Vaal University of Technology

IDENTIFICATION OF THE DOMINANT BACTERIA ASSOCIATED WITH THE SPOILAGE OF UHT FULL CREAM MILK

by

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DEDICATION

Dedicated to

My wonderful Mother (Ramokone Fredricka),

Whose love, support and perseverance

Show no boundaries.

My amazing life partner and husband (Esau Jonathan Daka),

Your love, support and encouragement are limitless.

My children (Boikanyo and Bonisile),

You are the definition of life for me.

DECLARATION

I declare that this dissertation is my own unaided work, except where specific acknowledgement is made in the form of a reference. The dissertation is being submitted for the degree of Magister Technologiae in the Department of Biotechnology, in the Faculty of Applied Sciences at the Vaal University of Technology, Vanderbijlpark. It has not been submitted before for any examination.

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ABSTRACT

The Organization for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations predict that milk production and the dairy sector will remain one of the fastest-growing agricultural subsectors over the coming decade. The global milk production is projected to expand over the 2011-2020 period at an annual rate of 2%. In South Africa alone, approximately 14 – 15 million litres of milk are wasted annually due to microbial spoilage. Therefore, the identification of the spoilage microorganisms in the milk products is necessary. This will contribute towards the design of appropriate measures to prevent wastage due to spoilage and in turn contribute towards sustainability of the sector. Accordingly, one hundred samples of spoiled full cream UHT milk were collected from two plants of each of the two largest milk processors. These samples were examined visually, and the pH was measured. A presumptive identification up to genus level was conducted by examining morphological features and conducting Gram-stain, catalase and oxidase tests. Species-specific identification was done by using the Analytical Profile Index and Biolog system. Molecular profiling was done by sequencing the rDNA genes. The main spoilage organisms identified in the samples were *Pseudomonas*, *Micrococcus*, *Bacillus*, *Enterococcus* and *Lactobacillus*. All organisms belonging to the five genera were psychrotrophs, which are commonly found in biofilms in UHT milk processing equipment. Therefore, according to the study, the spoilage bacteria apparently entered into the milk due to inadequate cleaning-in-place (CIP) processes. More importantly, further studies should be conducted in order to identify the spoilage microbes and how CIP processes can be improved.

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ABBREVIATIONS

API	Analytical Profile Index
BLASTN	Basic Local Alignment Search Tool for Nucleotide Sequences
CIP	Cleaning-in-place
CL_2	Chlorine
CLO_2	Chlorine Dioxide
CT	Cape Town
DNA	Deoxyribonucleic Acid
DMPD	N,N-Dimethyl-p-phenylenediamine
EC	Eastern Cape
EC	European Commission
EEC	European Economic Council
FAO	Food and Agriculture Organization
GP	Gauteng Province
HACCP	Hazard Analysis and Critical Control Point
HRS	Heat Resistant Spore formers
HTST	High Temperature Short Time
IDF	International Dairy Federation
IF	Inoculating Fluid
KZN	KwaZulu-Natal
L	Litre
LAB	Lactic Acid Bacteria

LTLT	Low Temperature Long Time
ML	Millilitre
NCBI	National Centre for Biotechnology Information
ng	Nanogram
PCR	Polymerase Chain Reaction
PE	Port Elizabeth
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SAMPRO	South African Milk Processors' Organisation
TBE	Tris/Borate/EDTA
TMPD	N,N,N',N'- Tetra methyl-p-phenylenediamine
UHT	Ultra High Temperature
µL	Microliter
µm	Micrometre
WC	Western Cape
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 General Background

Milk is composed of water, lipids, proteins and minerals and is considered a valuable inclusion in the human diet. However, these components can also support the growth of microorganisms (Ledenbach & Marshall, 2010). Microbial activity, in some cases, may be advantageous as observed in the production of dairy products, such as yoghurt and cheese. However, if milk is the desired product then any microbial growth and activity in it is undesirable. Microbial growth compromises the quality of the milk and in addition may also compromise the health of the consumers (Banks & Dalgleish, 1990). Therefore, the elimination of microbial contaminants from milk is critical for attaining a longer shelf life and a healthier product.

In order to improve the shelf-life of milk various technological processes have been developed and integrated in the production and packaging practices. These processes rely on the inhibition of the entry and/or growth of microorganisms. Processes such as pasteurization, ultra-high temperature (UHT) treatment, and aseptic packaging have played an important role in controlling the entry and/or growth of spoilage microorganisms (Gerosa & Skoet, 2012). Pasteurization was initially developed to kill food-borne pathogens, but with the development of milk processing procedures came the realization that it could improve the shelf-life of milk without compromising quality (Holsinger *et al*., 1997). During pasteurization and UHT treatment, microbial growth and activity in milk is controlled by the use of temperature. Currently, pasteurization coupled with refrigeration is the most commonly used technique to increase the shelf-life of milk. Although pasteurization does not kill all the microorganisms (or spores) in milk, it can efficiently eliminate most of the pathogenic bacteria that cause infections such as tuberculosis, brucellosis, and typhoid (Banks & Dalgleish, 1990).

Pasteurization of milk was introduced at a time when milk was collected from farms in churns, at ambient temperatures. Then, the dominant bacterial flora consisted of lactic acid producing, Gram-positive bacteria, which caused souring (Banks & Dalgleish, 1990). However, since current practices have incorporated refrigeration, there has

been a shift in the dominant bacterial flora. Low temperatures have favoured the emergence of psychrotrophic Gram negative rods (Samaržija *et al*., 2012). There have also been adjustments in the actual heat treatment for pasteurization. Originally, the milk was held at a temperature of 64ºC for 30 minutes, but this method has been replaced by the high temperature-short time (HTST) technique. During HTST treatment, milk is held at 74ºC for not less than 15 seconds (Muir, 1990). Ultra-high temperature (UHT) has also been introduced to effectively control microbial growth in milk. During UHT treatment, milk products are heated at 137ºC for at least 2 seconds, and packaged aseptically. UHT treatment is able to destroy a wide range of microorganisms and spores without degrading the taste or nutritional content of the product. However, it should be understood that the aim of UHT treatment is not to achieve complete sterility, but to achieve commercial sterility. The *Codex Alimentarius* World Health Organization/ Food and Agriculture Organization (WHO/FAO) definition of commercial sterility is:

> *"The condition achieved by application of heat sufficient, alone or in combination with other appropriate treatments, to render the food free from microorganisms capable of growing in the food under normal non-refrigerated conditions at which the food is likely to be held during distribution and storage"* (Jay, 2000).

