisoning reporting and record management is generally poor in developing countries hence no information is available pertaining *B. cereus* poisoning in Africa. Although no exact data is available on the effective dose of *B. cereus*, several researchers have suggested doses between 10^5-10^8 cfu/ml (Arnesen *et al*. 2008; Naranjo *et al.* 2011).

2.7 Advances in detection and identification of microorganisms in the food industry

2.7.1 Protein based methods

2.7.1.1 Matrix Assisted Laser Desorption / Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS)

First introduced in 1985, matrix-assisted laser desorption ionization (MALDI), when it was found that the amino acid alanine could be ionized more easily if it was mixed with the amino acid tryptophan and irradiated with a pulsed 266 nm laser (Karas *et al*. 1985). The technique had a breakthrough when biomolecules as large as the 34 472 Da protein carboxypeptidase-A was able to be ionized in 1987. Since then improvements to the technique have been made and it has since found use as a rapid method of microorganisms' identification because it requires minimal sample pretreatment (Fox 2006). The technique is based on three main steps. First, the sample is mixed with a suitable matrix material and applied to a metal plate. This is followed by a pulsed laser that irradiates the sample, triggering ablation and desorption of the sample and matrix material.

Finally, the analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases, and can then be accelerated into a mass spectrometer used to analyse them. The mass spectra of whole cells provide a 'fingerprint' of biomolecular composition and thus constitute a strain-specific spectral pattern (Wahl *et al.* 2002) that can be applied to differentiate micro-organisms even down to the strain level (Liu *et al.* 2007). The technique has shown high reproducibility in both intralaboratory and interlaboratory tests (Mellmann *et al*. 2008). It has

been used to identify microflora of water, vegetables, milk and milk processing equipment, among others in the food industry (Muller 2014; Khoza 2016; du Plessis *et al.* 2015).

2.7.2 DNA based methods

2.7.2.1 End point PCR

Developed in 1983 by Kary Mullis, PCR has become an indispensable technique in modern microbiology. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand using a primer to which it can add the first nucleotide (Bartlett and Stirling 2003). This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify (Erlich 2015). At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies often referred to as amplicons. Typically, agarose gel electrophoresis, followed by staining with fluorescent ethidium bromide is used to detect the amplified DNA fragments (Innis *et al*. 2012). This method is time consuming and not sensitive enough to measure the accumulated DNA copies accurately, so can only give a qualitative result. It has been used in the detection of virulence genes in pathogens (Aijuka *et al*. 2015) as well as identification of different organisms related to spoilage in the food industry (Muller 2014).

2.7.2.2 rep-PCR

The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive sequence elements dispersed throughout the bacterial genome. Repetitive element sequence-based PCR (rep-PCR) is a typing method that enables the

generation of DNA fingerprinting that discriminates bacterial strains, (Spigaglia and Mastrantonio 2003). It differentiates microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements (Woods *et al*. 1993; Versalovic *et al*. 1994; Olive and Bean 1999). Multiple amplicons of different sizes can be fractioned by electrophoresis, and the resulting DNA fingerprint patterns, specific for individual bacterial clones, can be compared. The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of *E. coli* and *Salmonella* spp. (Hiett and Seal 2009). The family of REP elements is generally between 33 and 40 bp in length and has 500-1 000 copies per genome. The method has been used for epidemiological studies and discrimination of pathogens in food (Hiett and Seal 2009; Muller 2014).

2.7.2.3 16S sequencing

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. The use of 16S rDNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used because of its presence in almost all bacteria, often existing as a multigene family or operons (Patel 2001). Furthermore, the function of the 16S rDNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and the 16S rDNA gene (1 500 bp) is large enough for informatics purposes (Janda and Abott 2007). For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and

culture-negative infections. Not only has it provided insights into aetiologies of infectious disease, but it is also useful in identifying novel species (Wu *et al*. 2009).

2.7.3.4 Multilocus sequence typing (MLST)

Proposed in 1998 as universal and definitive method for characterising bacteria, using the human pathogen *Neisseria meningitidis* as an example (Maiden 2006). MLST is a nucleotide sequence based approach for the unambiguous characterisation of isolates of bacteria and other organisms via the internet (http://www.mlst.net). MLST has been described as a generic typing method, employed to date, principally but not solely, bacterial pathogens, that aims to be a robust and portable method for the characterisation of bacterial isolates at the molecular level. It differs from many other former and current approaches to isolate characterisation in that it is based explicitly on the population genetic concepts that underpinned the earlier technique of multilocus enzyme electrophoresis (Maiden 2006). It is based on concepts of multilocus enzyme electrophoresis (MLEE) and has adapted them so that alleles at each locus are defined directly, by nucleotide sequencing, rather than indirectly from the electrophoretic mobility of their gene products (http://www.mlst.net). The procedure characterises isolates of microbial species using the DNA sequences of internal fragments of multiple housekeeping genes on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (Maiden *et al*. 1998). Each isolate of a species is therefore unambiguously characterised by a series of seven integers which correspond to the alleles at the seven housekeeping loci. In MLST the number of nucleotide differences

between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites (Urwin and Maiden 2003). The aim of MLST is to provide a portable, accurate, and highly discriminating typing system that can be used for most bacteria and some other organisms (Maiden 2006). MLST has the additional aims of providing an unified bacterial isolate characterisation approach that generates data that can also be an electronic data repository, the quality of which is maintained by active curation of the submitted data used for evolutionary and population studies of a wide range of bacteria, regardless of their diversity, population structure, or evolution (Urwin and Maiden 2003). At the core of the MLST concept is the provision of freely accessible, curated databases of nucleotide sequence data (Chan *et al*. 2001). MLST has been used for a number of bacteria that includes *B. cereus*, *S. epidermidis*, *E. coli* and *S. typhi* among others. Currently applications of MLST include examination of the evolution of virulence, population and evolutionary analyses and isolate characterisation and population structure analyses (Maiden 2006).

2.7.3.5 Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is the process of determining the complete DNA sequence of an organism's genome at a single time. This entails sequencing all of an organism's chromosomal DNA as well as DNA contained in the mitochondria. WGS overcame the obstacle faced by other typing methods of failing to sufficiently discriminate closely related strains. WGS represents a relatively new and increasingly accessible means for tracking disease outbreaks that has garnered success in multiple applied contexts. Using massively parallel (or "next-generation") DNA sequencing technologies, it is now possible to examine the complete or

nearly complete genomes of bacterial isolates (Salipantea *et al*. 2015). WGS has provided greater resolution in studies where direct comparisons were made with PFGE (Pendleton *et al*. 2013; Leekitcharoenphon *et al.* 2014). Theoretically WGS is said to be able to distinguish strains which differ at only a single nucleotide. Furthermore, genomic differences distinguishing strains can be precisely measured and are highly reproducible, allowing high-resolution inference of phylogenomic relationships (Didelot *et al*. 2013). WGS can enable exploration of isolates' virulence genes, antibiotic resistance mechanisms, and other medically relevant factors, concordantly with molecular epidemiology investigation. WGS has successfully been used in source tracking studies of *S. aureus* and *E. coli* among other bacteria (Rasko *et al*. 2011; Harris *et al*. 2013). The use of WGS in the food safety and microbiology has bright prospects considering the high resolution the technique offers. However, current limitation to its wide application in the food industry is the cost price per sample.

CHAPTER THREE

HYPOTHESES AND OBJECTIVES

3.1 Hypothesis 1

Extended shelf life milk processing will reduce both the bacterial counts and diversity of spore forming bacteria in ESL milk. Although *Bacillus* spp. will be isolated throughout the ESL milk process, *Paenibacillus* spp. will dominate the spore-formers population during cold storage to the end of ESL milk shelf life. Pasteurisation temperatures destroys spore forming bacteria in their vegetative state (Tammine 2009), however, addition of a non-thermal process to HTST pasteurisation has shown to be effective in reducing the spore-formers population (Sepulveda *et al*. 2005; Schmidt *et al*. 2012; Caplan and Barbano 2013). *Paenibacillus* spp. are low in numbers in raw milk and early in pasteurised milk, however, they reproduce during chilled temperatures cold storage to outcompete most microorganisms (Ranieri *et al*. 2012); this could be because *Paenibacillus* spp. spores use L-tryosine as one of its germinants (Alvarado *et al.* 2012). The *Bacillus cereus* enzyme increase the amount of free tryosine in milk during protein degradation (Jan·tová 2006). *Paenibacillus* spp. a microaerophile organism (Owayss 2007), also requires small amounts of oxygen for its spores to germinate.

3.1.1 Objective

To identify and characterise the spore-forming bacteria population associated with ESL milk during processing and chilled storage with the aim of validating the effectiveness of heat and bactofugation based ESL milk process on the spore-formers.

3.2 Hypothesis 2

ESL milk product contamination by *B. cereus* will be from both filler nozzles and raw milk among other sources. *B. cereus*, a ubiquitous spore-forming bacteria, has been identified as the main spoilage organism in pasteurised milk, frequently isolated in the dairy products and processing environment (Ranieri *et al.* 2012; Aouadhi *et al.* 2014). While raw milk has been implicated as an important source of endospores in milk products (Miller *et al*. 2015), filling equipment is a common source of psychrotrophic bacteria in packaged milk (Scott *et al.* 2007; Burgess *et al.* 2010; Hill and Smythe 2012). Some microorganisms are able to attach to processing equipment and these adhered bacteria can detach and contaminate the product as it passes the processing surfaces (Malek *et al.* 2012).

3.2.1 Objective

To characterise *B. cereus* isolates obtained from ESL milk processing and during storage with the aim of understanding the potential of ESL milk filler nozzles and raw milk as a sources of contamination in ESL milk.

CHAPTER FOUR

Bacillus and *Paenibacillus* spp. associated with extended shelf life milk during processing and storage

Redrafted from:

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4.1.1 Abstract

Characterisation of spore-formers associated with extended shelf life milk was done by analysing bacteriological quality of milk samples collected at various processing stages and during storage. Isolates were identified with MALDI-TOF-MS. Milk had spore counts < 2 $\log_{10}$ cfu/ml and 4 $\log_{10}$ cfu/ml during processing and storage respectively. *Bacillus pumilus* dominated the bacterial population. Bacterial species were inoculated in sterile milk for a shelf life study and population change observed over 42 days at 7 ^{o}C. Although ESL milk process was effective in the reduction of bacterial counts and species diversity, the presence of *Bacillus cereus* shows a potential safety problem in ESL milk.

4.1.2 Introduction

Extended shelf life (ESL) milk bridges the gap between high temperature short time (HTST) pasteurised milk with a shelf life of around 10 days under refrigeration and ultra-heat treated (UHT) milk with a shelf life of at least 3 months at ambient storage temperature. Over the past years, various processing methods for the production of ESL milk with sensory characteristics of pasteurised milk have been developed and implemented (Hoffman *et al.* 2006). In addition to high heat treatment at 123-127 ^{o}C for 1-5 s (Mayr *et al*. 2004; Schmidt *et al*. 2012) which has been reported to cause sensory quality problems in the final product (Schmidt *et al*. 2012), another method is a combination of HTST pasteurisation and a non-thermal process such as microfiltration (Hoffman *et al.* 2006) and bactofugation (Fox and McSweeney 1998).

Psychrotolerant endospore-forming spoilage bacteria have been identified as the greatest biological barrier currently limiting shelf life extension of fluid milk and related products (Huck

et al. 2007; Huck *et al*. 2008). Various authors have reported the diversity of endospore-forming bacteria in HTST pasteurised milk (Fromm and Boor 2004; Aouadhi *et al*. 2014). Among the psychrotolerant endospore-forming bacteria that have been reported is *Bacillus* spp. which is not only of spoilage concern but safety as well. Although regarded as a mesophile, various researchers have reported the existence of psychrotolerant strains of *Bacillus cereus* (Stenfors and Granum 2001). These strains have been reported to have the ability to germinate at temperatures between 4-6 ^{o}C and grow well at temperatures below 10 ^{o}C (Langveld and Cuperus 1993; Larsen and Jørgensen 1997). While some authors have described *B. cereus* as the common contaminant in HTST pasteurised milk, it has also been reported that *Paenibacillus* spp. is the dominating endospore forming bacteria at the end of shelf life in ESL milk under refrigeration temperatures (Larsen and Jorgensen 1997; Larsen and Jorgensen 1999; Ranieri *et al.* 2012). The existence of *Bacillus pumilus* in milk has also been reported (Coorevits *et al.* 2008; De Jonghe *et al.* 2010), however, no research focusing on *B. pumilus* in ESL milk has been done despite the fact that some of its strains have shown the ability to produce toxins such as pumilacidins which pose risk to human health (From *et al*. 2007; Nieminem *et al.* 2007; De Jonghe *et al.* 2010). The objective of this study was to characterise the spore-forming population associated with ESL milk spoilage during processing and chilled storage with the aim of validating the effectiveness of heat and bactofugation based ESL milk process on the spore-formers.

4.1.3 Materials and methods

4.1.3.1 Milk sampling

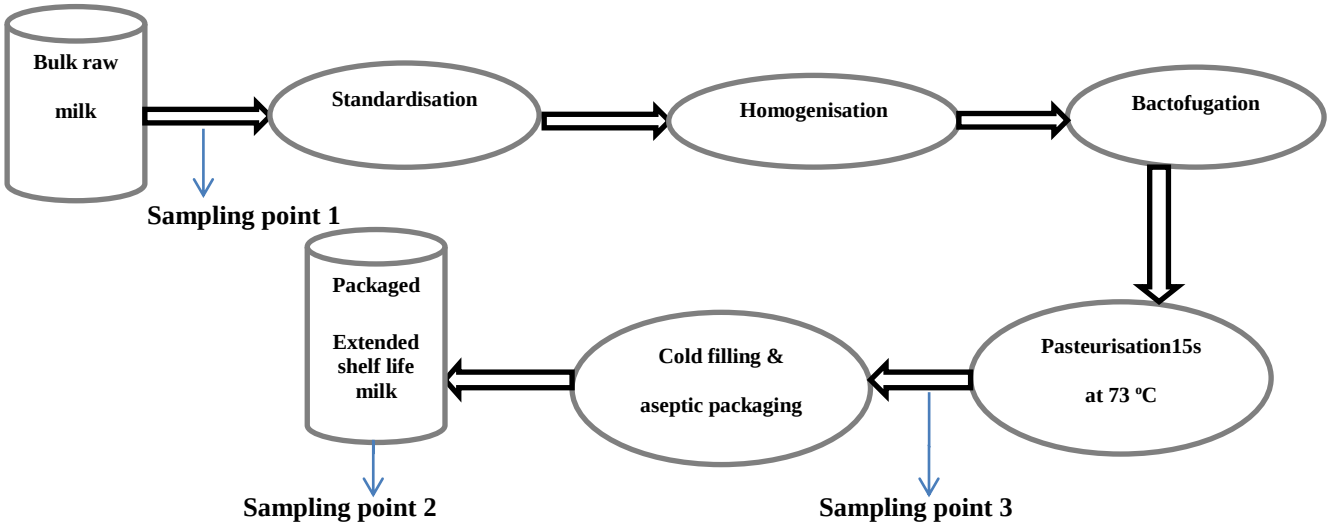

Figure 4.1.1: Schematic diagram of the extended shelf life milk manufacturing process and the sampling points

Milk samples (n = 72) were collected from an ESL milk processing plant (Gauteng, South Africa). Samples were selected from the same process line ensuring the raw milk is tracked to the final product. Samples of both raw milk (2 x 250 ml) and pasteurised milk before packaging (2 x 250 ml) were collected together with samples of packaged ESL milk (2 x 500 ml) during 4 visits. Samples were transported to the Department of Food Science, University of Pretoria at 4 ^{o}C. All the samples were analysed for psychrotrophs, mesophiles, thermophiles as well as spore-formers as described in Table 4.1.1 within 4 hours of collection. An additional 16 samples of packaged ESL milk samples were used for shelf life studies.

Table 4.1.1: Methodology for enumeration of bacterial organisms in raw milk, pasteurised milk and packaged extended shelf life milk

Organism	Media	Incubation Temperature (°C)	Incubation time (Hours)	References
Psychrotrophs, mesophiles & thermophiles	Plate count agar, Oxoid, Basingstoke United Kingdom	25, 30, 55	48	Duncan *et al.* 2004
Thermophilic & mesophilic spore formers	Plate count agar (Oxoid)	30 & 55	48	Ivy *et al.* 2012

4.1.3.2 Isolation and identification of *Bacillus* spp. and *Paenibacillus* spp.

Using enumeration plates described in the previous section with colonies in the range 1-50, spore formers' colonies were grouped according to morphology, colour and size. A total of 84 Isolates were obtained and combined with 33 isolates *B. pumilus* (2), *B. cereus* (29) *Paenibacillus* spp. (2) that was obtained from ESL milk filler nozzles in another research (Khoza 2016). Each isolate was subjected to catalase test (Koneman 2006). KOH test to determine Gram positives and negatives was done as previously described (Halebian *et al.* 1981; Buck 1982). Endospore staining was done following the description of Sumbali and Mehrotra (2009).

4.1.3.3 Determination of heamolysis, proteolytic and lipolytic enzyme activity

Ninety eight isolates from this study of presumptive *B. pumilus, B. cereus, B. subtilis* and *Paenibacillus* spp. isolates were analysed for their enzymatic activity. The enzyme activity of proteases, lipases and haemolysis were performed using skim milk agar (Oxoid Basingstoke, UK), tributyrin agar (Oxoid) and Columbia + 5% sheep blood agar plates (Bio-Rad, Marnes-la-Conquette, France) respectively following previously described procedures (Lucking *et al*. 2013). *B. cereus* ATCC 10876 was used as a positive control.

4.1.3.4 Shelf life studies

Eight samples of ESL milk were stored at 4 ^{o}C and the other 8 samples at 7 ^{o}C. Two samples of the ESL milk from each storage temperature were analysed for total bacterial count as described in Table 4.1.1 at days 7, 14, 18 and 21.

4.1.3.5 Growth profiles at 7 ^{o}C

One isolate that originated from milk stored at 7 ^{o}C was selected for each species (*B. pumilus*, *Bacillus subtilis, B. cereus* and *Paenibacillus* spp.). Eighteen hour old cultures of these isolates were standardised to 0.5 McFarland standards in saline solution. The 4 cultures were diluted in buffered peptone water then each inoculated in 500 ml of sterile milk to achieve a concentration range of 3 – 3.5 $\log_{10}$ cfu/ml. A cocktail of the 4 isolates was also prepared and inoculated in commercial UHT milk in the same concentration as the single isolates. The milk was sealed under sterile conditions, stored at 7 ^{o}C and population change observed at days 0, 14, 28 and 42 by analysing the total bacterial count as described in Table 1 coupled with Matrix-assisted laser

desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) identification. The growth rate of each strain and the strain cocktail was calculated using DMFit in ComBase (www.combase.cc)

4.1.3.6 Isolates identification by MALDI-TOF-MS

All isolates presumptively identified as *Bacillus* and *Paenibacillus* spp. by phenotypic identification were further analysed using MALDI-TOF-MS. A total of 117 isolates were streaked on plate count agar and incubated for 24 h at 30 ^{o}C. Samples for MALDI-TOF-MS analysis were prepared using ethanol treatment followed by extraction with formic acid and acetonitrile as described by Drevinek *et al*. (2012). They were identified using MALDI-TOF-MS software (MALDI biotyper 3.0 Bruker Daltonics) after a mass spectra was obtained using a MALDI-TOF-MS (Bruker Daltonics) following a procedure by Dybwad *et al*. (2012) and Fykse *et al.* (2015). The peak lists generated were used directly to obtain matches against the reference library (SARAMIS database). Using the spectra generated the relatedness of the isolates was determined through creation of MSP dendrograms using Bruker Daltonics MALDI Biotyper 3.0 software (Bruker Daltonics) according to the manufacturer's instructions.

4.1.3.7 Statistical analysis

ANOVA ($p \leq 0.05$) with multiple comparisons (Tukey's HSD test) was used to compare the log counts for the different groups of organisms in raw milk, pasteurised milk and ESL milk. Analysis was done using GraphPad Prism 7.0 (GraphPad Software, Inc., USA).

4.1.4 Results

4.1.4.1 Bacterial counts of milk during ESL milk processing and shelf life

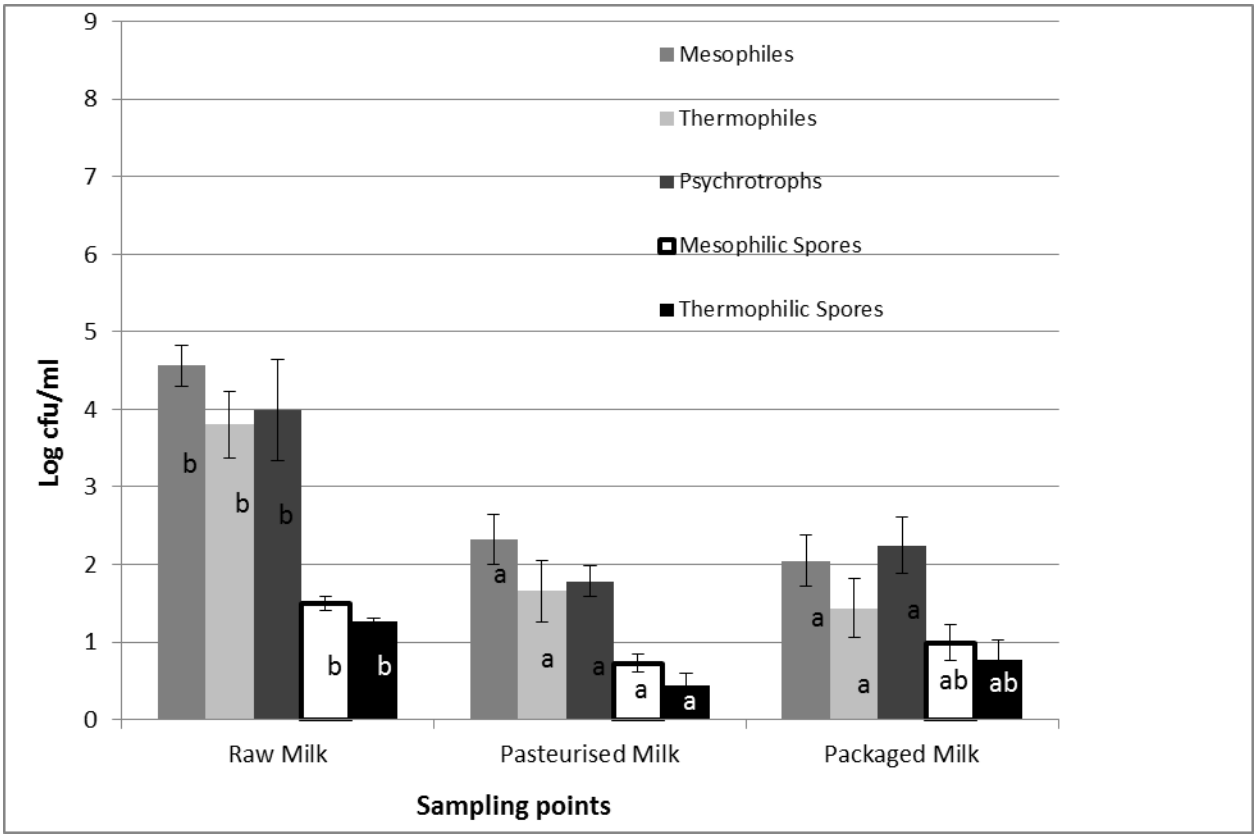

Figure 4.1.2: Bacterial counts of milk at different sampling points during extended shelf life milk processing. Sampling points with a different letter for the same bacterial group have means that are significantly different ($p \leq 0.05$) (n = 6)

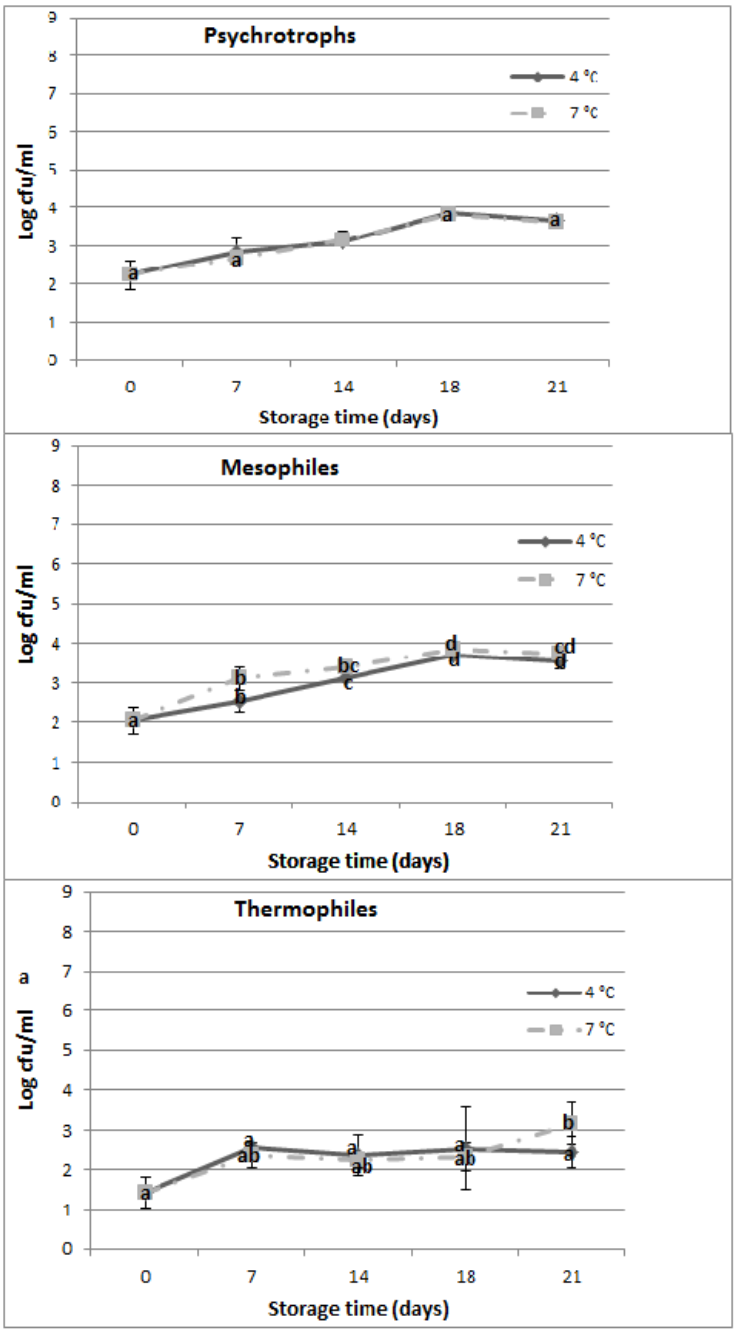

Figure 4.1.3: Effect of storage temperature on the bacterial composition of ESL milk during storage for 21 days at 4 and 7 ºC. Storage days with a different letter for the same bacterial group have means that are significantly different ($p \leq 0.05$) (n = 72)

ESL milk processing significantly ($p \leq 0.05$) reduced the mesophiles, thermophiles and psychrotrophs in raw milk by at least 2 $\log_{10}$ cfu/ml. The process also significantly ($p \leq 0.05$) reduced the counts of spore forming bacteria (Figure 4.1.2).

The psychrotrophic bacterial count increased by more than 1 $\log_{10}$ cfu/ml at both storage temperatures between the initial day of storage and day 21, (Figure 4.1.3). Mesophiles increased significantly ($p \leq 0.05$) by 1.5 and 1.7 $\log_{10}$ cfu/ml during storage at both 4 and 7 ^{o}C respectively. The initial thermophilic bacterial count was low, < 2 $\log_{10}$ cfu/ml, and increased by 1 $\log_{10}$ cfu/ml at storage temperature of 4 ^{o}C and by 1.7 $\log_{10}$ cfu/ml at 7 ^{o}C**.**

4.1.4.2 Bacterial identification, species distribution and enzymatic activity of isolates from ESL milk processing and storage

Table 4.1.2: Enzymatic activity (%) of *Bacillus pumilus, B. subtilis, B. cereus* and *Paenibacillus* species isolated from ESL milk processing and storage

Bacterial species (number of Isolates)	Proteolysis	Lipolysis	Beta haemolysis
B. pumilus (n=39)	89	56	Nd
B. subtilis (n=18)	94	Nd	Nd
B. cereus (n=35)	100	Nd	100
Paenibacillus spp. (n=6)	67	33	Nd

Nd = Not detected

Table 4.1.3: Prevalence of spore forming bacteria isolated during extended shelf life milk processing and storage

Bacterial species	% Isolates				Total isolates (%)
	Raw milk (n=26)	Pasteurised milk (n=16)	Packaged ESL milk (n=20)	ESL milk stored at 4 and 7 °C (n =22)	
Bacillus pumilus	35	50	25	68	**44**
B. subtilis	27	19	30	9	**21**
B. licheniformis	27	13	20		**16**
B. sonorensis	8	6	5		**5**
B. circulans		6			**1**
B. cereus			20	9	**7**
Paenibacillus spp.		6			**1**
Paenibacillus amylolyticus				14	**4**
Aneurinibacillus migulanus	4				**1**
					100

Total isolates (N) = 84

There was a high diversity of spore forming bacteria during processing. The diversity reduced during storage where only 4 species were isolated. *B. pumilus* was the dominating spore-former during ESL milk processing and throughout the shelf life study, (Table 4.1.3). *Bacillus licheniformis*, *Bacillus soronenis*, *Bacillus circulans* and *Aneurinibacillus migulanus* were only isolated during processing and *Paenibacillus amylolyticus* was only isolated during ESL milk storage. *B. cereus* and *B. subtilis* were isolated during processing and storage, although their

prevalence was lower than that of *B. pumilus*. Eighty seven percent of all isolates showed proteolytic activity. All *B. cereus* isolates were proteolytic and haemolytic. Only 22% of all the isolates were lipolytic and these belonged to *B. pumilus* and *Paenibacillus* spp. (Table 4.1.2)

4.1.4.3 *B. pumilus* MALDI-TOF-MS cluster analysis

B. pumilus isolates clustered into 5 main groups with sub groups (Figure 4.1.4). Isolates that originated from raw milk, pasteurised milk and packaged ESL milk before storage clustered together in Group 1 and 2. Isolates from all the sources except 4 ^{o}C storage clustered together in group 3. Group 4 contained isolates from filler nozzles, ESL milk stored at 4 ^{o}C and pasteurised milk. Group 5 was the only group where isolates from both storage temperatures clustered together showing a close relationship.

4.1.4.4 *B. subtilis* MALDI-TOF-MS cluster analysis

B. subtilis clustered into 5 main groups (Figure 4.1.5). All groups except group 3 had isolates from raw milk, pasteurised milk and packaged ESL milk before storage. Group 3 showed close similarities of isolates from both storage temperatures (4 and 7 ^{o}C).

4.1.4.5 *B. cereus* MALDI-TOF-MS cluster analysis

B. cereus showed 8 distinct groups in the clustering (Figure 4.1.6). Groups 1 and 3 showed a close relationship between isolates from ESL milk stored at 7 ^{o}C and isolates from ESL milk filler nozzles while group 2 showed a relationship between isolates from ESL milk filler nozzles

and isolates from packaged ESL milk. Group 7 was made up of isolates from packaged ESL milk before storage only. The other groups comprised of isolates from ESL milk filler nozzles only.