Since 1961, the global per capital consumption of milk has rapidly grown in percentage when compared to other food items such as meat and maize (Gerosa & Skoet, 2012). According to the FAO (2013), the world's milk production for 2014 was estimated to grow by 1.9%, a rate comparable to previous years (0.8 % and 1.0% in 2011 and 2012 respectively). The total milk production for 2014 was forecasted to be 10.78 billion tonnes. The total South African milk production during 2013 was slightly higher (2.2%) than during 2012 (1.651 million litres for 2012 and 1.684 million litres for 2013, respectively). In South Africa, the total demand for packaged pasteurized milk during 2014 was 645 million litres. The total packaged demand for UHT milk for the same period was 957 million litres (SAMPRO, 2014). There is strong demand for UHT milk in South Africa. However, UHT milk processing and packaging suffers from spoilage due to the entry of microbes into the system but in some cases the microbes may be present in the milk and not be destroyed during processing. The total volume of milk

lost due to microbial spoilage is calculated to be in the region of 14.36 million litres per annum (TetraPak, 2013).

The conditions in milk that might enable the growth of microorganisms are interdependent and include pH, temperature, moisture, oxygen level and the level of other gases, nutrient availability, and the presence of inhibitors. Bacterial growth varies from one species to another. Under the same conditions one species may weaken, while another will thrive (Lu *et al*., 2013). In order to eliminate microbial growth in milk, there is a need to prevent the entry of microorganisms.

A substantial percentage of the bacteria in raw milk include psychrotrophic microorganisms. Typically, 65–70% of the psychrotrophs isolated from raw milk are *Pseudomonas* species (Griffiths & Phillips, 1981). Other important psychrotrophs associated with raw milk include members of the family *Enterobacteriaceae* and genera such as *Bacillus, Micrococcus, Aerococcus,* and *Lactococcus* (Ledenbach & Marshall, 2010). Besides the actively growing microorganisms, the presence of spores presents a major challenge to UHT processing. High temperatures (137°C for minimum of 2 seconds) destroy microorganisms in the vegetative form. However, bacterial spores are more difficult to destroy due to their thick protective walls. For this reason, spores pose the biggest challenge to UHT processing, and hence play a major role when determining the parameters for sterilization (Tabit & Buys, 2011). Raw milk is often the source of spore-forming bacteria isolated in UHT milk (Griffiths & Phillips, 1981).

The most common bacteria found in dairy products are *Brucella, Pseudomonas, Enterobacteriaceae, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus thermophiles, Streptococcus. lactis, Streptococcus lactis-diacetyllatic, Streptococcus cremoris, Bacillus cereus, Leuconostoc lactis, Lactococcus lactis, Lactobacillus bulgaricus, Lactobacillus acidophilus, Mycobacterium tuberculosis and Propionibacterium* (Tetrapak, 2008). These microorganisms represent just a small number of all the possible microoganisms that could possibly exist in environmental milk. This could probally be ascribed to the fact that a large portion of the microorganisms are not culturable (for example *Planctomycelates* and *Symbiobacterium thermophilum*). Selectivity of media and lack of knowledge about the

conditions under which most microorganisms grow, viability and sampling are critical limitations to gaining an understanding of microbial diversity and community structure (Muyzer, 1999; Kozdrój & Elsas, 2000).

Besides microorganisms, microbial enzymes such as proteases, phospholipases, and lipases, have been implicated in the spoilage of dairy products. These enzymes may remain active in the milk after the enzyme-producing microbes have been destroyed. Populations of psychrotrophs ranging from 10^6 to 10^7 CFU/ml can produce sufficient amounts of extracellular enzymes to cause defects in milk that are detectable by sensory tests (Kozdrój & Elsas, 2000). Ledenbach and Marshall (2010) cite Griffiths *et al*. (1987) who reported that 70–90% of raw milk samples tested contained psychrotrophic bacteria capable of producing proteinases that were active after UHT treatment. Extracellular proteases influence the quality of milk through the production of bitter peptides. Thermal resistant proteases have been implicated as the main causes of spoilage of UHT treated milk. Although most gram-negative psychrotrophic bacteria produce lipases, *Pseudomonas fluorescens* is regarded as the most common producer of lipase in dairy products (Ledenbach & Marshall, 2010).

Besides raw milk, packaging and unsterile filling lines may be responsible for microbial contamination in the milk. TetraPak is a food processing and packaging company that produces and supplies filling machines. TetraPak also provides processing and packaging solutions such as TetraBrik, which introduced aseptic processing technology. Aseptic processing is defined as the process where a sterile product is packaged in a sterile container in a way that maintains sterility. Packaging material that is predominately made of paperboard, aluminium and various layers of polyethylene. During aseptic processing, commercial sterility is achieved through a flash heating process which ensures that the preservation and integrity of the nutrients is maintained as long as the packaging is not opened. One of the applications of flash heating is in the production of UHT milk (TetraPak, 2013).

The objective of aseptic technology is to produce a commercially sterile product with good taste, texture and stability over the entire shelf-life. Aseptic processing entails the production of a commercially sterile product as well as its packaging in a hermetically sealed container. Dairy products produced through aseptic processing

are able to remain stable at room temperature. Despite technological advances in UHT treatment and aseptic packaging, contamination of milk still occurs. Contamination can come from various sources, such as:

- the environment
- processing equipment
- packaging material
- human interaction
- failed cleaning operations (TetraPak, 2008).

The commercial sterility of the finished UHT processed product depends on excluding microbial contamination from two sources. These are:

- Raw milk which may harbour heat-resistant spores that could survive the UHT process
- Post-process contamination due to unsterile packaging and/or a failure in the integrity of the aseptic filling system (Gilmour & Rowe, 1990).

If the flora at spoilage is dominated by Gram-negative rods then post-pasteurization contamination is indicated. In addition, the presence of Gram-positive organisms suggests that spore-forming bacteria have survived heat treatment (Gilmour & Rowe, 1990).

In modern UHT food plants, sterility is largely achieved by robust process design and by Hazard Analysis Critical Control Point (HACCP)-based controls, rather than by routine microbiological testing of finished products. Despite modern technological processes, such as UHT treatment and aseptic packaging, milk products are contaminated by spoilage organisms (TetraPak, 2008). Therefore, the identification of the spoilage microorganisms is required to design appropriate containment measures and subsequently prevent wastage due to spoilage. Reduction in waste will reduce the effects of the increasing production of milk on the environment and result in higher revenue for the producers.

The most sensitive and simple method of testing for commercial sterility is by incubating product samples in their final packaging at elevated temperatures (typically

30ºC) before examining them for evidence of microbial growth. The European Commission Regulation (EC) 2074/2005 states that a UHT milk treatment must be:

> "sufficient to ensure that the products remain microbiologically stable after incubating for 15 days at 30ºC in closed containers or for 7 days at 55ºC in closed containers or after any other method demonstrating that the appropriate heat treatment has been applied" (EC, 2005).

Non-sterility of UHT milk may result in gas formation, thereby causing inflated packages which can easily be detected in the storage area. When the milk has only an altered pH change, turbidity, coagulation or separation, the result is not visible by examination of the packaging alone. Following incubation, the product samples are examined for signs of microbial growth by plating onto appropriate non-selective agar media (TetraPak, 2013).