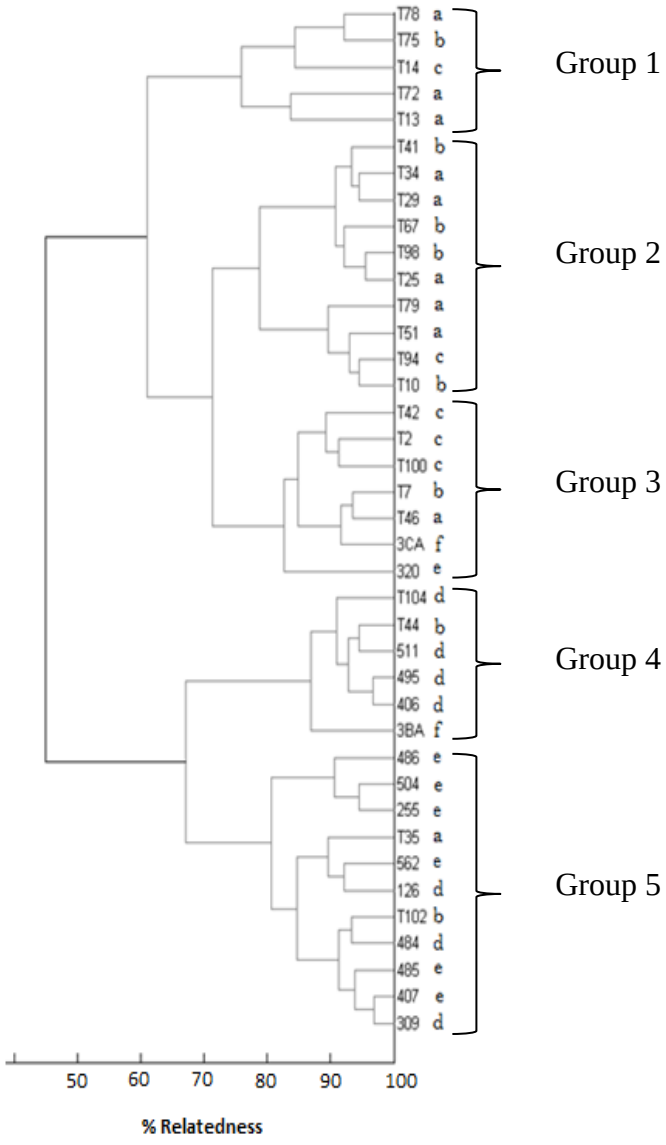

Figure 4.1.4: ***Bacillus pumilus*** **cluster analysis of MALDI-TOF-MS data derived from isolates obtained in extended shelf life milk processing, extended shelf life milk stored at 4 ºC and 7 ºC over a period of 21 days and extended shelf life milk filler nozzles after CIP process.**

Isolate sources: a – Raw milk, b – Pasteurised milk, c – Packaged ESL milk on the day of sampling, d- Stored ESL milk at 4 °C, e – Stored ESL milk at 7 °C and f – ESL milk filler nozzles

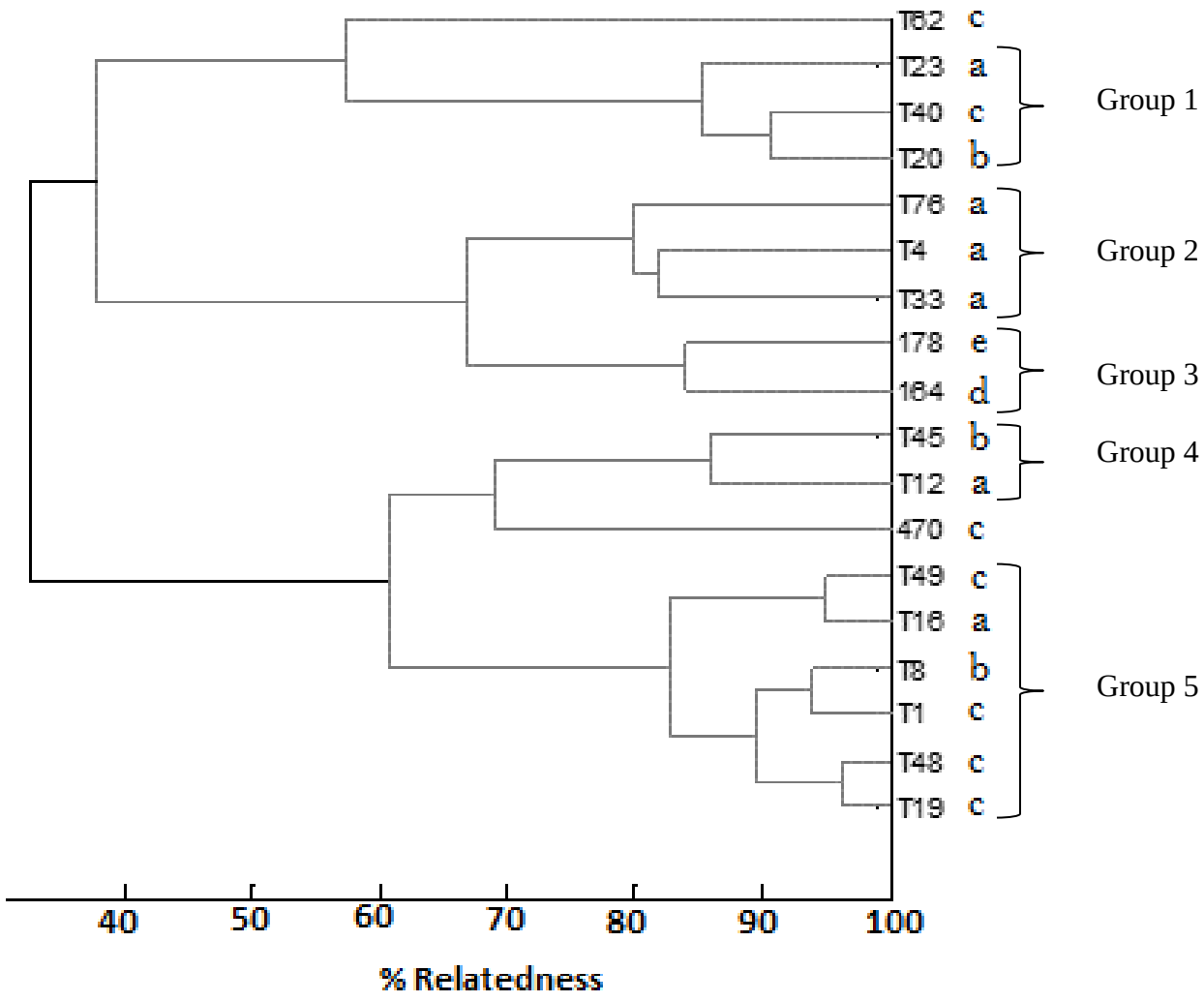

Figure 4.1.5: *Bacillus subtilis* cluster analysis of MALDI-TOF-MS data derived from isolates obtained in extended shelf life milk processing, extended shelf life milk stored at 4 °C and 7 °C over a period of 21 days and Extended Shelf Life milk filler nozzles after CIP process.

Isolate sources: a – Raw milk, b – Pasteurised milk, c – Packaged ESL milk on the day of sampling, d- Stored ESL milk at 4 ºC*, e – Stored ESL milk at* 7 ºC

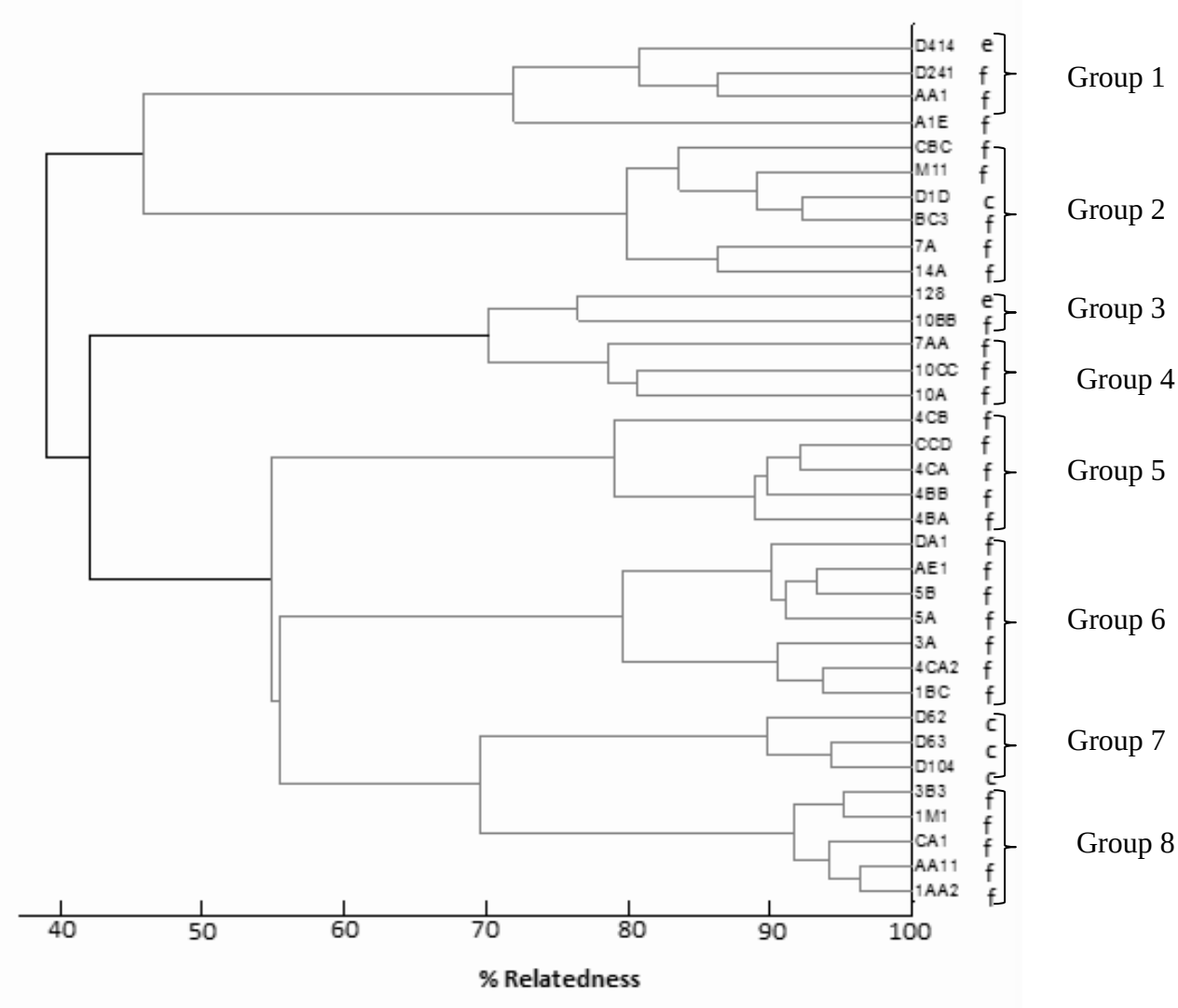

Figure 4.1.6: *Bacillus cereus* cluster analysis of MALDI-TOF-MS data derived from isolates obtained in extended shelf life milk processing, extended shelf life milk stored at 4 ºC and 7 ºC over a period of 21 days and extended shelf life milk filler nozzles after CIP process.

Isolate sources: c – Packaged ESL milk on the day of sampling, e – Stored ESL milk at 7 ºC *and f – ESL milk filler nozzles*

4.1.4.6 *Paenibacillus* spp. MALDI-TOF-MS cluster analysis

Paenibacillus spp. showed one distinct cluster (4.1.7). Isolates from pasteurised milk and ESL milk filler nozzles did not have a close relationship. Group 1 had isolates identified as *P. amylolyticus* and these were isolated from ESL milk stored at 7 ºC.

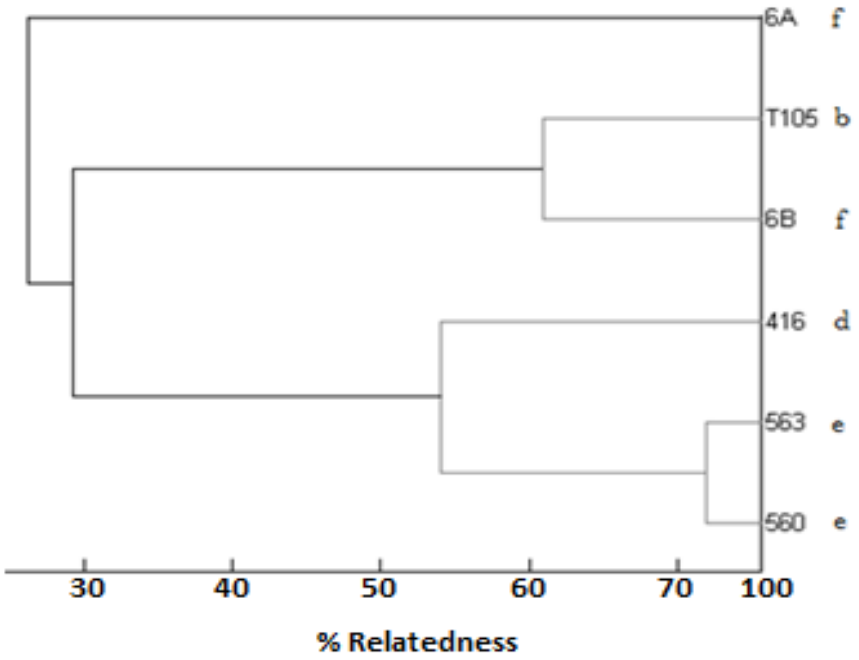

Figure 4.1.7: *Paenibacillus* spp. cluster analysis of MALDI-TOF-MS data derived from isolates obtained in extended shelf life milk processing extended shelf life milk stored at 4 ºC and 7 ºC over a period of 21 days and extended shelf life milk filler nozzles after CIP process.

Isolate sources: b – Pasteurised milk, d – Stored ESL milk at 4 ºC, *e – Stored ESL milk at* 7 ºC *and f – ESL milk filler nozzles*

4.1.4.7 *Bacillus* spp. and *Paenibacillus* spp. growth profiles at 7 ºC

Paenibacillus spp. had the highest growth rate followed by *B. pumilus*. *B. subtilis* had the lowest growth rate while the growth rate of *B. cereus* was not different from the cocktail growth rate (Figure 4.1.8). *Paenibacillus* spp. dominated the cocktail population. *Paenibacillus* spp. constituted half of the total cocktail population at 14 days while no other bacteria was enumerated after 42 days of storage.

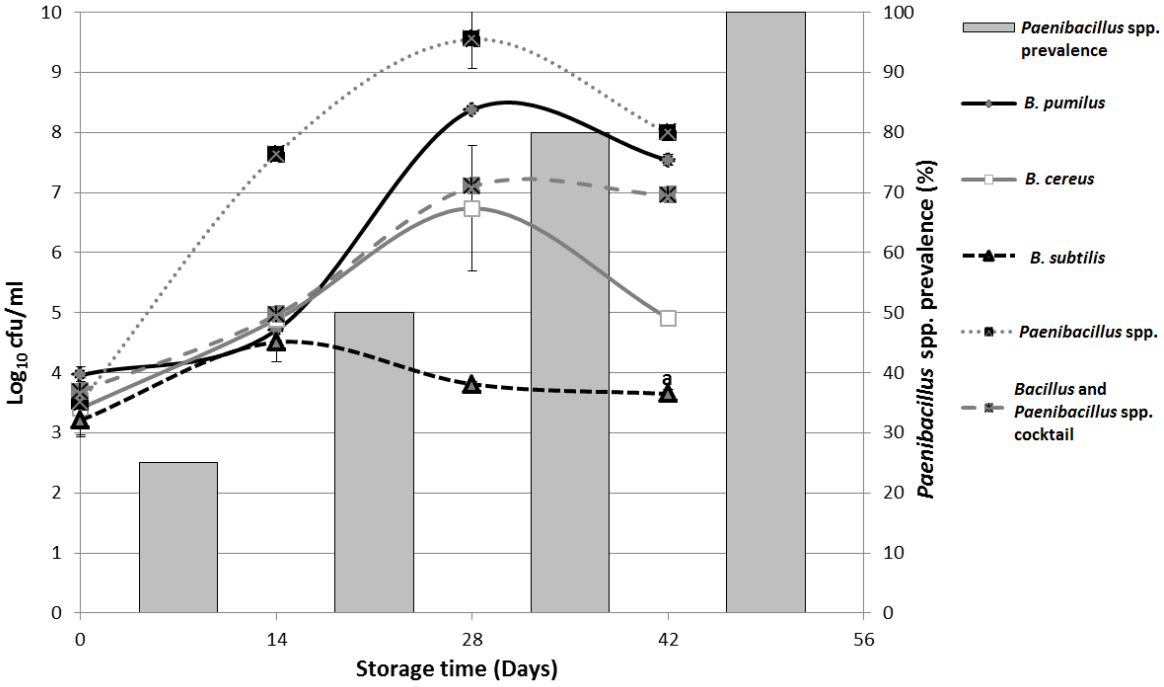

Figure 4.1.8: Changes in the population of inoculated *B. cereus*, *B. subtilis*, *Paenibacillus* spp. *B. pumilus* and bacteria cocktail of *B. cereus*, *B. subtilis*, *Paenibacillus* spp. and *B. pumilus* in milk over a 42 day shelf life at 7 ºC.

Bacteria species with a different letter have growth rates that are significantly different at 7 ^{o}C ($p \leq 0.05$). (Growth rates: *B. subtilis* 0.004±0.000, *B. cereus* 0.006±0.003; *B. pumilus* 0.011±0.000; *Paenibacillus* spp. 0.006±0.001)

4.1.5 Discussion

Similar to other reported ESL milk processes (Fromm and Boor 2004; Sepulveda *et al*. 2005; Schmidt *et al*. 2012; Caplan and Barbano 2013), the ESL milk process under investigation, which involves bactofugation, reduced vegetative bacterial counts significantly. This reduction was also facilitated by the raw milk which had low bacterial counts (< 5 $\log_{10}$ cfu/ml, Foodstuffs, cosmetics and disinfectants Act 54 of 1972) (DAFF 1972). Despite the process significantly reducing the bacterial count of spore-formers after bactofugation, there was an increase of the spore formers in the packaged product, possibly due to post process contamination and growth of survivors a trend that is similar to findings of Eneroth *et al*. (2001), who in their study concluded contamination by filling machines. The dominance of mesophilic bacteria at 7 ^{o}C storage is likely to have been a result of mesophilic bacteria that may exhibit psychrotrophic characteristics due to the presence of the 16S psychrotrophic signature, (von Stetten *et al*. 1998). Stenfors and Granum (2001) reported the presence of both mesophilic and psychrotrophic characteristics in majority of *B. cereus* strains in their study while other studies have also shown that minimum growth temperature for spore-forming *Bacillus* and *Paenibacillus* spp. range from 5-20 ^{o}C, and maximum range from 35-55 ^{o}C (De Vos *et al*. 2009). The increase in thermophilic counts under refrigeration (Figure 4.1.3) may be an indication that they are neither true thermophiles nor thermotrophs. Thermophilic organisms do not grow at temperatures below 40 ^{o}C while

thermotrophs have a growth range of 20-60 ^{o}C (Deák and Farkas 2013). These organisms could possibly have been mesophiles that contain psychrotrophic signatures (Stenfors and Granum 2001). However more elaborate studies are needed to verify this hypothesis especially in *B. pumilus* that dominated this group. The bacterial counts obtained from the ESL milk product in this study were lower than those previously reported for pasteurised milk stored at 4-7 ^{o}C (Larsen and Jorgensen 1999; Fromm and Boor 2004; Petrus *et al*. 2010), by more than 1 $\log_{10}$ cfu/ml. The presence of the bactofugation step accounts for the difference in microbial load with pasteurised milk from previous studies. However, the bacterial count observed in this study was higher than that reported for microfiltered ESL milk (Schmidt *et al*. 2012). The difference in the bacterial count of the two processes maybe due to a number of factors described in previous researches that include raw milk quality, season of production, water and equipment among others (Coorevits *et al*. 2008; De Jonghe *et al*. 2010; Eneroth *et al*. 2001).