The rapid expansion and transformation of the global dairy sector requires continued development of better methods of preventing loss due to microbial contamination. Therefore, the identification of the spoilage organisms is a critical step towards reducing the contamination of UHT milk. This will result in lower manufacturing costs and fewer consumer complaints.

1.2 Rationale

In modern UHT food plants, sterility is largely achieved by robust process design and by HACCP-based controls, rather than by routine microbiological testing of finished products. Despite modern technological processes, such as UHT treatment and aseptic packaging, milk products are contaminated by spoilage organisms. In South Africa, approximately 1.740 billion litres of white cows' milk as opposed to goat or buffalo milk is packaged per annum. Of this volume, 0.1725 billion litres is reported to be wasted due to microbial spoilage (TetraPak, 2008). Therefore, identification of the spoilage microorganisms is necessary to design appropriate containment measures and subsequently prevent wastage due to spoilage.

1.3 Problem Statement

UHT milk processing and packaging suffer from spoilage due to the entry of microbes into the system. The top two manufacturers of UHT milk in South Africa produce a combined total volume of milk estimated to be between 500 – 950 million litres of UHT milk per annum. The total production of milk in South Africa is estimated at 957 million litres UHT milk and 645 million litres pasteurised milk. The total volume of milk that is lost due to spoilage for UHT is therefore calculated to be in the region of 14.36 million litres per annum (TetraPak, 2013). Identifying the spoilage organisms in the UHT milk is the first step towards reducing the contamination of UHT milk, resultant processing costs, and consumer complaints.

1.4 Research Aim

The aim of this research was to identify the dominant bacteria associated with the spoilage of UHT full cream milk packaged by four different processing and packaging plants of the two leading processors of UHT milk in South Africa.

1.5 Research Objectives

The research objectives were:

1. To collect 110 samples of 1 litre full cream UHT milk from four different milk processing and packaging plants in South Africa during two different seasons of the year.
2. To examine the 110 samples from each of the four UHT full cream milk processors, incubated at 30ºC for seven days, that have been identified as non-sterile due to a pH that falls outside the normal range for the specific customer, and/or that have other visible signs, such as inflation of the packs, or coagulation and cream separation of the milk.
3. To test the pH of 440 UHT milk samples.
4. To identify the genera of the spoilage bacteria in 100 of the 110 samples as this would be manageable (400 in total) through microbiological testing using the Gram-stain, oxidase and catalase tests.

5. To characterize the bacteria using the API (Analytical Profile Index) and Biolog Identification System.
6. To identify the dominant isolates to species level using nucleotide sequencing of the ribosomal genes.

CHAPTER 2
LITERATURE REVIEW

2.1 The Composition of Milk

Milk from a cow is a complex colloidal dispersion consisting of water, lipids, proteins, carbohydrates and trace minerals (Haug *et al*., 2007). Of these components, water is the most abundant (Table 1). The protein fraction of milk is mainly composed of casein, and to a lesser extent, enzymes and blood plasma. Lipids in milk are mainly in the form of small globules surrounded by a membrane that separates the fat core from the aqueous phase. The physical form of the proteins and lipids in milk play a critical role during milk processing (Banks & Dalgleish, 1990; McCarthy & Singh, 1997).

Table 1: The composition of milk (Caffarelli *et al*., 2010)

Cow milk component	Approximate percentage (%)
Water	86.5
Milk sugar (lactose)	4.8
Fat	4.5
Proteins	3.5
Vitamins and minerals	0.7

The composition of the different constituents of milk may vary according to the breed of the animal. They may also be influenced by the diet and stage of lactation. However, during production, milk from different breeds of cows is usually combined and stored together in bulk tanks. This often provides a relatively consistent composition of milk year around (Caffarelli *et al*., 2010)

The constituents of milk make it a valuable inclusion in the human diet. However, the same constituents provide an excellent growth medium for various microbes because of milk's (i) neutral pH, (ii) high water content, and (iii) complex biochemical composition (Frank & Hassan, 2003).

2.2 Temperature and Microbial Growth

The use of temperature to control or prevent microbial growth in milk is based on the optimum temperature requirements of the presumed contaminating microorganisms. Microorganisms can be placed into four categorizes based on their temperature requirements, as shown in Fig. 1. These categories include: (i) Psychrophilic bacteria, which grow at temperatures below 7ºC; (ii) Psychrotrophic bacteria which grow at temperatures below 20°C; (iii) Mesophilic bacteria which grow best at temperatures between 20 and 45°C and, (iv) Thermophilic bacteria which grow best at temperatures above 45°C (Todar, 2012).

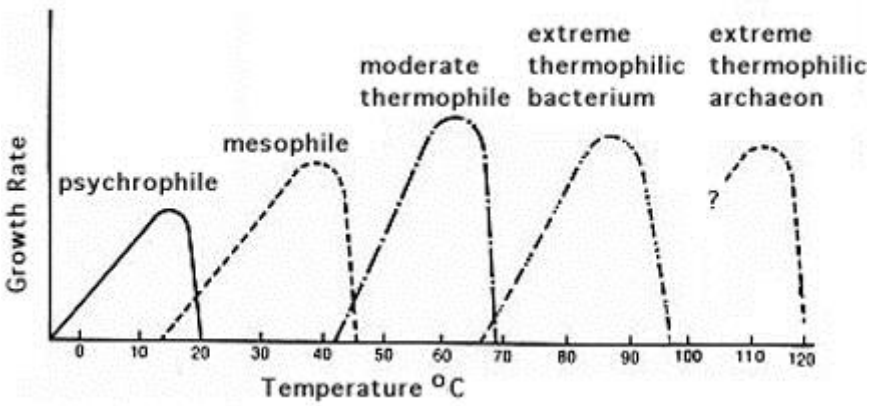

Figure 1: Growth rate vs temperature (Todar, 2012)

2.2.1 Psychrophiles and Psychrotrophs

Psychrophiles are defined by their ability to grow at 0ºC. A variant of a psychrophile is a psychrotroph. Psychrotrophs are able to grow at 0ºC but display an optimum temperature in the mesophilic range. It is important to note that psychrotrophic bacteria are not a specific taxonomic group of microorganisms, but a group of different bacterial species that are able to grow at 7ºC or less regardless of their optimal growth temperature (IDF Bulletin, 1976).

Psychrophile proteins and/or membranes, which adapt them to low temperatures, do not function at the body temperatures of warm-blooded animals (37ºC), which is in the mesophilic range (IDF Bulletin, 1976). According to D'Amico *et al.* (2006), psychrophiles appear to have evolved and overcome key barriers inherent to permanently cold environments in order to survive and proliferate at these temperatures. Some of these barriers include reduced enzyme activity, decreased membrane fluidity, altered transport of nutrients and waste products, decreased rates of cell division, protein cold-denaturation, inappropriate protein folding, and intracellular ice formation. Microorganisms that are adapted to low temperatures have successfully evolved genotypic and/or phenotypic features that enable them to grow in these environments.

One of the phenotypic features that has enabled the adaptation and survival of psychrophilic bacteria to cold environments is the amount of unsaturated fatty acids in their plasma membranes. This is due to the correlation between the degree of unsaturation of a fatty acid and its solidification temperature. The fluidity of a membrane is determined by the state of matter (liquid or solid) of the fatty acids in the membrane. The state of matter then affects the ability of the membrane to function (D'Amico *et al.,* 2006). The enzymes of psychrophiles also continue to function at temperatures at or near 0ºC (Todar, 2012). However, they may not be able to function at elevated temperatures above their optimum and hence retard microbial growth at high temperatures (D'Amico *et al.,* 2006).

Psychrotrophs play a significant role in food spoilage and safety. This could be attributed to the fact that storage at cold temperatures is a routine practice during the production, transportation, processing and post-purchase of food products (Beales, 2004). Psychrotrophic bacteria have consequently become a major problem in the dairy industry as the leading cause of spoilage and significant economic losses (Samaržija, 2012). Due to the impact that psychrotrophic bacteria may have on the economy, scientists worldwide extensively study these microorganisms with the aim of developing effective control measures to ensure the quality and safety of milk and dairy products (De Oliveira *et al*., 2015).

2.2.2 Thermophiles

Thermophiles, unlike the psychrophiles, are adapted to temperatures above 60ºC in a variety of ways. For instance, thermophiles contain an unusually high GC content in their DNA. This implies that the microorganism's maximum growth temperature may be high if one is to consider the melting point of the DNA containing a high GC content. However, there is no perfect correlation between the maximum growth temperature of a thermophile and the GC content (Dutta & Chaudhuri, 2010).

Unlike the psychrophiles, the fatty acids in the membranes of thermophilic bacteria are highly saturated (Reese *et al*., 2014). This enables the cell membranes of the thermophiles to remain stable and functional at high temperatures. The structural and transport proteins, as well as the enzymes, of thermophiles are more heat stable in comparison to those of the mesophiles. This could be attributed to posttranslational modifications in the primary structure of the proteins (Todar, 2012).

2.3 Microbial Spoilage of Milk

Milk in the alveolar tissue of a healthy cow's udder does not carry any microorganisms (Mantere-Alhonen, 1995; Chen *et al*., 2003). However, raw milk provides a physicochemical environment that is favourable for the multiplication of a broad spectrum of microorganisms that contaminate milk during collection, processing and storage (De Oliveira *et al*., 2015). The microbial composition of milk following the milking process is often made up of micrococci and streptococci. This is usually the case because these groups of microorganisms are normal flora in the udder and teat skin (Bramley & McKinnon, 1990).

Spoilage is a term used to describe the deterioration of the texture, colour, odour or flavour of food to the point where it is unappetizing or unsuitable for consumption. Microbial spoilage of food often involves the degradation of protein, carbohydrates, and fats by the microorganisms or their enzymes (Vanetti, 2009). As a result, microbiologists are continuously challenged to find innovative ways to eliminate the entry of microorganisms along with their enzymes during processing and to destroy those that survive processing treatments (Ledenbach & Marshall, 2010).

The characteristic of microbial populations in raw milk has a significant influence on the shelf life, organoleptic quality and spoilage of raw and processed milk, as well as dairy products (Samaržija *et al*., 2012). There are five groups of micro-organisms responsible for the spoilage of milk. These include bacteria, fungi (moulds and yeast), viruses, protozoa and algae. Of these, bacteria are the most prevalent contaminants of milk. Abiotic factors that affect bacterial growth are temperature, nutrient, water and oxygen availability as well as the pH of the medium (Gilmour & Rowe, 1990). As mentioned in the previous section, milk is an ideal medium that can support the growth of a variety of microorganisms. However, not every microorganism can grow at any temperature (Chirlague, 2011). Temperature has therefore been used to reduce and control microbial numbers in order to preserve and protect the quality and safety of milk. Refrigeration alone or in combination with other methods, such as the addition of preservatives, is the most commonly used means of preserving food, including milk and dairy products (Beales, 2004). Reduced temperatures inhibit growth of mesophiles and thermophiles and reduce the activity of degradative enzymes (Todar, 2012).

The current trend in the dairy industry is to reduce the frequency of milk collection; thus, the refrigerated storage of milk has increased from two to five days prior to heat treatment (O'Brien & Guinee, 2011). As a result, modern dairy farms use refrigerated bulk storage tanks to maintain milk at 4°C or below (Hayes & Boor, 2001) prior to its transportation to dairy processing plants. The reduction in the frequency of milk collection is due to the desire for a 5-day workweek, and in response to a decreased milk supply at certain times of the year (McPhee & Griffiths, 2011).

However, the extended refrigerated storage of raw milk has enabled the dominance of psychrotrophic bacteria within the milk industry. Psychrotrophs are usually introduced from their mesophilic habitats and continue to grow, albeit at a slower rate, in the refrigerated environment where they cause spoilage (Todar, 2012). Various researchers have documented the emergence of psychrotrophic microbial dominance in milk. Lafarge *et al.* (2004) showed that a difference occurred in the bacterial composition during the storage of raw milk samples for 24 hours at 4ºC and that a significant increase in the psychrotrophic bacterial community was observed. McPhee and Griffiths (2011) made a similar observation when they refrigerated milk at 6ºC for

48 hours. McPhee and Griffiths (2011) showed that on average the number of psychrotrophic aerobic bacteria in milk silos in southwest Scotland was approximately 1.3×10^5 cfu mL^{-1}. Based on these studies, it can be deduced that current practices for collection and cold storage have improved the quality of milk and milk products, but have led to a selection of psychrotrophic bacteria (Lafarge *et al.,* 2004).

Psychrotrophic microorganisms develop over time during the refrigeration of raw milk, and as a result reduce the shelf life of milk to less than 5 days (Chen *et al.*, 2003; McPhee & Griffiths, 2011; Raats *et al.*, 2011). A variety of psychrotrophic bacterial species, especially *Pseudomonas*, found in raw milk produce heat-stable lipases (Chen *et al.*, 2003), proteases (Liu *et al.*, 2007), and phospholipases (Ledenbach & Marshall, 2010), usually during the late log or early stationary growth phases. Many of the enzymes remain active after pasteurisation (72–75°C/15–20 seconds) and even UHT treatment (130–150°C/2–4 seconds), and cause spoilage by degrading fats and proteins in milk (Dunstall *et al.*, 2005; Barbano *et al.*, 2006; De Jonghe *et al.*, 2011). In addition, psychrotrophic bacteria are reported to cause age gelation in UHT milk after 20 weeks of storage (De Oliveira *et al*., 2015). 'Age gelation' is a change in the physical state of the milk that manifests in an increase in viscosity of more than 10 mPa.S at 20°C, followed by the formation of a gel, and loss of fluidity (Datta & Deeth, 2001).