B. pumilus isolates had the highest prevalence among the endospore forming bacteria, contributing 45% of the total isolated endospore forming bacteria. This is contrary to most reported results on endospore forming bacteria in heat treated milk. Aoudhi *et al.* (2014) reported *B. cereus* as the dominating *Bacillus* spp. in pasteurised milk hence, concurs with Larsen and Jørgensen (1999) and Ranieri *et al.* (2012) who also observed similar results. However, our results showed similarity with those obtained from microfiltered ESL milk by Schmidit *et al*. (2012) who observed 48% prevalence of *B. pumilus*. Apart from raw milk composition (Coorevits *et al*. 2008) the dominance of *B. pumilus* can be attributed to its resistance to a number of stresses including UV light exposure and presence of oxidisers such as H_2O_2 among others as well as the ubiquitous nature of its endospore (Benardini *et al.* 2003; Link *et al*. 2004;

Dickinson *et al*. 2004). In line with available data (Ranieri and Boor 2009; Ranieri *et al.* 2012; Schmidt *et al*. 2012), our results showed the presence of *Paenibacillus* spp. *B. cereus* as well as *B. licheniformis* although the percentage prevalence we observed was lower than that reported. *B. licheniformis* did not grow in the ESL milk product stored at both 4 and 7 ºC because it is a mesophile (Heyndrickx and Scheldeman 2002). The few *B. cereus* isolates obtained in our study are in agreement with previous research findings, (Larsen and Jorgensen 1997; Larsen and Jorgensen 1999). Apart from the fact that *B. cereus* is a poor competitor, good raw milk quality also accounts for the low prevalence levels of *B. cereus* and *Paenibacillus* spp. also suggesting that good hygienic processing conditions existed in this processing plant. When inoculated as single organisms, *Paenibacillus* spp. had the highest growth rate. It dominated the cocktail composition mainly because of its ability to be competitive against accompanying microbiota at refrigerated conditions. *Paenibacillus* spp. requires L-tyrosine as one of its spore germinants (Alvarado *et al.* 2012). It is likely that the proteolytic activity of *B. cereus* increases the amount of free tyrosine in milk thereby aiding the growth of *Paenibacillus* spp. (Jan·tová *et al*. 2006). *Paenibacillus* spp. is also a microaerophile (Owayss 2007) that requires small amounts of oxygen for its spores to germinate thus helping more spores to germinate as milk ages.

Although there is no direct correlation between enzyme activity and milk spoilage, the fact that more than 70% of isolates under study were proteolytic, shows a great potential of these organisms to be the main causes of bacterial spoilage in ESL milk. These results show a similar trend to previous studies (De Jonghe *et al*. 2010; Lucking *et al.* 2013). Our study observed low lypolytic activity in *B. cereus* and *Paenibacillus* spp. a result that is similar to previous research

findings (Lucking *et al.* 2013). This implies that *B. cereus* and *Paenibacillus* spp. pose low risk of ESL milk spoilage by lipid hydrolysis. However, contrary to previous milk studies (De Jonghe *et al*. 2010; Lucking *et al.* 2013), none of the *B. subtilis* isolates under investigation were lypolytic. With the highest lipolytic activity (62%), *B. pumilus* has potential to contribute to ESL milk spoilage by lipolysis. All *B. cereus* isolates showed haemolytic activity similar to previous results (Lucking *et al.* 2013). Despite being isolated in low numbers this shows that *B. cereus* poses a potential safety risk to ESL milk consumers.

B. pumilus exhibited psychrotrophic characteristics as they managed to grow both at 4 and 7 ^{o}C and dominate the microflora of ESL milk. In this research it was observed that *B. pumilus* isolates that could grow at both 4 and 7 ^{o}C showed more than 75% similarity with isolates of *B. pumilus* isolated from the filler nozzles after Cleaning In Place (CIP) at the same dairy plant. This suggests post pasteurisation contamination of ESL milk by possible psychrotrophic *B. pumilus.* A previous study on pasteurised milk has also demonstrated the ability of processing equipment to contaminate the product, (Eneroth *et al.* 2001). Although the *Paenibacillus* spp. isolates did not show close similarities, *P. amylolyticus* showed ability to grow at both 4 and 7 ^{o}C possibly owing to its psychrotrophic nature (Ivy *et al*. 2012). The existence of most *B. subtilis* isolates during processing compared to storage shows that the available strains could not exhibit psychrotrophic properties as it is a well-known mesophile (Heyndrickx and Scheldeman 2002). The presence of *B. cereus* in stored milk cements reports that some of its strains have psychrotrophic properties, (Heyndrickx and Scheldeman 2002) and the similarity of isolates from stored ESL milk and fillers is in line with the findings of Eneroth *et al*. (2001) suggesting post

pasteurisation contamination by the fillers. While the growth temperature of psychrotrophic endospores of *B. cereus* have been reported to be in the range between 4-11 ^{o}C (Larsen and Jørgensen 1999), it has also been shown that they germinate below their minimum growth temperatures (Larsen and Jørgensen 1999).

4.1.6 Conclusions

The study indicated that bactofugation and pasteurisation based ESL milk process is effective in reduction of both bacterial count and species diversity. While *B. pumilus* dominated the spore-formers in ESL milk process, *Paenibacillus* spp. showed ability to outgrow other spore-formers when the initial population is approximately the same. Although a few *B. cereus* isolates were obtained, its presence is a potential safety problem in ESL milk. *Paenibacillus* spp. and *Bacillus* spp. have great potential to cause spoilage of ESL milk through lipid and protein hydrolysis.

CHAPTER FIVE

Diversity of *Bacillus cereus* strains in extended shelf life milk

Redrafted from:

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5.1.1 Abstract

Characterisation of 49 *Bacillus cereus* strains obtained from extended shelf life (ESL) milk processing and filler nozzles was done using $(GTG)_5$ Rep PCR fingerprinting; determining the presence of virulence genes; *cytK*, *nheA*, *cer* and *hblA* and discrimination of psychrotrophic and mesophilic strains with 16S rDNA. Fourteen isolates were selected for 16S partial sequencing. Fingerprinting and sequencing showed evidence of filler nozzles contaminating ESL milk despite high heterogeneity existing between the isolates. While there is high prevalence of *cer*, *hblA* and *nheA; cytK* was not widely distributed. There was 100% and 8% prevalence of mesophilic and psychrotrophic signatures respectively. Despite the large diversity of the *B. cereus* strains in this study, there is evidence that filler nozzles and raw milk are a source of contamination of *B. cereus* in ESL milk.

5.1.2 Introduction

Bacillus cereus, a ubiquitous spore-forming bacteria has been identified as the main spoilage organism in pasteurised milk (Aouadhi *et al.* 2014). Its presence in milk products does not only cause spoilage concern, but safety too as it is known to produce a number of toxins (Hansen and Hendriksen 2001; Bartoszewicz *et al*. 2008). Extended shelf life (ESL) milk is a product bridging the gap between UHT and pasteurised milk that has gained popularity over the past few years. Although previous studies have reported that *B. cereus* was not the dominating organism in ESL milk (Schmidit *et al*. 2012; Mugadza and Buys 2014), its ability to grow at 7 ^{o}C (Mugadza and Buys 2017a), makes it a significant organism in ESL milk.

While raw milk has been implicated as an important source of endospores in milk products (Miller *et al*. 2015) other studies have shown that a different population of endospore forming microorganisms exists in raw milk and milk products made from the same raw milk. This population change has been attributed to a number of reasons that include post process contamination among others (Scott *et al.* 2007; Burgess *et al.* 2010; Hill and Smythe 2012). Post heat treatment contamination by processing equipment such as fillers has also proved to cause bacterial population variations between raw milk and milk products (Scott *et al.* 2007; Burgess *et al.* 2010; Hill and Smythe 2012). Other studies have shown that *B. cereus* isolates from the environments (soil, food, the dairy production chain) have a higher degree of heterogeneity compared to clinical isolates (Helgason *et al*. 2000; Ehling-Schulz *et al*. 2005). Banyko´ and Vyletelova 2009 concluded that *B. cereus* does not originate exclusively in either the raw or pasteurised milk but from post-pasteurisation contamination and propagation during the manufacturing process.

A full understanding of the characteristics and relationship between *B. cereus* strains isolated in the ESL milk processing environment becomes important in determining the source of product contamination. The objective of this study was to characterise *B. cereus* isolates obtained from ESL milk processing and during shelf life under refrigeration with the aim of determining cross contamination routes within the ESL milk processing plant.

5.1.3 Materials and methods

5.1.3.1 Isolates and DNA extraction

Samples of raw milk, pasteurised milk before packaging and packaged ESL milk were collected together with filler nozzles swabs during 3 visits at an ESL milk processing plant (Gauteng, South Africa). Samples were selected from the same process line ensuring the raw milk is tracked to the final product. Furthermore, some packaged ESL milk samples were stored at 4 and 7 ^{o}C over a 21 day shelf life period. *B. cereus* isolates were obtained from these samples as described elsewhere (Khoza 2016; Mugadza and Buys 2017a). Forty nine *B. cereus* isolates that were obtained from raw milk (2), pasteurised milk (4), filler nozzles (40) and ESL milk during storage at 7 ^{o}C (3), were grown in nutrient agar at 30 °C together with a reference strain, *B. cereus* ATCC 10876 until the late exponential phase which required an incubation period of 16-24 h. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, California, USA) according to the manufacturer's instructions for use in the PCR reactions.

5.1.3.2 $(GTG)_5$ Rep PCR Fingerprinting

Fingerprinting PCR was carried out in 20 µL reaction tubes in a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Singapore, Singapore). The reaction mixture consisted of 10 µL KAPA BIOSYSTEMS 2X KAPA Taq Ready Mix (Kapa Biosystems Cape Town, South Africa), 0.3 µL GTGGTGGTGGTGGTG oligonucleotide primer (Versalovic *et al*. 1994), 2 µL DNA template, 7.3 µL PCR-grade water and 0.4 µL Dimethyl sulfoxide (DMSO). The PCR program was as follows: 95 ^{o}C for 5 min, 95 ^{o}C for 1 min, annealing at 52 ^{o}C for 1 min, extension at 72 ^{o}C for 3

min. The program was repeated for 34 cycles and a final extension at 72 ^{o}C for 10 minutes. PCR products were separated by Gel electrophoresis using a 1.5% Agarose gel (55 V for 4 h) and the image viewed using a Bio-Rad Gel Doc™ EZ (Bio-Rad, California, USA). The $(GTG)_5$ fingerprints were analysed using Gel-Compar II version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of digitised band patterns was calculated using Pearson's correlation coefficient, and unweighted pair group method with arithmetic means. Complete linkage algorithms were used to construct an average linkage dendrogram to show relationship of isolates. Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt *et al*. 2002). However, due to the close similarities that existed between the isolates sub clusters were further considered at 95%.

5.1.3.3 PCR to determine virulence genes and discriminate psychrotrophic from mesophilic *B. cereus*

Pathogenicity of the *B. cereus* was determined by observing the presence of the following genes; cytotoxin K (*cytK*), nonhemolytic enterotoxin A (*nheA*), emetic toxin cereulide (*cer*) and enterotoxin hemolysin BL (*hblA*). Discrimination of psychrotrophic and mesophilic *B. cereus* was done by targeting the 16S rDNA signatures. The primers used are described in Table 5.1.1. All PCRs were carried out in 10µL reaction tubes in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Singapore, Singapore). The reaction mix consisted of 1 µL bacterial DNA template, 5 µL KAPA BIOSYSTEMS 2X KAPA Taq Ready Mix (Cape Town, South Africa), 3.4 µL PCR-grade water and 0.3 µL of each primer. The PCR conditions for *cytK, cer, hblA and nheA* are described in a previous study (Swiecicka and Mahillon 2006), while conditions for mesophilic

and psychrotrophic *B. cereus* PCR are also previously described (von Stetten *et al*. 1998). Correlation between isolates was also determined by assigning a score of 1 and 2 for the presence and absence of a gene respectively and similarity calculated using principle component analysis (PCA) and agglomerative hierarchical clustering (AHC) in XLSTAT an add-in programme of Microsoft Excel.

5.1.3.4 *B. cereus* 16S rRNA Sequencing

Sixteen *B. cereus* isolates were randomly selected, ensuring representation of each sampling point for 16S sequencing. Using the ZR Fungal/Bacterial DNA Kit™(Zymo Research, California, USA), DNA was obtained from the cultures. The 16S target region was amplified using DreamTaq™ DNA polymerase (Thermo Scientific™, Johannesburg, South Africa) and the primers, (Amplicon size 1465 bp) shown in Table 5.1.2. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, California, USA), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, California, USA) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI).

Table 5.1.1: Primers used to detect virulence, 16S mesophilic and psychrotrophic genes in *B. cereus* strains isolated from extended shelf life milk processing and during shelf life

Primer name	Sequence	Amplicon size (bp)	Gene amplified	Reference
Emetic toxin cereulide	F. ACGTCACCAGTNGATATWTC R. CTCCACCATTCCCAWGCAAG	1146	*Cer*	Swiecicka *et al*. 2006
Non hemolytic enterotoxin (A)	F. TACGCTAAGGAGGGGCA R. TTTTTATTGCTTCATCGGCT	499	*nheA*	Swiecicka *et al.* 2006
Hemolysin BL (B)	F. GTGCAGATGTTGATGCCGAT R. ATGCCACTGCGTGGACATAT	1154	*hblA*	Swiecicka *et al.* 2006 Hansen and Hendriksen 2001
Cytotoxin K	F. ACAGATATCGGTCAAAATG R. CAACCCAGTTACCAGTT	1011	*cytK*	Fagerlund *et al.* 2004 Swiecicka *et al.* 2006
Mesophilic MF/UR	MF – ATAACATTTTGAACCGCATG UR – CTTCATCACTCACGCGGC	249	16S rDNA	Stenfors and Granum 2001
Psychrotrophic PR/UF	PR – GAGAAGCTCTATCTCTAGA UF – CAAGGCTGAAACTCAAAGGA	132	16S rDNA	Stenfors and Granum 2001

Table 5.1.2: 16S Primers sequences used for *B. cereus* 16S sequencing

Name of primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

5.1.4 Results

5.1.4.1 $(GTG)_5$ Rep PCR Fingerprinting of *B. cereus* strains isolated from ESL milk processing and during storage

All the *B. cereus* isolates under study showed close similarities above 70%. However they clustered into 6 groups at 95% similarity level (Figure 5.1.1). Groups 1, 3 and 5 comprised of isolates from filler nozzles only. Groups 2 and 4 showed evidence of a very close relationship between raw milk and filler nozzles originating isolates. Group 6 showed very close similarities between isolates from filler nozzles and pasteurised milk. All the isolates from ESL milk stored at 7 ^{o}C and the reference strain *B. cereus* ATCC 10876 did not cluster with any isolates at 95% although it showed a relationship with the other isolates at similarity level above 70%.

5.1.4.2 Detection of virulence, mesophilic and psychrotrophic genes in *B. cereus* strains isolated from ESL milk processing and during storage

While 65% of the isolates showed the presence of *cer* none were from ESL milk stored at 7 ºC (Table 5.1.3). The gene *cyt*K was present in 33% of the isolates from all the sources and 88% of the isolates showed the presence of *hblA*. Ninety-eight percent of the isolates were positive for *nhe*A while all the isolates showed the presence of the mesophilic 16S signature (MU/MF). Only 8% of the isolates showed the presence of psychrotrophic 16S signature of which the isolates originated from ESL milk stored at 7 ºC, raw milk and pasteurised milk. Six groups were observed when isolates were clustered based on these genes (Figure 5.1.2). Group 1 comprised isolates originating from raw milk, pasteurised milk and ESL milk filler nozzles that were positive to all genes under investigation except *cytK* and the psychrotrophic 16S signature. Groups 2, 3 and 4 comprised entirely of isolates from filler nozzles. Group 2 isolates were positive only for *nheA, hblA* and the mesophilic 16S signature while group 3 was only negative for the psychrotrophic 16S signature. Furthermore group 4 was negative for the psychrotrophic 16S signature and *hblA*. Group 5 was made up of isolates from ESL milk stored at 7 ºC only. Group 6 comprised isolates from ESL milk stored at 7 ºC and ESL milk filler nozzles isolates. Both groups 5 and 6 were positive for *nheA* as well as mesophilic and psychrotrophic 16S signatures. In addition group 5 was positive for *cytK*. However, *cer* and *hblA* were not detected in both groups. The principal component analysis (Figure 5.1.3) showed that *cytK* present in psychrotrophic strains was absent in over 70% of mesophilic strains hence these two groups did not cluster together. Mesophilic strains showed 4 distinct clusters with the biggest cluster

showing the presence of *hblA* and *cer* genes. *Cer* and *nheA* dominated the second cluster while *hblA* dominated the other cluster. The smallest cluster was more aligned to the presence of *cytK* and *nheA*.