Although the procedure of cooling and low temperature storage of raw milk effectively controls the growth of mesophilic spoilage organisms, it provides selective advantage for the growth of psychrotrophic bacteria (Barbano *et al.*, 2006; De Jonghe *et al.*, 2011; Samaržija *et al.*, 2012). According to McPhee and Griffiths (2011), the culturable psychrotrophic bacteria in milk are predominantly Gram-negative, including *Pseudomonas*, and to a lesser extent Gram-positive, including *Bacillus*, *Lactobacillus* and *Microbacterium* spp.

2.3.1 Pseudomonas and Milk

Pseudomonas are a diverse and ecologically significant group of bacteria. They are catalase and oxidase positive and their metabolism is respiratory, never fermentative. These microorganisms are psychrotrophic and have an optimum growth temperature of 37ºC (Gilmour & Rowe, 1990). Due to their simple nutritional requirements, *Pseudomonas* spp. have been detected in virtually all natural habitats such as soil, fresh water, house dust, and clouds (Franzetti & Scarpellini, 2007). The ubiquitous distribution of *Pseudomonas* indicates their remarkable physiological and genetic adaptability (Samaržija *et al*., 2012).

The high spoilage potential of *Pseudomonas* spp. is due to their ability to multiply at refrigeration temperatures. In addition, *Pseudomonas* spp. produce thermostable proteases, lipases (Sorhaug & Stepaniak, 1997) and phospholipases that may remain active after the enzyme-producing microbes have been destroyed (De Oliveira *et al.*, 2015). Lipase is responsible for hydrolysing fats at temperatures below 0ºC, and can also act on dehydrated substrates. The proteinase action on the other hand leads to the development of gelation or bitter flavours of UHT milk (Gilmour & Rowe, 1990). In particular, *P. fluorescens* is generally considered the principal spoilage agent of stored milk (McPhee & Griffiths 2011; De Oliveira *et al*., 2015). Adams *et al*. (1975) reported that 70–90% of raw milk samples tested contained psychrotrophic bacteria that could produce proteinases that were active after heating at 149ºC for 10 seconds. This observation was also made by Griffiths *et al*. (1981). Although *Pseudomonas* spp. metabolise optimally at temperatures between 20 and 30°C, they can proliferate at low temperatures through an enrichment of polyunsaturated fatty acid in their membrane lipids (Samaržija *et al*., 2012).

De Oliveira *et al.* (2015) cite Jaspe *et al.* (1995) who observed that *Pseudomonas* isolated from milk stored at 7°C had enhanced growth rates, as well as proteolytic and lipolytic activity in comparison to *Pseudomonas* isolated from freshly drawn milk. This observation may provide justification for the suggestion made by Ledenbach (2010) regarding the shelf life of milk. According to Ledenbach (2010), the extent of recontamination of pasteurized fluid milk products with psychrotrophic bacteria is a

major determinant of their shelf life due to the large amounts of extracellular hydrolytic enzymes they produce.

Another reason for the observation that species of *Pseudomonas* are the predominant group of psychrotrophic bacteria present in spoiled refrigerated milk (Chen *et al.*, 2003), is that these microorganisms are prevalent in the crevices of cleaned milk processing equipment (Cleto *et al.*, 2012). Their ability to survive in milk processing lines is attributed to their tendency to form biofilms (Simões *et al.*, 2008; Bai & Rai, 2011). The complex and multi-layered structures of biofilms allow the bacterial communities to live in a sessile and protected environment. When bacterial population densities in the biofilms increase, bacteria are released into the environment. This results in a continuous source of planktonic bacteria that have the ability to replicate within milk (Bai & Rai, 2011).

2.3.2 Coliforms and Milk

Coliforms are facultative anaerobes with an optimum growth temperature of 37°C. They are rod-shaped, Gram-negative and non-spore forming, and may be either motile or non-motile. Coliforms are isolated from water, soil, vegetation and in large numbers from the faeces of warm-blooded animals. Although coliforms are not themselves pathogenic, their presence is used to indicate the presence of other pathogenic organisms of faecal origin and disease-causing bacteria, viruses, or protozoa and a variety of multicellular parasites (Lu *et al*., 2013).

Enterobacter aerogenes is a coliform generally found in the human gastrointestinal tract. Its presence in UHT-treated milk could therefore be considered as an indication of post-production contamination as a result of poor hygiene in the processing plant. The presence of coliforms is also an indication of poor herd hygiene, contaminated water, unsanitary milking or processing practices, or inadequate maintenance of processing equipment (D'Aoust *et al*., 2007). Coliforms can cause rapid spoilage in milk due to their ability to ferment lactose and to degrade milk proteins (Lu *et al*., 2013).

2.3.3 Lactic Acid Bacteria and Milk

Although lactic acid bacteria (LAB) are genetically diverse, common characteristics of this group include being Gram-positive, nonsporulating, aero-tolerant cocci or rods. LAB are typically about 1.2 µm by 1.5 µm, occurring in pairs and short chains. They are generally non-respiratory and lack catalase. They ferment glucose primarily to lactic acid, or to lactic acid, CO_2 and ethanol. All LAB grow anaerobically (Todar, 2012). Lactose present in milk is readily fermented by lactic acid bacteria, resulting in sour flavour notes and, if the pH of milk drops below 4.6, precipitation of casein proteins (Bylund, 1995; Jay, 2000).

Although many genera of bacteria produce lactic acid as a primary or secondary end-product of fermentation, the term “Lactic Acid Bacteria” is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Streptococcus,* in addition to *Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus,* and *Weisella* (Todar, 2012). The lactobacilli can produce a pH of 4.0 in foods that contain a fermentable carbohydrate, and they can group up to a pH of about 7.1 which is a neutral range. Some can grow at a pH as low as 3.2, some as high as 9.6, and most grow in the pH range 4.0 – 4.5. Although they are mesophilic, some can grow below 5ºC and some as high as 45ºC (Jay, 2000).