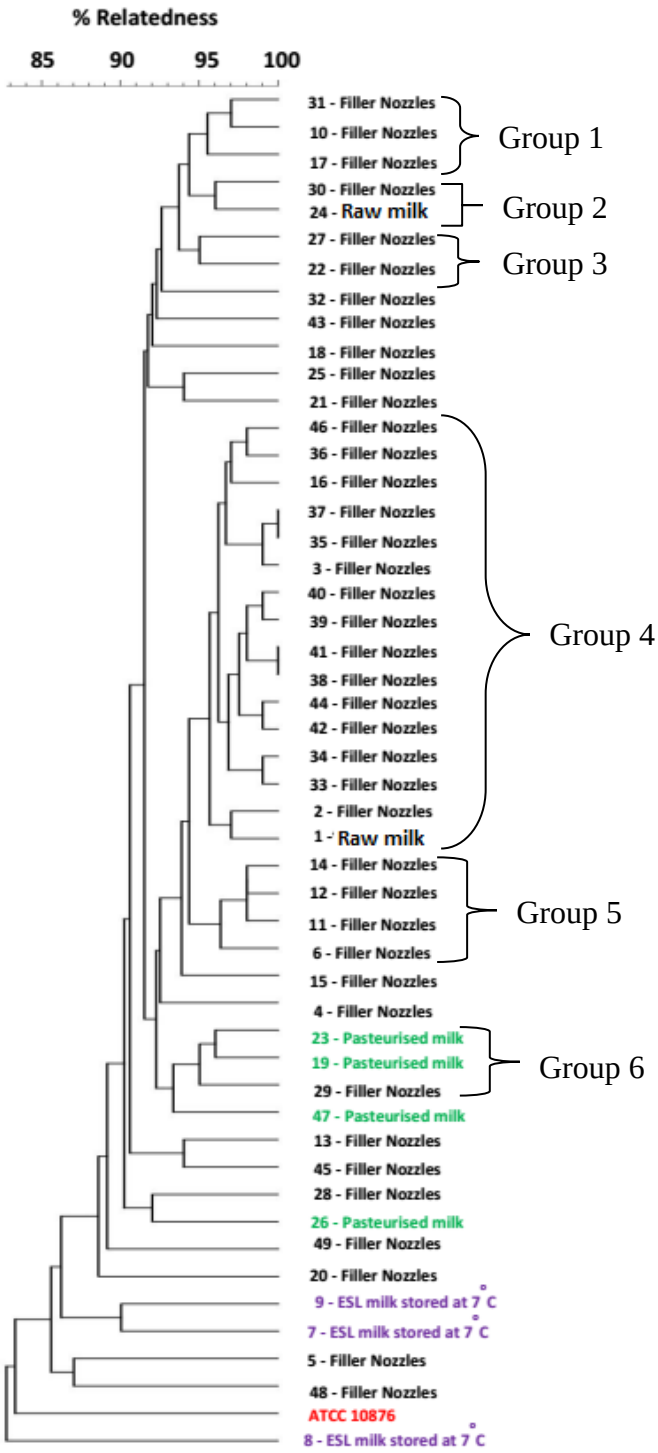

Figure 5.1.1: Dendrogram of Rep-PCR $(GTG)_5$ fingerprint patterns in *B. cereus* isolates from raw milk, ESL milk processing and during shelf life.

Table 5.1.3: Detection of virulence, mesophilic and psychrotrophic genes in *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life

Isolate source	Isolate (%)	Gene					
		Cer	***nheA***	***hblA***	***CytK***	**MF/UR**	**PR/UF**
Raw Milk	2	+	+	+	-	+	-
Pasteurised Milk	4	+	+	+	-	+	-
Filler Nozzles	33	+	+	+	-	+	-
Pasteurised Milk	2	+	+	+	+	+	-
Filler Nozzles	20	+	+	+	+	+	-
Filler Nozzles	22	-	+	+	-	+	-
Filler Nozzles	4	+	+	-	+	+	-
Filler Nozzles	2	-	+	-	-	+	+
ESL Milk	2	-	+	-	-	+	+
ESL Milk	4	-	+	-	+	+	+
Raw Milk	2	-	+	+	+	+	-
Pasteurised Milk	2	-	-	+	-	+	-
ATCC 10876	-	+	+	+	+	+	+

+ means positive, - means negative. * Total percentage is 99 % due to rounding off

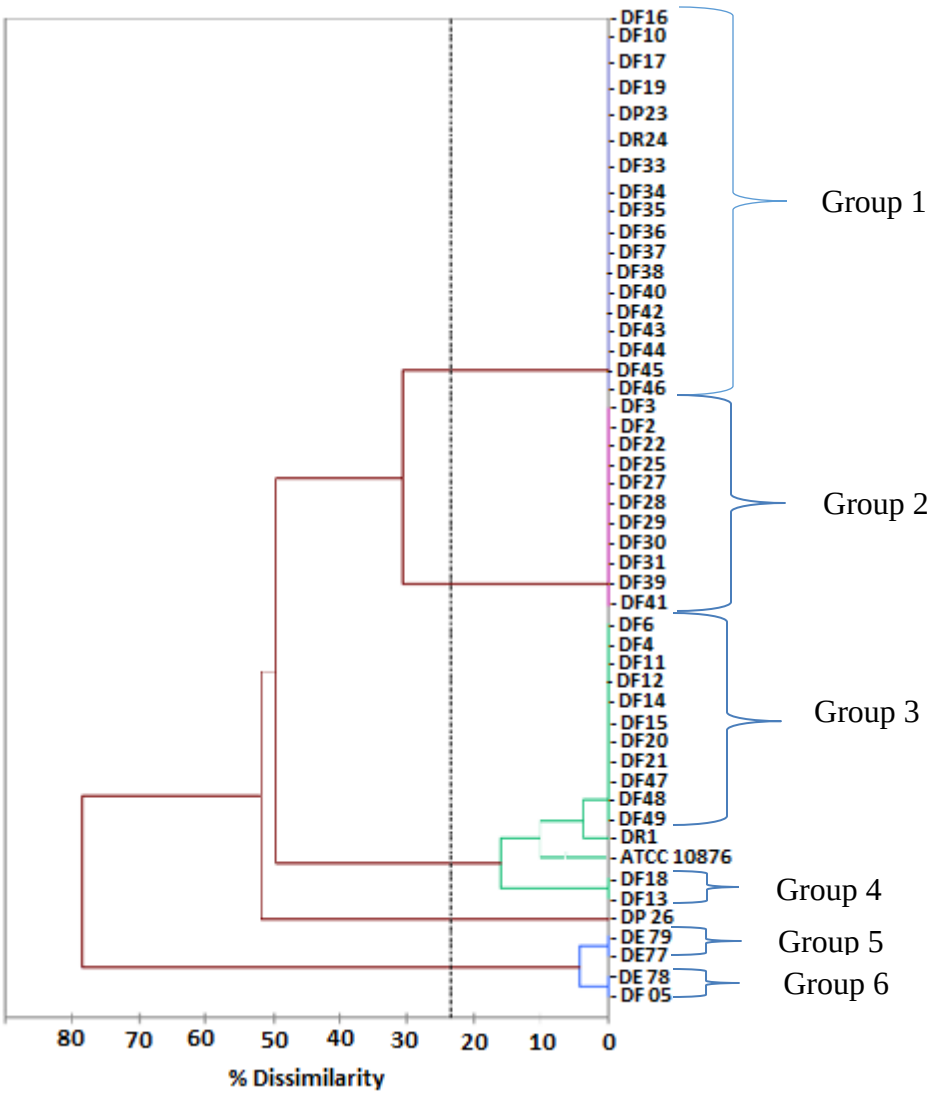

Figure 5.1.2: Agglomerative Hierarchical clustering of *B. cereus* isolates from ESL milk processing and during shelf life based on presence of virulent genes, 16S mesophilic and psychrotrophic signatures.

(Sources: DR – Raw milk, DF – Filler nozzles, DP – Pasteurised milk, DE – ESL milk)

5.1.4.3 *B. cereus* 16S Sequencing

While the isolates had 2 distinct clusters (Figure 5.1.4), the biggest cluster showed that seven isolates from filler nozzles, clustered together with two from ESL milk stored at 7 ^{o}C and one from pasteurised milk. Nine isolates out of the total 16 (56.25%) possessed a 16S rDNA sequence with 64% similarity to that of a previously characterised clinical and environmental bacterial species. The second cluster comprised of three isolates from filler nozzles, two from raw milk and one from pasteurised milk. The relatedness in this second cluster is high enough to prove that these isolates are the same strain. Although all the isolates did not show close relationship with previously characterized strains, 4 of them possessed a 16S rDNA sequence with ≥ 99% similarity.

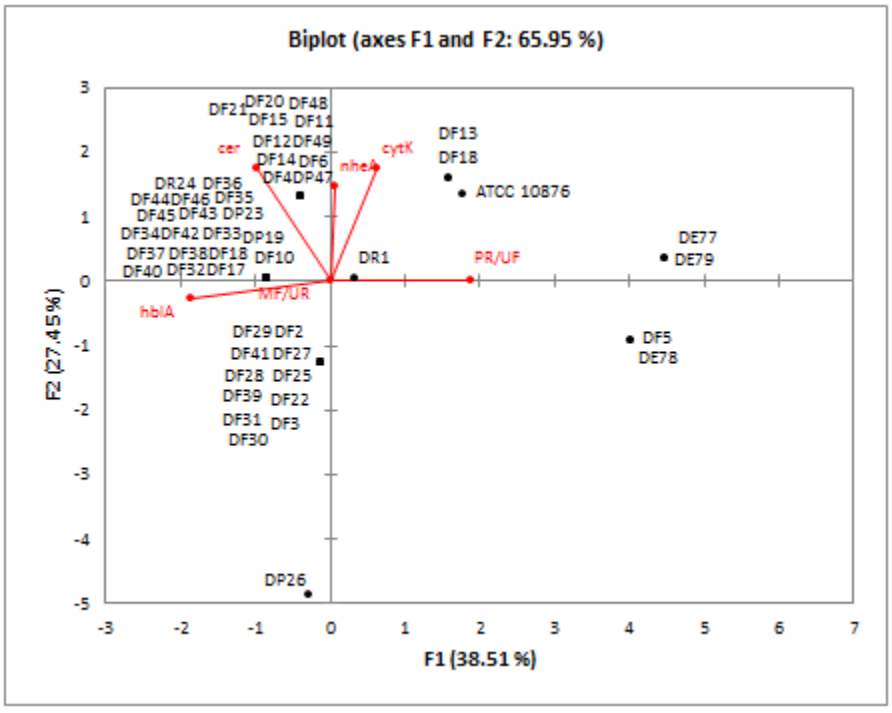

Figure 5.1.3: Principal component analysis of virulent genes, 16S mesophilic and psychrotrophic signature data set consisting of 49 *B. cereus* isolates obtained from ESL milk processing and during storage.

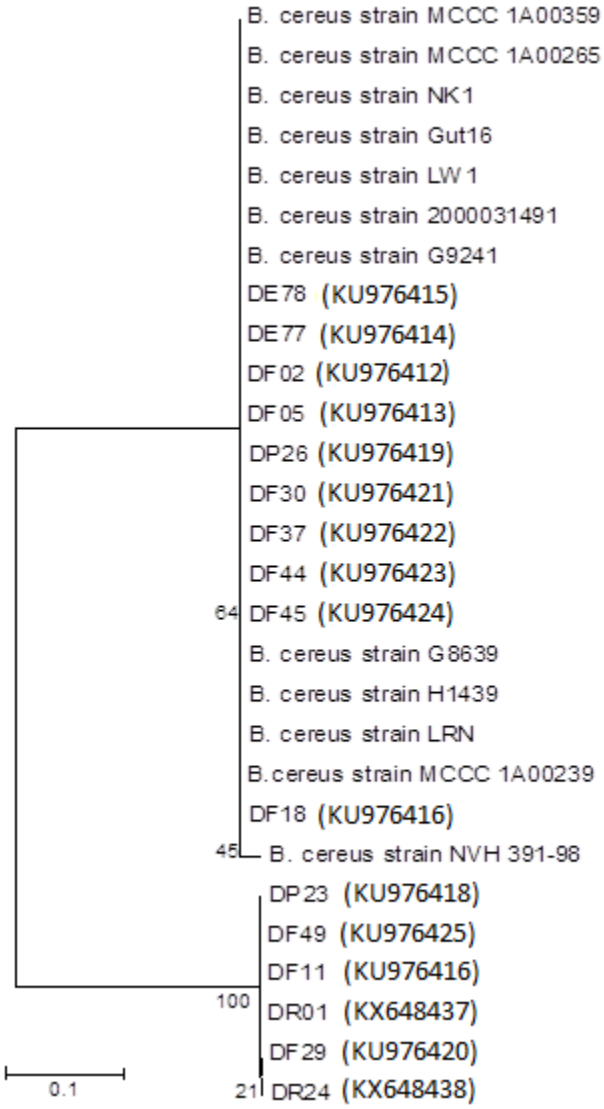

Figure 5.1.4: 16S rDNA sequence based maximum likelihood phylogenetic tree of *B. cereus* from ESL milk processing and during shelf life and other *B. cereus* strains.

*Gene Bank accession numbers are given in parentheses. (Sources: DR – Raw milk, DF – Filler nozzles, DP – Pasteurised milk, DE – ESL milk)

5.1.5 Discussion

In this present study the genetic diversity of *B. cereus* isolates from an ESL milk processing factory was determined. The results showed that there was relatedness amongst all isolates at 70% similarity level. Although this clustering does not mean the isolates belong to one strain, the similarity can be attributed to isolates belonging to the same species. Furthermore, as previously described (Aminov 2011; Böhm *et al*. 2015), evolution and transfer of genetic material through horizontal gene transfer may also have contributed to these similarities. Although the isolates under study belonged to the same cluster at 70%, they exhibited high levels of genetic polymorphism resulting in several sub clusters. These results were similar to previous studies on *B. cereus* (Helgason *et al*. 2000; Ehling-Schulz *et al*. 2005; Savic *et al*. 2015), that revealed high heterogeneity among the *B. cereus* isolates from milk environment. This suggests that ESL milk contamination is not exclusively from one source but rather a number of them including raw milk and processing equipment among others (Faille *et al.* 2001; Jan·Tová *et al.* 2004; Miller *et al*. 2015). These results were consistent with previous studies on *B. cereus* in pasteurised milk processing and farm environment that provided evidence for additional contamination of pasteurised milk in production lines (Christiansson *et al*. 1999; Svensson *et al*. 2000). Despite the high heterogeneity observed among sub-clusters, high similarities were also observed within sub-clusters on isolates from filler nozzles and from the milk. This confirms the hypothesis of raw milk being a source of contamination among many sources (Svensson *et al.* 2000; 2004). In

an earlier study, it was revealed that pasteurised milk and final products were contaminated by *B. cereus* strains that germinated from spores in raw milk (Lin *et al.* 1998). The presence of the similar isolates of *B. cereus* in raw milk, filler nozzles and commercially pasteurised milk samples, further confirms the role of raw milk and filling machines as sources of *B. cereus* contamination (Eneroth *et al*. 2001; Huck *et al*. 2007; Banyko´ and Vyletelova 2009).

B. cereus contaminants associated with food borne illness are reported to be mesophilic strains (Larsen and Jørgensen 1997; Francis *et al*. 1998; Stenfors and Granum 2001; Guinebretire *et al*. 2008). This shows that the risk posed by psychrotrophic *B. cereus* in food is obviously low. However, in the present trial psychrothrophic strains isolated from ESL milk contained some virulence genes. This difference could indicate that virulence genes are closely associated with mesophilic strains. Furthermore, the coexistence of mesophilic and psychrotophic signatures may have resulted in the presence of virulence genes in psychrotrophic strains. However, scientific evidence is needed to support this hypothesis.

The study also revealed that all *B. cereus* isolates linked to ESL milk contamination showed mesophilic characteristics. This was in line with a previous study that demonstrated a clustering of *B. cereus* strains from a dairy plant and suggested contamination caused by mesophilic *Bacillus* strains. It was concluded that contamination occurred early in the production chain probably in raw milk silos through biofilms (Svensson *et al*. 1999).