LAB such as *Lactobacillus fermentum* are associated with plant material, especially grasses. It is from such plant material that they are introduced into the milk and cause souring (Todar, 2012). Souring of milk may not be detrimental depending on the desired product. For instance, fermentation of lactose to lactic acid is the key reaction in the manufacturing of a variety of fermented milk products such as cheese and yoghurt. The presence of lactic acid causes the pH value to drop from the normal value of 6.6 to <4.0 (in the case of yoghurt). This acts as a natural way of preserving fermented milk products such as yoghurt (Onilude *et al*., 2005). Several studies have shown that LAB improve technological and sensory properties in food systems, and that they also help to inhibit the development of spoilage and pathogenic microorganisms (Riley and Wertz, 2002; Chen and Hoover, 2003; Deegan *et al.*,

2006). It should be noted that spontaneous fermentation of milk is not encouraged, hence starter cultures, containing several species of LAB are used (Cogan & Accolas, 1990). These bacteria produce acid and continue to do so until the pH of the milk falls to below 4.6, at which point they gradually die off (Chirlaque, 2011), although some species can grow at a pH as low as 3.2, as noted by Jay (2000).

Enterococci are gram-positive, catalase-negative cocci, produce lactic acid homofermentatively from glucose, and also derive energy from degradation of amino acids. (Klein *et al*., 1998). *Enterococcus faecalis* is the new proposed name for *Streptococcus faecalis* (De Vos *et al., 2009)*. The cells are usually spherical or ovoid, <2*µ*m in diameter. Enterococci can grow at temperature ranging from 10 - 50ºC, with the optimum growth temperature at 37ºC (Erkman & Bozoglu, 2016). Isolated from the intestines of mammals, its presence in food or water is associated with faecal contamination. It is considered to be more resistant to freezing, low pH, moderate heat treatment and marginal chlorination than *Escherichia coli* (Jay, 2000). *Enterococcus faecalis* is a common indicator organism. Their presence in foods indicates faecal contamination, improper sanitation, and possible presence of pathogens. The relation of *Enterococcus* to enteric pathogens is lower than coliforms. Their ability to survive at pasteurization temperature and in dried, frozen, refrigerated, and low pH foods can place them as indicators for these types of foods (Gilmour & Rowe (1990).

2.3.4 Micrococcaceae and Milk

Micrococcus is a genus of thermoduric spherical bacteria in the family *Micrococcaceae*. The family comprises four genera: *Micrococcus*, *Planococus*, *Stomatococcus* and *Staphylococcus* (Gilmour & Rowe, 1990). *Micrococci* are Gram-positive, catalase positive and 0.5 - 1.5 *µ*m in diameter. They are usually non-motile, and are either aerobic or facultatively anaerobic. Their metabolism is respiratory and/or fermentative. *Micrococci* are usually not pathogenic. They are normal flora on the human body and play an essential role in keeping the balance among the various microbial flora of the skin. *Micrococci* can be isolated from the dust in the air (*M. roseus*), from the soil (*M. denitrificans*), in marine waters (*M. colpogenes*), and on the skin or in skin glands (Gilmour & Rowe, 1990). Those species found in milk, such as *M. luteus, M. varians*, and *M. freudenreichii*, are sometimes referred to as milk

micrococci, and can result in spoilage of milk products (Epstein, 2015). *Micrococcus luteus* and *M. varians* are thermoduric sugar fermenters that occur frequently on dairy equipment and in pasteurized milk. *Micrococcus luteus* is an obligate aerobe found in soil, dust, water and air, and as part of the normal flora of the mammalian skin, from where they easily enter into milk. Although once regarded as non-pathogenic, it is now considered an opportunistic pathogen, especially in immunocompromised patients, and is responsible for nosocomial infections (GBIF, 2012).

2.3.5 Bacillus and Milk

Bacillus is a genus of Gram-positive, rod-shaped, bacteria and a member of the phylum *Firmicutes. Bacillus* species can be obligate aerobes or facultative anaerobes (Němečková *et al.*, 2011). *Bacillus* exibit a broad variability in colony morphology. *Bacillus* spp. are a heterogeneous group of bacteria characterised by a high degree of variability in their nutritional requirements. This genus is able to grow at a wide range of temperatures and pH values. *Bacillus* is abundant in nature, and includes both non-parasitic and parasitic pathogenic species. Some species of *Bacillus* are important pathogens, causing anthrax and food poisoning (Tetrapak, 2008). *Bacillus* spp. are considered the main cause of microbial spoilage of milk and milk products and also pose a significant economic risk in the dairy industry. Among the microbial species that can survive UHT treatment of milk, *Bacillus* spp. are the most common isolated Gram-positive bacteria. These include *stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. circulans* which are the most commonly isolated species of *Bacillus* from raw, heat treated milk and dairy products. This genus is able to cause spoilage of milk through the production of endospores as well as spores (Samaržija *et al*., 2012). Under stressful environmental conditions, the bacteria can produce oval endospores (Němečková *et al.*, 2011). These are not true spores but they house the bacteria in a dormant state for long periods of time. Spores produced by *Bacillus* spp. are activated by heat following the UHT treatment. *Bacillus subtilis, B. licheniformis and B. cereus* are the most common sporeformers in raw milk (Tetrapak, 2008).

The vegetative cells of *Bacillus* spp. have a greater capacity to produce thermostable extracellular and intracellular hydrolytic enzymes that are broader in spectrum when

compared to those produced by *Pseudomonas* (Samaržija *et al*., 2012). Furthermore, *Bacillus* spp. show more diverse proteolytic activity when compared to *Pseudomonas* spp. Various *Bacillus* spp. are capable of forming more than one type of extracellular and intracellular proteinases (Nabrdalik *et al*., 2010). However, the intensity of protolytic activity is dependent on the species and strain as well as the temperature. The lipolytic activity of *Bacillus* spp. is also significant at temperatures higher than 4°C, and results in an increase in the concentration of free fatty acids in milk. The lipases of *Bacillus* spp. are more thermostable than the proteinases, but both types of enzymes are sufficiently thermostable at all temperatures of milk heat treatment (Samaržija *et al.*, 2012).

Bacillus cereus has been recognized as an agent of food poisoning since 1955 due to its ability to produce several enterotoxins (Samaržija *et al*., 2012; Todar, 2012). The presence of *B. cereus* often results in the coagulation of milk as a result of lipolytic and proteolytic enzymatic action. Sweet curdling in pasteurised milk is caused B. by which also produces rennet-like enzymes that coagulate milk without souring, resulting in sweet curdling (Ryser, 2001). Fermentation of milk by *B. cereus* may not result in gas production nor change in pH (Todar, 2012). The ability of *Bacillus* spp. to grow in milk at low temperatures as well as produce thermostable enzymes poses a challenge to the milk industry (Griffiths & Phillips, 1990). *Bacillus cereus* is destroyed when heated above 100ºC. However, when left in a system after a weak cleaning-in-place (CIP), *B. cereus* can grow to large populations that are very heat resistant at temperature higher than 100ºC (Adams & Moss, 2008). Due to the capacity of its extensive lipolysis and proteolysis, it is considered that *Bacillus c*an reduce the shelf-life of UHT milk by 25% (Samaržija *et al*., 2012).