This study revealed that only 8% (4) isolates showed the presence of the 16S psychrotrophic signature. Similarly, a previous study reported that most *B. cereus* strains obtained from the food chain environment were mesophilic while a few exhibited psychrotrophic properties (Stenfors

and Granum 2001). Although previous studies have indicated that most food contaminants and virulent *B. cereus* are mesophilic strains (Larsen and Jørgensen 1997; Francis *et al*. 1998; Stenfors and Granum 2001; Guinebretire *et al*. 2008), this study revealed a contrary trend. This may be a result of the emergence of psychrotrophic *B. cereus* strains resulting from increased cold exposure to cold environments leading to increased adaptation and evolution (Guinebretire *et al*. 2008). This indicates that the most problematic strains in ESL milk are not psychrotrophic strains of *B. cereus* only but mesophilic that have psychrotrophic signatures or vice versa.

Contrary to a previous study which concluded that emetic toxin, cereulide producing *B. cereus* are rare in the environment (Altayar and Sutherland 2005) our results showed 65% prevalence of the emetic toxin producing *B. cereus*. However, in agreement with previous studies (Carlin *et al.* 2006; Hoton *et al*. 2009), all cereulide positive isolates were mesophilic. The increased presence of the *cer* gene is probably due to adaptation as most *cer*-containing strains usually propagate at low incidences in milk (Bartoszewicz *et al*. 2008). Furthermore, the fact that toxigenic determinants spread horizontally among *Bacillus* species (Bartoszewicz *et al.* 2008) may have contributed to increased *cer* prevalence. Although cereulide synthesis is known to occur at a temperature range of 12-37 ^{o}C (Finlay *et al.* 2000; Häggblom *et al*. 2002), depending on strain and media too (Apetroaie-Constantin *et al.* 2008) cold chain abuse of ESL milk product therefore puts consumers at risk of this emetic toxin.

The distribution of enterotoxin gene *hblA* did not follow a definitive trend. Nevertheless, it has been hypothesised that many food-borne strains of *B. cereus* are less prone to cause diarrhoea (in't Veld *et al*. 2001; Guinebretière and Broussolle 2002). The high prevalence of the

enterotoxin genes *hblA* and *nheA* in the total isolates and low prevalence of the genes in the final ESL milk product could be result of failure of the *hblA* and *nheA* possessing strains to withstand processing conditions and proliferate during cold storage (Hansen and Hendriksen 2001; Ehling-Schulz *et al*. 2006b; Bartoszewicz *et al*. 2008). The presence of the *hblA* does not directly translate to production of the toxin since other components are required to be present (in't Veld *et al*. 2001; Arnesen *et al.* 2008). However, the presence of this toxin determinant in ESL milk shows that consumers of cold-stored ESL milk product may be at risk of the diarrhoeal causing toxins.

Although *cytK* is regarded as highly toxic to humans (Hardy *et al.* Lund and Granum 2001; Fagerlund *et al*. 2004), it is not widely distributed in *B. cereus* (Lund *et al*. 2000; Stenfors and Granum 2001; Fagerlund *et al*. 2004; Swiecicka and Mahillon 2006). While the reason for low frequency of *cytK* in food related *B. cereus* isolates is not clear, a possible explanation could be the failure of *cytK* containing strains to propagate in the food matrix. However studies need to be done to prove this hypothesis. Despite the fact that enterotoxins are heat labile and prone to degradation by pH changes (Hansen and Hendriksen 2001) the presence of the *cytK* in psychrotolerant isolates from the ESL milk poses a potential safety threat to consumers.

5.1.6 Conclusion

The simultaneous presence of psychrotrophic, mesophilic and at least 2 virulence genes in some of the isolates shows large diversity of *B. cereus* strains in ESL milk process that does not follow any specific pattern. This shows that contamination of ESL milk with *B. cereus* follows several

routes. This study provided evidence that filler nozzles and raw milk are sources of contamination of ESL milk.

CHAPTER SIX

Source tracking *Bacillus cereus* in an extended shelf life milk processing plant using partial sequencing of *rpo*B and multilocus sequence typing

6.1.1 Abstract

Characterisation of seven *B. cereus* strains obtained from extended shelf life (ESL) milk stored at 7 ^{o}C, pasteurised milk, raw milk and filler nozzles after CIP was performed using *rpoB* partial sequencing and multilocus sequence typing. The objective of the study was to determine relatedness within *B. cereus* isolates from several sampling points with the aim of source tracking *B. cereus* in an ESL milk processing plant. The study revealed that some isolates from pasteurised milk shared 100% similarity with isolates from raw milk and ESL milk using *rpoB* sequencing. It also revealed very close similarity between isolates from filler nozzles and ESL milk using MLST. This study showed that *B. cereus* contamination of ESL milk is through raw milk and biofilms from filler nozzles. Furthermore, the study also proposes at least 3 routes of *B. cereus* contamination in ESL milk. In addition, the study also showed that *rpoB* partial sequencing and MLST can be used as tools for source tracking in ESL milk processing

6.1.2 Introduction

Despite advancement in preservation and processing technologies, *Bacillus cereus* remains a shelf life and consumer safety challenge in the dairy industry (Huck *et al*. 2007a; Ranieri *et al.* 2012; Aouadhi *et al.* 2014; Mugadza and Buys 2017a). *B. cereus* is a ubiquitous spore-forming bacterium (Ranieri *et al.* 2012; Aouadhi *et al*. 2014) that is reported to produce various toxins responsible for diarrhoeal and emetic food poison (Hansen and Hendriksen 2001; Arnesen *et al*. 2008; Bartoszewicz *et al*. 2008). Studies conducted at an ESL milk processing plant revealed the presence of psychrotrophic *B. cereus* in milk during processing as well as during storage and in filler nozzles after CIP (Khoza 2016; Mugadza and Buys 2017a). A follow up study on these *B.*

cereus strains then showed close relatedness between these strains using repPCR and 16S partial sequencing (Mugadza and Buys 2017b).

Furthermore, *B. cereus* endospores can germinate at refrigeration temperatures. In addition, some strains can also grow, as well as produce toxins under these refrigeration conditions (Larsen and Jørgensen 1997; Stenfors and Granum 2001; Thorsen *et al*. 2006). *B. cereus* has also been reported to be able to attach to stainless steel (Eneroth *et al*. 1998, 2001; Khoza 2016) the main material used in manufacturing processing equipment, while other studies have isolated *B. cereus* on the processing equipment, suggesting the potential to form biofilms which can later be responsible for post process contamination of processed milk (Eneroth *et al.* 1998, 2001).

While raw milk has been implicated as a source of *B. cereus* contamination in pasteurised milk (Huck *et al*. 2007a,b), post processing contamination by processing plant equipment has also been described (te Giffel *et al*. 1997; Svensson *et al*. 2000; Huck *et al*. 2007a,b). Identification of points of entry for these bacteria may allow the development of effective strategies for reducing or eliminating their presence in milk production systems (Huck *et al*. 2008).

While several methods have been suggested for source tracking (Fu and Li 2014), multilocus sequence typing (MLST) has been described as a source tracking tool that determines exact nucleotide differences for conserved loci (Cardazzo *et al*. 2008). MLST studies have previously been used to examine the phylogeny of the *B. cereus* complex (Helgason *et al*. 2000, 2004; Barker *et al*. 2005), identifying three distinct lineages that largely correspond to the species distribution (Cardazzo *et al*. 2008). In addition to MLST, partial sequencing of protein coding genes such as *rpoB* have also been successfully used to discriminate closely related species

difficult to distinguish with other methods that are based on the 16S rRNA (Adékambi *et al*. 2009; Jiménez *et al*. 2013).

The objective of this research was to source track *B. cereus* in an extended shelf life milk processing plant using partial sequencing of *rpoB* and multilocus sequence typing with the aim of determining route of *B. cereus* contamination in ESL milk.

6.1.3 Materials and methods

6.1.3.1 Bacteria strains and DNA preparation

Seven *B. cereus* isolates (BC7, BC8, BC5, BC29, BC23, BC26, BC24) obtained from ESL milk stored at 7 ^{o}C, pasteurised milk, raw milk as well as filler nozzles after CIP and described in previous studies (Khoza 2015; Mugadza and Buys 2017a), were selected for sequencing. The isolates were selected based on previously described $(GTG)_5$ fingerprints (Mugadza and Buys 2017b), ensuring representation of each sampling point. Description and characteristics of the isolates is in Table 6.1.1. Bacterial cultures were grown on nutrient agar at 30 ^{o}C for period of 16-24 h. DNA was extracted from the *B. cereus* isolates using the ZR Fungal/Bacterial DNA MiniPrep (California, USA) according to manufacturer's instructions for use in the PCR reactions.

Table 6.1.1: Description of virulence, mesophilic and psychrotrophic genes in *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life (Khoza 2016[*]; Mugadza and Buys 2017b)

Isolate	Source	Genes						% Adherence[*]	
		Cer	***nheA***	***HblA***	***cytK***	**MF/ UR**	**PR/ UF**	**Chloroform**	**Xylene**
BC7	ESL milk	-	+	-	+	+	+	80	90
BC8	ESL milk	-	+	-	-	+	+	80	90
BC5	Filler nozzles	-	+	-	-	+	+	80	90
BC29	Filler nozzles	-	+	+	-	+	-	80	90
BC23	Pasteurised milk	+	+	+	-	+	-	80	90
BC26	Pasteurised milk	-	-	+	-	+	-	80	90
BC24	Raw milk	+	+	+	-	+	-	80	90

(+) means present, (-) means absent

DNA Amplification and Sequencing

Protein coding gene *rpoB* together with 5 housekeeping genes; glycerol uptake facilitator protein (glpF), guanylate kinase, putative (gmk), dihydroxy-acid dehydratase (ilvD), phosphate acetyltransferase (pta) and phosphoribosylaminoimidazolecarboxamide (pur) distributed around the chromosome of *B. cereus* were chosen for partial sequencing and MLST respectively and amplified using a Bio-Rad T100 Thermal Cycler (Singapore, Singapore). Details of primers are described in Table 6.1.2. The reaction mixture consisted of 8 µL distilled PCR grade water, 1 µL

each of the forward and reverse primer, 10 µL PCR mix (containing dNTPs, buffer, $MgCl_2$ and Taq polymerase) 1 µL gDNA. PCR protocol was as follows; 95 ^oC for 5 min, 95 ^oC for 30 s, 50 ^oC for 30 s, 45 Cycles, 72 ^oC for 30 s, 72 ^oC for 10 min, 4 ^oC hold. The PCR product was cleaned using USB ExoSAP-IT PCR Product Cleanup (Singapore, Singapore) according to manufacturer's instructions. Gel electrophoresis was conducted using a 1% agarose gel (with 5 µl of EZ-Vision In-Gel solution (Solon, Ohio USA) for every 50 ml of melted agarose). PCR product was mixed with loading dye. The gel was run for 30 min at 100 V. DNA sequencing was done using Big Dye V3.1 as per manufacturer's instructions on the ABI 3500 XL with POP-7 and a 50 cm array.

Table 6.1.2: Sequence description and annealing temperatures for MLST and *rpoB* genes used for sequencing *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life.

Gene	Sequence	Annealing Temperature (^oC)
Glp-F	GCGTTTGTGCTGGTGTAAGT	59
Glp-R	CTGCAATCGGAAGGAAGAAG	59
Gmk-F	ATTTAAGTGAGGAAGGGTAGG	56
Gmk-R	GCAATGTTCACCAACCACAA	56
IlvD-F	CGGGGCAAACATTAAGAGAA	58
IlvD-R	GGTTCTGGTCGTTTCCATTC	58
Pta-F	GCAGAGCGTTTAGCAAAAGAA	58
Pta-R	TGCAATGCGAGTTGCTTCTA	58
Pur-F	CTGCTGCGAAAAATCACAAA	56
Pur-R	CTCACGATTCGCTGCAATAA	56
rpoB-F	CCGAACCGTTCCGCGAACATCGCGCTGG	50
rpoB-R	CCAGCAGATCCAGGCTCAGCTCCATGTT	50

(Miyoshi-Akiyama *et al*. 2013; www.mlst.net Accessed 30 May 2016)

***rpoB* gene Phylogenetic Analysis**

Sequence analysis was performed at Inqaba Biotechnologies, Pretoria, South Africa and the chromatograms from the ABI 3100 sequences were exported, visually examined and gene sequences were analyzed using FinchTV version 1.4.0 (Geospiza). The *rpoB* gene sequences were aligned using BIOEDIT version 7.2.1 (Hall 1999). Multiple sequence alignment was performed using Clustal Omega (EMBL-EBI, Hinxton). A phylogenetic tree was inferred using the neighbor-joining and maximum-likelihood algorithm in CLC Genomics workbench version 10.0.0 (Qiagen Bioinformatics, Aarhus). The strength of the internal branches of the phylogenetic tree was statistically estimated by bootstrap analysis of 1000 bootstrap replications.

MLST Data analysis

The chromatograms from the ABI 3100 sequences were exported, visually examined and gene sequences were edited using FinchTV version 1.4.0 (Geospiza). The sequences of the five housekeeping genes (*gmk, ilv, pur, pyc, tpi*) were edited to the previous allele lengths (between 348 and 504 bp). The genes sequences were then assigned allele numbers based on the already described alleles of *B. cereus* MLST database (http://www.pubmlst.org/cereus). Isolates were assigned sequence type (ST) based on the combination of 5 alleles. The five gene fragments of each of the 7 isolates were concatenated and downloaded from the MLST website. A phylogenetic tree was derived using the neighbor joining method, with 1000 step bootstrap analysis of the data using CLC Genomics Workbench version 10.0.0 (Qiagen Bioinformatics, Aarhus).

6.1.4 Results and discussion

MLST showed 75% similarities between 4 isolates from ESL milk (1), raw milk (2) and pasteurised milk (1). With the exception of 1 isolate from raw milk, the other 3 isolates showed 95% similarity while 2 of the 3 isolates (ESL milk and Pasteurised milk) showed 97% similarity. Two isolates (Filler nozzles and Pasteurised milk) showed 85% similarity while the other isolate from ESL milk did not show any close relatedness with any of the other isolates (Figure 6.1.1).

Figure 6.1.1: MLST profiles using five housekeeping alleles showing relationship among *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life.

These results show that *B. cereus* in ESL milk originated from raw milk among other sources. This is similar to previous studies that concluded raw milk as the major source of *B. cereus* contamination in milk products (Huck *et al*. 2007a,b). The *B. cereus* strain from raw milk was closely related to strains from pasteurised milk as well as ESL milk stored at 7 ^{o}C. This could mean that these strains are surviving from raw milk throughout the ESL milk process until they

get into the final product. *B. cereus* strains have successfully used endospores as a strategy to survive processing hurdles as compared to their vegetative forms (Lin *et al*. 2017).

MLST also showed relatedness between *B. cereus* isolates from filler nozzles and pasteurised milk suggesting possible post processing contamination by processing equipment. This is consistent with previous studies that showed the contribution of processing equipment to milk contamination (Svensson *et al*. 2000; Huck *et al*. 2007a,b). These results were also consistent with previous studies on *B. cereus* in pasteurised milk processing and farm environment that provided evidence for additional contamination of pasteurised milk in production lines, (Christiansson *et al.* 1999; Svensson *et al*. 2000). In addition to endospore formation some *B. cereus* strains also use biofilm formation together with resistance to acids and alkali as strategies of survival during CIP (Lin *et al*. 2017). The fact that one isolate from ESL milk did not cluster with any of the isolates under study shows genetic diversity of *B. cereus*. In addition, this may also show that contamination of ESL milk is from a diverse range of sources apart from raw milk and ESL milk filler nozzles. This is in line with previous studies which concluded that ESL milk contamination is not exclusively from one source but rather a number of them including raw milk and processing equipment among others, (Faille *et al.* 2001; Jan·Tová *et al.* 2004; Miller *et al*. 2015).

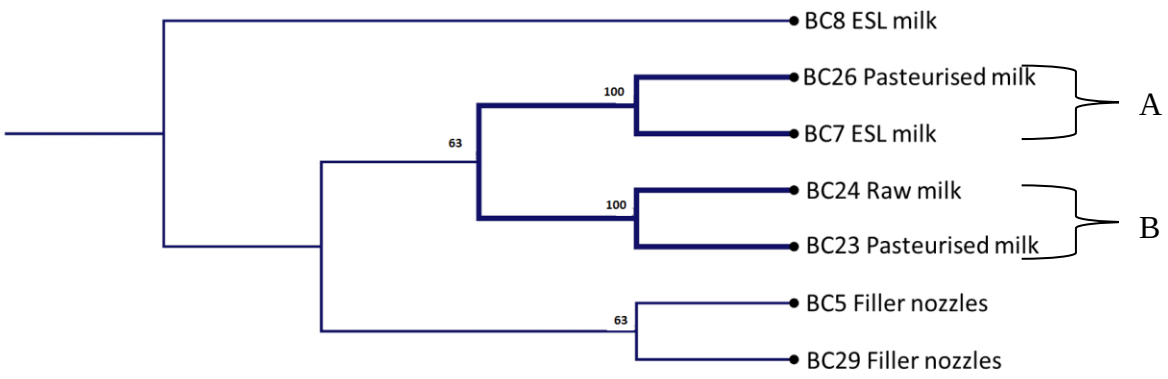

Figure 6.1.2: Neighbor-joining *rpoB* dendrogram representing the phylogenetic relationships of *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life.

The *rpoB* partial sequencing showed that 2 pairs of isolates with 100% similarity existed. Group A consisted of isolates from pasteurised milk and ESL milk stored at 7 ^{o}C while group B consisted of isolates from raw milk and pasteurised milk. Isolates from filler nozzles showed similarities at 64% (Figure 6.1.2). Partial sequencing of *rpoB* further confirmed that *B. cereus* raw milk and pasteurised milk contributed to ESL milk contamination. This route of transmission from raw milk to pasteurised products is consistent with previous studies tracking spore-forming microbial contaminants from raw milk to finished fluid milk products (Huck *et al*. 2007a, 2008). Although *rpoB* has high discriminatory power it is important to highlight that supplementary methods might be necessary for those species that cannot be delineated by sequence comparison of a single gene (Spanu *et al.* 2010).

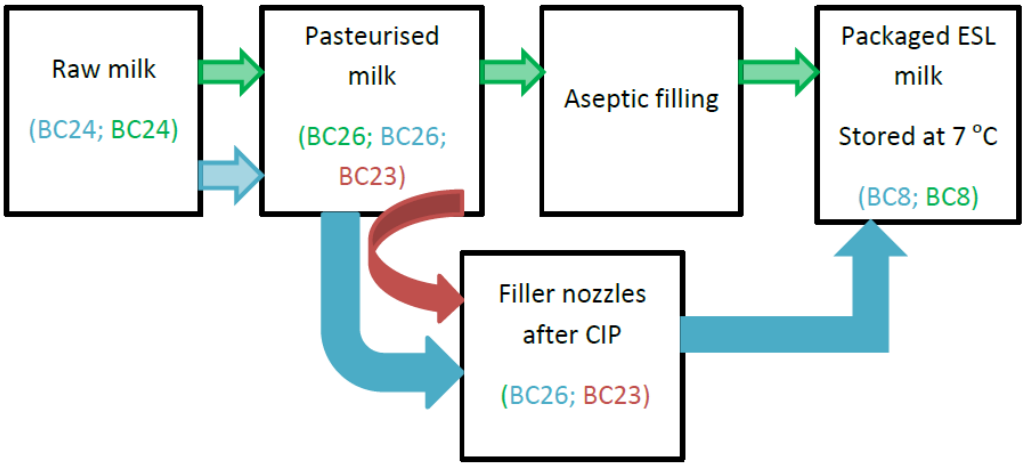

Figure 6.1.3: Routes and sources of ESL milk contamination by *B. cereus* in a processing plant based on similar MLST sequence type

Isolates of the same color belong to the same MLST sequence type and arrows of the same color represent a possible route of B. cereus contamination in ESL milk processing plant.

Despite showing relatedness using *rpoB* and MLST, isolates under study showed some variation in the presence of virulence genes (Table 6.1.1) as well the 16S psychrotrophic signature (Mugadza and Buys 2017b). Based on these results isolate BC7 possessed a 16S rDNA psychrotrophic signature and *nheA* gene that was missing in BC26. However, BC26 also possessed *hblA* which was lacking in BC7. If *rpoB* and MLST results are proving contamination then the difference in the virulence and psychrotrophic genes may be due to evolution, adaptation as well as horizontal gene transfer and deletion of some determinants (Bartoszewicz *et al*. 2008; Aminov 2011; Böhm *et al*. 2015), most likely over a long period of time in the presence of biofilms. Furthermore, previous studies have indicated that increased exposure to

cold environments leading to increased adaptation and evolution resulted in the emergence of psychrotrophic *B. cereus* strains (Guinebretire *et al*. 2008).

6.1.6 Conclusions

B. cereus contamination of ESL milk maybe through raw milk and biofilms associated with filler nozzles after CIP. The study also established three possible routes of *B. cereus* contamination in ESL milk processing as shown in Figure 6.1.3. However, further work will be needed using more isolates to get a more robust picture about these possible routes of *B. cereus* contamination in ESL milk. In addition, the study has also shown that *rpoB* partial sequencing and MLST can be used as a tool for source tracking in ESL milk processing.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 Methodological considerations

In this study spore-formers were isolated using a method previously described (Ivy *et al*. 2012) in which samples are preheated at 80 ºC for 10-12 min. This method is effective in reducing the background flora and vegetative cells that may outcompete spore-formers thus reducing isolation efficiency of spore-formers. *Bacillus* spp. is a non-competitive bacterium, hence they are difficult to isolate in the presence of non-spore-formers such as *Pseudomonas* spp. The isolation method has been successfully used in previous studies (Huck *et al*. 2007; Ranieri 2009; Ivy *et al*. 2012). While other scholars have used the filtration method (Svensson *et al*. 2003; Bartoszewicz *et al*. 2008) to successfully harvest spores, we believe the method we used was the best for the purpose of enumeration and further identification. The enumeration method was followed by grouping colonies according to colour, morphology and size. Representative colonies were purified by streaking on fresh nutrient agar. This technique has been used in previous studies (Fromm and Boor 2004; Ranieri 2009) and has proved to be a cost effective way of successfully screening isolates.

Purified isolates were confirmed as spore-forming bacteria by the Schaeffer-Fulton endospore staining method previously described (Schaeffer and Fulton 1933; Hussey and Zayaitz 2012). The method is based on the ability of a primary stain-malachite green to be forced into the endospore by steaming the bacterial emulsion.

Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells may be decolourized with water (Vasanthakumari 2009). Safranin is then applied to counterstain any cells which have been decolorized. At the end of the staining

process, vegetative cells will be pink, and endospores will be dark green. However, the only challenge of this stain is that when staining *Mycobacterium* the bacteria may look positive for endospores because some cells will stain green due to the its thick, wax coats (Hussey and Zayaitz 2012).

Isolates were further identified as Gram positives using the KOH method described in previous studies (Halebian *et al.* 1981; Buck 1982). This is a non-staining method that bases on the ability of Gram negative bacterial cell wall to dissolve with 3% KOH and not in Gram positive cell walls. Dissolved cell wall releases the intercellular material and the liberated cellular DNA makes the mixture viscous or "stringy." The positive string test indicates a Gram negative organism. This method has been shown to be effective in previous studies (Halebian *et al.* 1981; Buck 1982; Fromm and Boor 2004) and has time and cost advantages over the microscope based Gram staining method.

To aid identification of the isolates, their pathogenicity was also examined. This was done by determining haemolysis on 5% blood agar (Lucking *et al*. 2013). Blood agar, which is a mixture of tryptic soy agar and sheep blood, allows differentiation of bacteria based on their ability to haemolyse red blood cells. The haemolysin (extotoxin) radially diffuses outwards from the colony causing complete or partial destruction of the red cells in the medium and complete denaturation of haemoglobin within the cells to colourless products. Haemolysis method is semi-quantitative and there are three major types of haemolysis namely beta haemolysis, alpha haemolysis and gamma haemolysis (Sood 2006). Beta haemolysis is the complete destruction of red blood cells and haemoglobin, and results in a clearing of the medium around the colonies.

The method has been used successfully for *Bacillus* spp. (Fromm and Boor 2004; Lucking *et al*. 2013).

Enzymes activity was also used in the identification process. Catalase test that determines the ability to degrade H_2O_2 was done following procedures in previous studies (Koneman 2006). Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites such as H_2O_2. The catalase enzyme neutralises the bactericidal effects of H_2O_2 and protects them. Anaerobes generally lack the catalase enzyme (Góth *et al*. 2004). Another enzyme that was used for identification as well as to study the potential of the isolates to participate in ESL milk spoilage was lipase. Lipase is an exoenzyme which allows the organisms that produce it to break down lipids into smaller fragments of triglycerides, fatty acids and glycerol (Mobarak-Qamsari *et al*. 2011). The tributyrin agar based method was used as previously described (Lucking *et al*. 2013). Despite giving a true indication of the isolates with regards to lipase the method, however, cannot quantify the amount and rate at which lipid hydrolysis can occur in a milk product. Several methods have since been developed for quantification purposes (Thomson *et al*. 1999). However, the method applied in this study was enough to fulfil the objective. Following lipase activity of the isolates was determination of the protease activity in the isolates as described by Lucking *et al*. (2013). The protease under study was casease an exoenzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk. This test is conducted on milk agar which is a complex media containing casien, peptone and beef extract. If an organism can produce casein, then there will be a zone of clearing

around the bacterial growth. Similar to the lipase activity test, the casease activity test is qualitative.

MALDI-TOF-MS was used to characterise and identify the genus and species of the isolates (Drevinek *et al*. 2012). MALDI-TOF-MS has been introduced in bacterial taxonomy (Van Baar 2000; Fenselau and Demirev 2001; Lay and Liyanage 2006) and successfully applied to a number of taxa (Vargha *et al*. 2006; Barbuddhe *et al*. 2008; Grosse-Herrenthey *et al*. 2008). It has the ability to measure peptides and other compounds in the presence of salts and to analyze complex peptide mixtures, making it an ideal method for measuring non-purified extracts and intact bacterial cells (De Bruyne *et al*. 2011). MALDI-TOF-MS relies on proteomic profiling of highly conserved proteins generated from direct ionization of a colony of intact organisms or bacterial protein extract, and correlates this spectral signature to a database of spectra collected from reference strains (Kaleta *et al*. 2011). Different experimental factors, including sample preparation, cell lysis method, matrix solutions and organic solvents, affect the quality and reproducibility of bacterial MALDI-TOF-MS fingerprints (Ruelle *et al*. 2004; Vargha *et al*. 2006; Jaskolla *et al*. 2011). Also, differences in instrumental performance, mass range, and mass resolution have profound effects on the obtained spectra (Wunschel *et al*. 2005; De Bruyne *et al*. 2011). Although MALDI-TOF-MS has proved to be an effective identification tool that has been used to distinguish different strains of *Escherichia coli* (Holland *et al.* 1999) and also to identify rare bacterial species implicated in human infectious diseases (Seng *et al.* 2013), for very accurate results it must be accompanied by traditional identification methods such as biochemical tests and API system among other as other studies have noted disparities of the

system on *Staphylococcus aureus* (Risch *et al*. 2010). The system has the potential to establish a complementary tool for phenotypic analysis of microbial taxa, especially for physiologically inert species such as *Porphyromonas*, *Acinetobacter*, *Kingella*, *Moraxella* and those such as *Bacillus* species that are difficult to separate by rDNA analysis due to their evolutionary relatedness (Shah *et al*. 2002; Keys *et al*. 2004). A further advantage of the system is that only a loopful of cells is needed for MALDI-TOF-MS analysis and the profile is generated in minutes with minimal consumables, cost and operational manipulation (Keys *et al*. 2004). The power of MALDI-TOF-MS is also inseparable to its database hence it is important to create a microbial database and standardise analysis protocols and pattern matching systems (Keys *et al*. 2004).

Pathogenicity of *B. cereus* was done by determining the presence of virulence genes. While *B. cereus* has been linked to several toxins (Arnesen *et al*. 2008), in this study we focused on *cytK, cer, hblA and nheA* that are described in a previous study (Swiecicka and Mahillon 2006). *Cer* is the emetic toxin responsible for vomiting (Arnesen *et al*. 2008). Various methods have been used to detect *cer* including; monkey feeding (Melling *et al*. 1976), cell culture assays (Hughes *et al*. 1988; Sakurai *et al*. 1994; Finlay *et al*. 1999), boar sperm biological assay (Hoornstra *et al*. 2003; Anderson *et al*. 2004) and rat liver utilisation (Kawamura-Sato *et al*. 2005). These methods however, do not specifically detect *cer* (Arnesen *et al*. 2008), hence the introduction of the laborious and costly HPLC-MS (Häggblom *et al*. 2002). Since the genes encoding the production of *cer* are restricted to emetic toxin producing strains (Ehling-Shulz *et al.* 2005; 2006) PCR method has become relevant and ideal for identifying potential harmful strains. The PCR method

used in this study has been used with success in previous studies (Ehling-Shulz *et al*. 2004; 2005; Swiecicka and Mahillon 2006; Fricker *et al*. 2007)

The three toxins that have mainly been implicated with the diarrheal disease are the pore-forming cytotoxin haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (*cytK*) (Lund *et al*. 2000; Arnesen *et al*. 2008). Although antibodies based detection kits have been developed for detection of the three-component toxins Nhe and Hbl (Buchanan and Schultz 1994; Day *et al*. 1994) none of the methods will confirm an active toxin as only one of the three components of the toxin is targeted. Currently there is no antibody based assay for the *cytK* (Arnesen *et al*. 2008). For non-specific detection and characterisation of *B. cereus* enterotoxins different laboratory animal and tissue assays have been developed (Buchanan and Schultz 1994; Hardy *et al*. 2001; Fletcher and Logan 1999; From *et al.* 2005). Although the detection of an encoding gene does not translate to expression or production of the toxin PCR is still widely used to give an indication of potential hazard in food. Several PCR assays for enterotoxins have been developed and have been useful in identifying potential food safety threat (Hansen and Hendriksen 2001; Guinebretière *et al*. 2002; Ehling-Shulz *et al*. 2005). In this study we chose similar PCR assay that were previously described (Hansen and Hendriksen 2001; Fagerlund *et al.* 2004; Swiecicka and Mahillon 2006) because it is the best method available to accurately indicate potential food safety threat of *B. cereus* regarding enterotoxins.

To discriminate between psychrotrophic and mesophilic *B. cereus,* a PCR assay that targets the 16S signature (von Stetten *et al.* 1998) was used. Studies have indicated that ribosomes (Condon *et al.* 1995; Graumann *et al.* 1996) and major cold shock proteins play a role in cold adaptation

of bacteria. In addition traditional microbiology relied on growth at 7 ^{o}C for detecting psychrotolerant strains (von von Stetten *et al*. 1998) which may take up to 14 days to be detected (Meer *et al*. 1991). PCR based assays have been developed targeting the cold shock protein *cspA* (Francis *et al*. 1998) and the 16S signature (von Stetten *et al.* 1998). Although the presence of a psychrotrophic 16S signature does not necessarily translate to phenotypic expression, it however gives an indication of what potential the strain has when subjected to favourable environmental conditions.

In this study rep-PCR was done using $(GTG)_5$ primer because it is an easy to perform method basing on primers complementary to certain repetitive sequences dispersed in bacterial genomes (Lupski and Weinstock 1992). In addition PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with a high discriminatory power, low cost, suitable for a high-throughput of strains, and considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Olive and Bean 1999; Gevers *et al*. 2001; Švec *et al*. 2008). Apart from the labour intensive techniques of discrimination such as 16S sequencing, randomly amplified polymorphic DNA (RAPD) fingerprinting is by far the most used PCR-based genomic technique for identification of bacteria (Khaled *et al*. 1997; Gevers *et al*. 2001). However, RAPD primers are not directed against a particular genetic locus, the resulting band patterns often exhibit poor reproducibility (Olive and Bean 1999) hence $(GTG)_5$ becomes a better method. It is frequently used in bacterial taxonomy and has been successfully applied for reliable and fast identification of different bacterial groups such as *Lactobacilli* (Gevers *et al*. 2001), *Staphylococci* (Wieser and

Busse 2000), *Mycobacteria* (Cangelosi *et al.* 2004), *Streptomycetes* (Lanoot *et al*. 2004) and *S. mutans* (Švec *et al*. 2008). Although the homogeneity of *B. licheniformis* in previous studies basing on $(GTG)_5$ raised questions on the discriminatory power of $(GTG)_5$-PCR (De Clerck and De Vos 2003), another study showed that it is a high discriminatory tool in *B. cereus* (Samapundo *et al*. 2011).

16S rDNA partial sequencing was used to confirm the identity of *B. cereus* as well as determine the relatedness among isolates from filler nozzles and ESL milk processing. Although it has been emphasised that 16S rDNA data may not be useful in defining species and strains especially in *B. cereus* group (Fox *et al*. 1992), it is still, in most instances, a powerful tool for determining to which species a strain probably belongs once the relevant species are represented in the 16S rDNA sequence data base. This molecular approach was used as it has been extensively used with success for bacterial phylogeny (Woese *et al*. 1990), leading to the establishment of large public domain databases (Maidack *et al*. 1996; Drancourt *et al*. 2000) and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms (Relman *et al*. 1992; Strous *et al*. 1999), unique or unusual isolates and collections of phenotypically identified isolates (Tang *et al.* 1998, 2000; Drancourt *et al*. 2000). 16S rDNA based molecular identification could achieve identification, for reasons including its universal distribution among bacteria and the presence of species-specific variable regions. In a previous study the overall performance of 16S rDNA sequence analysis proved excellent, since it was able to resolve almost 90% of identifications, when applied to a large collection of phenotypically unidentifiable bacterial isolates (Drancourt *et al*. 2000). Although the downside of this technique

is failure to discriminate between species of the *B. cereus* group, it was useful in confirming the identity as well as determining the relatedness of the isolates under study. A previous study found the 16S rDNAs of *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis* to have almost complete sequence identity and only two differences were found in the 23S rDNAs of *B. anthracis* and *B. cereus* (Ash *et al.* 1991).