2.4 Thermal Treatment of Milk

Heat treatment plays a critical role in controlling bacteria in processed milk products. Heat is used to treat raw milk in three different ways. These include pasteurization, ultra-pasteurization and UHT. These modes of heat treatment differ from one another based on their purpose. Pasteurization which is partial sterilization at a temperature normally 72-75ºC for a period of about 15 seconds that destroys objectionable

organisms without major chemical alteration, aims to eliminate the non-spore-forming pathogens that are most resistant to thermal destruction as well as reduce non-pathogenic bacterial numbers in milk. Ultra-pasteurization which is similar to pasteurization but a higher than normal temperature (about 85ºC) especially to extend the shelf life, is aimed at increasing the product's shelf life through further reduction in total bacterial numbers. UHT processing aims to achieve microbial sterility to create a shelf-stable fluid milk product (Hayes & Boor, 2001).

There are factors that may have an influence on the tolerability of the microorganisms towards heat treatment. According to Jay (2000) these are:

- water
- fat
- salts
- carbohydrates
- pH
- proteins and other substances
- numbers of organisms
- age of organisms
- growth temperature
- inhibitory compounds
- time and temperature
- effect of ultrasonic.

Bacteria have been reported to survive under extreme temperatures (between -250ºC and 160ºC) conditions. However, all microorganisms have a thermal death point. A thermal death point is the length of time needed to kill organisms at a given temperature in a given material (Zall, 1990). The thermal death point of each microorganism may vary based on the combination of time and temperature required for its destruction. The main factor that determines the severity of a thermal process is the acidity of the product (Potter & Hotchkiss, 2013).

Heat treatment kills bacteria at a rate that is proportionate to the number of bacteria present, which is referred to as a *logarithmic order of death* (Fig. 2). This means that

only a percentage of bacterial destruction takes place in a specified amount of time. The implication is that if 90% of a bacterial population is killed within the first minute of heating, 90% of the remaining population will be killed in the second minute, and 90% of the surviving bacteria in the third minute, and so on. This is called the *D-value*, which is an index of the time-temperature combination needed to reduce microbial numbers by one log cycle. The logarithmic order of death also applies to bacterial spores, but, due to the greater heat resistance of spores, the curve will differ from that of vegetative cells (Potter & Hotchkiss, 2013).

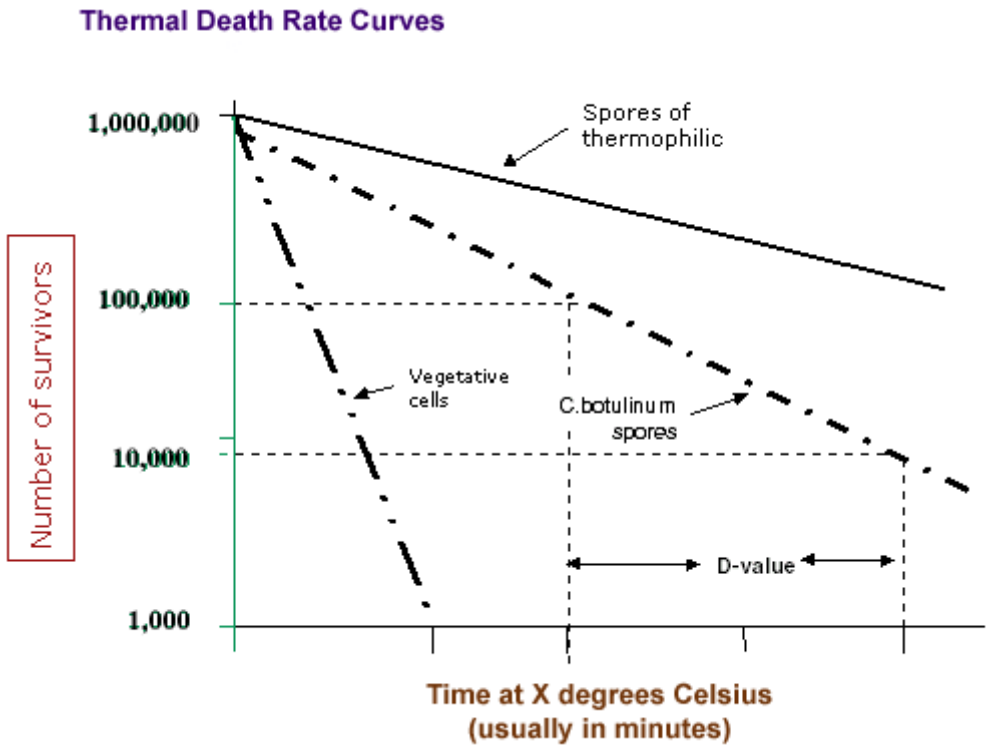

Figure 2: Bacterial destruction rate curve (Potter & Hotchkiss, 2013)

To select the most appropriate heat treatment, the following must be known:

i) Time and temperature combination needed to inactivate the most heat-resistant pathogens and spoilage organisms in a particular food.
ii) The heat penetration characteristics of a particular food, including the container of choice if it is packaged.

The most heat-resistant pathogen found in foods, especially those that are canned and held under anaerobic conditions, is *Clostridium botulinum*. However, there are non-pathogenic spore-forming spoilage bacteria, such as *Putrefactive anaerobe* 3679 (PA 3679) and *Bacillus stearothermophilus* (FS 1518), that are more heat resistant than *C. botulinum*. It is ideal to design the thermal treatment of food based on the parameters required to eliminate the most thermal resistant microorganisms that could exist in the food. For instance, if *P. anaerobe* and *B. stearothermophilus* are destroyed by heat treatment, then it is safe to assume that *C. botulinum* will be destroyed, together with all other pathogens (Potter & Hotchkiss, 2013). Currently, *Coxiella burnetii* is considered the most thermal resistant microorganism in the food and beverage industry (Hayes & Boor, 2001).

Different foods will support the growth of different microorganisms and hence there will be variations in terms of the heat and temperature combinations to achieve the desired sterility. Processors of UHT milk must therefore use the right time/temperature combination to ensure that each particle of milk is reached so that the most resistant microorganism is destroyed (Potter & Hotchkiss, 2013).

2.4.1 UHT Treatment of Milk

Milk is usually subjected to heat treatment to ensure microbiological safety before retail and consumption. There are three types of heat treatment, i) low temperature long time (LTLT) pasteurization, ii) high temperature short time (HTST) pasteurization, and iii) ultra-high temperature (UHT) treatment. One of the principal goals of milk preservation methods by short time treatment at increased temperatures is to obtain the desired degree of destruction of microorganisms and of inactivation of enzymes, and introducing the minimum undesired changes of physico-chemical and sensory properties, while preserving its nutritional value (Scott, 2008).

The UHT process involves heating milk at temperatures ranging from 135 – 148ºC for 2 - 4 seconds. At this temperature commercial sterility can be achieved without any negative impact on the taste or nutrition of the product. The aim of UHT processing is

commercial sterility, and not absolute sterility. As a logarithmic function can never reach zero, absolute sterility is not possible (TetraPak, 2013).