Sequencing the protein coding gene *rpoB* was done as a way of confirming the identity of *B. cereus* isolates as well as source tracking the organism. While 16S rDNA gene sequencing has been used as a framework for modern bacterial classification, it often shows limited variation for members of closely related taxa (Fox *et al*. 1992; Janda and Abbort 2007). The differences that do exist among *B. anthracis*, *B. cereus*, and *B. thuringiensis* are due largely to the presence of plasmids (McDowell and Mann 1991; Ombui *et al*. 1996; Yang *et al*. 2003). However, plasmids may be lost, making it difficult to differentiate rapidly among species (Hurtle *et al*. 2004). On the other hand, protein-coding genes exhibit much higher genetic variation, which can be used for classification and identification of closely related taxa (Mollet *et al*. 1997; Kim *et al*. 1999; Yamamoto *et al*. 1999; Chun and Bae 2000). The gene for the RNA polymerase beta subunit, *rpoB*, is suggested to be used as an alternative to 16S rDNA in species identification as it provides a much more efficient tool for molecular subtype analysis (Rantsiou *et al*. 2004; Huck *et al*. 2008; Ki *et al*. 2009). The *rpoB* gene has been reported to provide improved phylogenetic resolution over the 16S rDNA gene. This is essential when studying subspecies diversity in which analysis of the *rpoB* single copy gene could be used to complement the information gathered from the 16S rDNA gene (Adékambi *et al*. 2009). Several studies have indicated that

rpoB gene can be successfully used for identification at the species or subspecies level of bacteria (Adékambi *et al*. 2003; Khamis *et al*. 2009). This was therefore one of the best methods to use in this research.

To assist in source tracking, *B. cereus* was analysed using the multilocus sequencing technique that focuses on sequencing housekeeping genes (Urwin and Maiden 2003). It is based on concepts of multilocus enzyme electrophoresis (MLEE) and has adapted them so that alleles at each locus are defined directly, by nucleotide sequencing, rather than indirectly from the electrophoretic mobility of their gene products (http://www.mlst.net). The aim of MLST is to provide a portable, accurate, and highly discriminating typing system that can be used for most bacteria and some other organisms (Maiden 2006). Most MLST schemes are based on housekeeping genes, which are subject to purifying selection and slow evolution, and the variation within these genes is nearly neutral (Aanensen and Spratt 2005). Although there are normally fewer polymorphic sites in individual housekeeping genes compared with hyper variable genes, using the combined sequences of multiple housekeeping genes has been shown to provide high discriminatory power while retaining signatures of longer-term evolutionary relationships or clonal stability (Urwin and Maiden 2003; Pérez-Losada *et al.* 2013). Furthermore, analyses of multiple loci can buffer against potentially skewed evolutionary pictures obtained by single-locus analyses (Enright and Spratt 1999). Current applications of MLST include examination of the evolution of virulence, population and evolutionary analyses and isolate characterization and population structure analyses (Maiden 2006). Bacterial subtyping methods not only improve our ability to detect and track pathogen outbreaks, but also

provide tools to track sources of pathogens contamination throughout the food system. It also provides an opportunity to better understand the population genetics, epidemiology, and ecology of bacteria (Enright and Spratt 1999; Pérez-Losada *et al.* 2013). These features of MLST make it relevant to this section of the study where the objective was to source track *B. cereus* in an ESL milk processing plant. MLST has been used for a number of bacteria that includes *B. cereus*, (Helgason *et al*. 2004), *Staphylococcus* spp. (Thomas *et al*. 2007; Enright *et al*. 2000), *E. coli* (Gordon *et al*. 2008), *Streptococcus* spp. (Ahmad *et al*. 2009) and *Salmonella* spp. (Kotetishvili *et al*. 2002) among others.

7.2 Isolation of spore-formers in ESL milk and characterisation of *B. cereus* from ESL milk processing and during storage

The ESL milk process under study reduced vegetative bacterial counts significantly. This was also aided by the fact that the raw milk used also showed satisfactory microbial quality according to most international dairy standards (Marth and Steele 2001). Previous studies indicate that pasteurisation temperatures are sufficient to completely destroy vegetative bacteria if raw milk has good microbial quality (Ranieri and Boor 2009; Tammine 2009). Apart from reducing the vegetative population the process also reduced the spore-former population before packaging. The reduction of spore-forming bacteria is a combination of the pasteurisation effect on their vegetative form as well as bactofugation. Bactofugation has proven to be effective in reducing spore-formers and is believed to reduce 90-99% of bacterial cells and clostridal spores which cause late blowing of Swiss cheese (Fox and McSweeney 1998; Faccia *et al*. 2013). When isolates were identified by MALDI-TOF-MS it showed that apart from reducing the counts, the

process also reduced the diversity of organisms. This can be attributed to the inability of other spore-formers to continue with normal metabolic processes under cold storage. Previous studies have indicated that the ability to produce cold shock proteins (CSPs) enables organisms to protect themselves from cold conditions and hence continue with normal metabolic processes (Francis *et al*. 1998). Another study has also indicated that the presence of the psychrotrophic 16S signature plays a similar role too (von Stetten *et al*. 1998).

Although *B. pumilus* has not been reported as a psychrotroph, its dominance in the ESL milk process as well as during storage can be attributed to its resistance to a number of stresses including UV light exposure and presence of oxidisers such as H_2O_2 among others as well as the ubiquitous nature of its endospore (Benardini *et al.* 2003; Link *et al*. 2004; Dickinson *et al*. 2004). The dominance of *B. pumilus* even in ESL milk stored at 4 ^{o}C suggests possible presence of psychrotrophic features within the species. Previous studies have indicated the presence of mesophilic and psychrotrophic 16S signatures, a property which may allow mesophilic organisms to adapt well at low temperatures. Although no horizontal gene transfer has been reported in that regard it however, cannot be ruled out as a possibility. The presence *of Paenibacillus* spp. is in line with previous studies that have described it as a psychrotrophic microaerophile that dominate the spore-former population at the end of shelf life (Owayss 2007; Ranieri *et al*. 2012). *Paenibacillus* spp. had the highest growth rate compared to its *Bacillus* spp. competitors when inoculated as single organisms in sterile milk and it also dominated the cocktail composition probably because of its ability to be competitive against accompanying microbiota at refrigerated conditions. *Paenibacillus* spp. is also reported to require L-tryosine as one of its spore germinants (Alvarado *et al.* 2012), while the proteolytic activity of *B. cereus*

increases the amount of free tryosine in milk (Jan·tová 2006), thus aiding the growth of *Paenibacillus* spp.

Although the presence of both proteolytic and lipolytic enzymes does not directly translate to the ability to spoil milk, it is still an indication of the potential to cause spoilage, considering that more than 70% of isolates under study were proteolytic. *Bacillus* spp. and *Paenibacillus* spp. have been implicated as the major hurdles to shelf life extension of milk because of their ability to participate in hydrolysis of milk components such as lipids and proteins at refrigeration temperatures, (Boor *et al*. 1998; De Jonghe *et al.* 2010). *B. pumilus* has greatest potential to cause spoilage because it also had the highest percentage of isolates that were positive for proteolytic activity apart from dominating the spore-forming bacteria population in the whole ESL milk processes and during cold storage. While all *B. cereus* strains exhibited mesophilic characteristics by showing the presence of the 16S mesophilic signature the psychrotrophic signature was not widely distributed. This shows that *B. cereus* spoilage and possibly poisoning will greatly depend on storage practices exercised on the ESL milk.

Although the *B. cereus* population was low in this study, it remains a relevant bacterium in ESL milk as it showed the ability to hydrolyse casein as well as lipids. Furthermore, it was the only spore-former to be positive for haemolysis, exhibiting β haemolysis on 5% sheep blood agar. This shows the organism has the ability to produce toxins that target the red blood cells. However, a previous study has shown that the production of toxins is not only dependent on the strain but also the substrate and temperature among others (Apetroaie-Constantin *et al.* 2008). PCR results revealed that 3 toxin production genes were present in $\geq$ 65% of the *B. cereus* under study; these results confirmed the previously described potential of *B. cereus* to cause

gastrointestinal diseases (Hansen and Hendriksen 2001; Guinebretière and Broussolle 2002; Ehling-Schulz *et al*. 2006b). Our results concurred with previous studies that reported that all cereulide positive isolates were mesophilic although a recent study has shown that 2 isolates belonging to the psychrotolerant species *B. weihenstephanensis* were able to produce cereulide at 8 ^{o}C, (Thorsen *et al.* 2006). Contrary to previous reports which hypothesise that many food-borne strains of *B. cereus* are less prone to cause diarrhoea (Guinebretière and Broussolle 2002), our study showed high prevalence of the enterotoxin genes *hblA* and *nheA* showing that the ESL milk product consumers maybe at potential risk of the diarrheal causing toxins. Although *cytK* is not widley distributed in *B. cereus* despite being regarded as highly toxic to humans (Hardy *et al*. 2001; Fagerlund *et al*. 2004), it still remains important as this was present in psychrotophic strains too.

The increase in the spore-former population after packaging can be attributed to post-process contamination. While raw milk has always been implicated as the major source of contamination in modern dairy processing (Miller *et al*. 2015), research has also shown that processing equipment also contribute (Eneroth *et al.* 2001). MALDI-TOF-MS dendrograms showed high similarities within species (*B. pumilus*, *B. subtilis*, *B. cereus* and *Paenibacillus* spp.) that suggested ESL milk contamination from both raw milk and ESL milk filler nozzles. Further analysis on *B. cereus* using rep-PCR fingerprinting, 16S rDNA and *rpoB* partial sequencing as well as MLST confirmed the role of biofilms from ESL milk filler nozzles on contamination of ESL milk. This was in line with previous studies that suggested that milk contamination is not exclusively from one source but rather a number of them including raw milk and processing equipment among others, (Faille *et al*. 2001; Jan·Tová *et al.* 2004; Miller *et al*. 2015).

7.3 Future research

The study determined the dominating spore forming bacteria in ESL milk processing and during storage. Although *B. pumilus* showed the highest prevalence throughout the process and during storage followed by *B. subtilis* no further characterisation was done on the organisms to ascertain their contribution to ESL milk spoilage. It is therefore necessary to characterise *B. pumilus* and *B. subtilis* both at the genetic and phenotypic levels. Characterisation techniques to be used should also have potential to source-track the organisms as well as determine their psychrotrophic characteristics because some of the isolates were enumerated in milk stored at 4 and 7 ^{o}C. MALDI-TOF-MS revealed a close relationship between isolates from ESL milk process, during storage and ESL milk filler nozzles. The potential of *B. pumilus* and *B. subtilis* to attach and form biofilms on milking equipment that subsequently contaminate ESL milk must be investigated.

The presence of potentially psychrotrophic *B. cereus* shows that ESL milk can be a potential threat to consumers. It is therefore necessary to conduct a quantitative risk analysis of *B. cereus* in ESL milk so as to determine the level of risk posed by the product to consumers. Although source tracking of *B. cereus* was conducted within the ESL milk processing plant, it is necessary to use all the *B. cereus* obtained from this plant to have a better understanding of the contamination sources in this ESL milk processing plant. *Paenibacillus* spp. did not dominate the organisms isolated in the ESL milk process under study; nevertheless, it showed the highest growth rate and also outnumbered other major spore-formers when inoculated in sterile milk as a cocktail of microorganisms in approximately equal quantities. It is therefore ideal to study the

mechanism that allows it to outnumber other spore-formers as milk ages as well as determining its ability to participate in biofilm formation and subsequently spoil milk.

The spore-formers isolated in this ESL milk process under study do not exist in isolation but as a community of microorganisms. While filler nozzles have been shown to participate in contamination and most studies have focussed on attachment of single organisms on processing equipment, it is also important to study the attachment and possible biofilm formation of these spore-formers as a community as well as their release rate at any particular time.

CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATIONS

This study showed that apart from reducing the spore-forming population the ESL milk process reduces the diversity of spore-forming species. It also showed that *B. pumilus* is the dominating spore-former under study as well as during storage at 4 and 7 ^{o}C. While *Paenibacillus* spp. was isolated in very low quantities both in ESL processing and during storage it was also revealed that it dominates the spore-formers as milk ages during storage if the initial concentration is equal to other spore-formers.

B. pumilus, B. subtilis, B. cereus and *Paenibacillus* spp. strains in this study possessed at least one hydrolytic enzyme showing potential to cause spoilage of ESL milk. Although isolated in low levels, *B. cereus* is a safety threat as all isolates showed ability to participate in haemolysis and also contained at least one virulence gene. Although all *B. cereus* isolates were mesophilic, the presence of a psychrotrophic signature in some isolates further increases the safety threat of *B. cereus* in ESL milk

Despite MALDI-TOF-MS analysis showing great diversity within each spore-forming species, very close relatedness existed within each species especially in *B. pumilus* as well as *B. cereus.* A further repPCR based analysis on *B. cereus* isolates showed great diversity, as well as a very close relationship among isolates. The relatedness among isolates suggests that both raw milk and filler nozzles are contaminating ESL milk product with *B. cereus*.

Partial sequencing of *rpoB* and 16S rDNA as well as MLST further confirmed that raw milk and filler nozzles are contaminating ESL milk. The study also established three possible routes of *B. cereus* contamination in ESL milk. Furthermore, the study showed that MLST and *rpoB* can be used for source tracking spore-formers in ESL milk processing.

CHAPTER NINE

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CHAPTER TEN

PUBLICATIONS AND AWARDS

10.1 Peer reviewed journal articles

Mugadza, D.T. and Buys, E.M. 2017. *Bacillus* and *Paenibacillus* spp. associated with extended shelf life milk during processing and storage. *International Journal of Dairy Technology*,

Mugadza, D.T. and E.M. Buys. 2017. Diversity of *Bacillus cereus* strains in extended shelf life milk. *International Dairy Journal*, 73, 144-150

10.2 Popular publications

Mugadza, D.T. and Buys, E.M. 2014. Bacteria Diversity in ESL Milk. Dairy mail, September 2014, p 113

Mugadza, D.T and Buys, E.M. 2017. It's time to *B. cereus*. Dairy mail, August 2017, p 117

10.3 Conference presentations

10.3.1 Oral presentations

Mugadza, D.T. and Buys, E.M. 2017. Source tracking of *Bacillus cereus* in an extended shelf life (ESL) milk processing factory. South African Society of Dairy Technology (SASDT) 48th Annual AGM and Symposium. Kievits Kroon, Pretoria, South Africa. 8-11 May 2017.

Mugadza, D.T. and Buys, E.M. 2015. A Risk Assessment of *Bacillus cereus* extended shelf life milk, 21st SAAFoST International Congress and Exhibition. Durban, South Africa. 7-9 September 2015.

Mugadza, D.T. and Buys, E.M. 2015. Spoilage potential of *Bacillus* spp. & *Paenibacillus* spp. in extended shelf life milk, South African Society of Dairy Technology (SASDT) 48th Annual AGM and Symposium. Cape St Francis, South Africa. 23-24 April 2015.

Mugadza, D.T. and Buys, E.M. 2014. Spore-formers and non-spore-formers associated with extended shelf life milk, ASSAf Annual South African Young Scientists Conference. Pretoria, South Africa. 14-16 October 2014.

Mugadza, D.T. and Buys, E.M. 2014. Spore-formers and non-spore formers in extended shelf life milk, South African Society of Dairy Technology (SASDT) 47th Annual AGM and Symposium. Cape town, South Africa. 23-24 April 2014.

10.3.2 Poster presentations

Mugadza, D.T. and Buys, E.M. 2016. A Quantitative risk Assessment of *Bacillus cereus* in extended shelf life milk, 25th International ICFMH conference - FOOD MICRO 2016. Dublin, Ireland. 19-22 July 2016.

Mugadza, D.T. and Buys, E.M. 2015. *Bacillus* and *Paenibacillus* spp. associated with extended shelf life milk, International Dairy Federation (IDF) World Dairy Summit. Vilnius, Lithuania. 20-24 September 2015.

Mugadza, D.T. and Buys, E.M. 2015. Characterisation of *Bacillus cereus* in extended shelf life milk, International Association for Food Protection Annual Meeting, Portland, Oregon. 25-28 July 2015.

Mugadza, D.T. and Buys, E.M. 2014. '*Bacillus* spp. and *Paenibacillus* spp. associated with extend shelf life (ESL) milk, 24th International ICFMH conference - FOOD MICRO 2014. Nantes, France. 1-4 September 2014.

Mugadza, D.T. and Buys, E.M. 2013. Characterisation of *Bacillus* spp. and *Paenibacillus* spp. in extended shelf life milk, South African Association for Food Science and Technology (SAAFoST) 20th Biennial International Congress. Pretoria, South Africa. 7-9 October 2013.

10.4 Awards

3rd Best presenter, South African Society of Dairy Technology (SASDT) student night presentations. University of Pretoria 25 August 2016

International Association for Food Protection student travel scholarship. International Association for Food Protection Annual meeting 2015. Portland, Oregon, USA

Best presenter, South African Society of Dairy Technology (SASDT) student night presentations. 14 August 2014. University of Pretoria

ICFMH Food microbiologist travel grant – Food Micro conference 2014. Nantes, France.