The European Council Directive 92/46/EEC (European Economic Council 1992) definition is:

> "In random sampling checks, UHT must meet the following standard after incubation at 30ºC for 15 days: Plate Count at 30ºC $\leq$ 10 colonies per 0.1ml".

UHT processing can be achieved either directly or indirectly. During the direct UHT milk treatment process pressurized culinary steam is injected into the milk at 140ºC for 4 seconds. The product temperature increases almost instantly due to the latent heat of vaporization. The condensed steam dilutes the milk. After holding for 4 seconds the temperature is lowered rapidly by flash cooling in a vacuum chamber and steam escapes. The rapid heating and cooling gives minimal heat-load on the product, thus achieving the desired quality. In indirect heating, a stainless steel barrier is placed between the heating/cooling medium and the product. The heating and cooling media flow on the one side of the barrier, and the product on the other without direct contact with each other (Scott, 2008).

UHT processing uses continuous flow, which renders less chemical change to the product in comparison to retort processing. Minimum processing times and temperatures are determined by the inactivation of thermophilic bacterial spores (Datta & Deeth, 2007; Ledenbach & Marshall, 2010). Factors such as pH, water activity, viscosity, composition, and dissolved oxygen dictate the processing conditions necessary to achieve commercial sterility (Overman, 1998), but the selection criteria of UHT and aseptic packaging systems reflect customer preferences (Scott, 2008). The processor may reduce the failure rate during storage by increasing the severity of heat treatment, but at a cost. As the heat treatment increases, chemical changes occur in the milk giving rise to browning, and to the development of cooked and caramelized flavours. Consequently, processing conditions for the production of UHT milk are a compromise between the need to destroy bacteria and the desire to limit chemically-induced changes (Muir, 1990).

The production process must be designed to ensure commercial sterility and acceptable sensory attributes throughout shelf life. The selection criteria of UHT and aseptic packaging systems reflect customer preferences (Scott, 2008). The selection of a heat sterilization process depends on i) the initial number of microorganisms, ii) the decimal reduction time (heat resistance) of the microorganisms, and iii) the time and temperature of heat exposure (TetraPak, 2013).

Commercial sterility is achieved not only by heat treatment of a food product but also aseptic packaging. Aseptic processing uses separate systems; one to sterilize the product and the other to package the product. The sterile product is filled into sterile packages within the sterile zone of an aseptic packaging system. The UHT treatment and aseptic package protects dairy foods from bacteria and external contamination. The shelf life of milk is extended from 21 days in traditional pasteurization to over four months with UHT and aseptic technology (Von Bocklemann & Von Bocklemann, 1998).

There are several advantages to aseptic processing and packaging over traditional pasteurization. Advantages include lower energy costs, the elimination of required refrigeration during storage and distribution, and extended shelf life. The main challenges associated with UHT processing and aseptic packaging include product quality loss, such as age gelation, fat separation, and flavour loss, as well as manufacturing concerns such as limited production capacity, potential contamination, slow packaging speeds, and limited shelf life knowledge (Scott, 2008).

2.4.2 Spoilage of UHT Treated and Packaged Milk

Spoilage of pasteurized milk is due to one of two reasons:

- post-pasteurization contamination, or
- the growth of organisms that have survived the heat treatment (Banks & Dalgleish, 1990).

Post-pasteurization bacterial contamination provides a serious obstacle to maintaining commercial sterility. The biggest source of post-pasteurization contamination is

ineffective CIP procedures with the correct chemical, at the right interval and at the right temperatures achieving the correct flow rate of the interior of processing equipment. This creates milk residues and biofilms that allow bacteria to multiply and contaminate subsequent milk flow (Austin & Bergeron, 1995, Ralyea *et al*., 1998). Psychrotrophic bacteria associated with the spoilage of milk and dairy products have the ability to form exopolysaccharides and/or lipopeptides. These components enable bacteria to adhere to solid surfaces upon which they can form a biofilm (Marchand *et al*., 2012). Although rare, biofilm may consist of a single bacterial species that could eventually result in spoilage. Generally, the biofilm often consists of a mixture of microorganisms present in the actual environment (Samaržija *et al*., 2012).

Cells within the biofilm that are physiologically similar exhibit significantly different properties and changes in the bacterial physiology in comparison to their planktonic counterparts. In addition, they are more resistant to the majority of disinfectants than planktonic cells. What is of particular significance is that the bacterial species *B. cereus* and*, B. subtilis*, (facultative anaerobic bacteria) and *P. fluorescens* (aerobic bacteria) are capable of jointly forming a biofilm. In a binary biofilm, for example, the survival of *P. fluorescens* after exposure to chlorine dioxide ($C\ell O_2$) is higher in the presence of *B. cereus.* In addition, the bacterial cells within the biofilm develop a greater cell biomass and have the ability to temporarily separate from the formed biofilm. Consequently, biofilm formation can be difficult to resolve and may result in a long-lasting source of permanent product contamination with psychrotrophic bacteria that cause spoilage (Samaržija *et al*., 2012).

Biofilms are the predominant form of growth for bacteria in the majority of environments, including food processing settings (Bai & Rai, 2011; Cleto *et al.*, 2012). The critical stages for biofilm development are adherence, proliferation, and the dispersion phases (Mann & Wozniak, 2012). Each of these stages includes reinforcement by or modulation of the extracellular matrix. The functionality of biofilms depends on a complex web of symbiotic interactions and factors, including pH, nutrient availability, quorum sensing molecules, the presence of organic and inorganic compounds and temperature (Bai & Rai, 2011; De Oliveira *et al.*, 2015). Research on the production of spoiling enzymes by bacteria present in biofilms has only been conducted recently (Teh *et al.*, 2014).

Besides the formation of biofilms and an inefficient CIP process, the presence of Gram-negative rods is a sign of post-pasteurisation contamination (Fernandes, 2009), whereas the presence of Gram-positive organisms suggests that spore-forming bacteria have survived heat treatment. In general, the primary reason for the failure of fresh products is post-pasteurisation contamination (Banks & Dalgleish 1990).

2.5 The Identification of Spoilage Bacteria

2.5.1 pH of Milk

Fresh milk has a pH ranging between 6.5 and 6.7 and is therefore slightly acidic. Values higher than 6.7 denote mastitic milk and values below 6.5 indicate the presence of colostrum or bacterial deterioration. Since milk is a buffer solution, significant acid development may take place before the pH changes. pHs lower than 6.5 therefore indicates that considerable acid development occurred. This is normally due to bacterial activity. Measuring milk's acidity is an important test used to determine milk quality. A drop in the pH of milk is an indicator of the presence of lactic acid producing microorganisms. However, no change in pH does not indicate a sterile package, since certain bacteria do not change the pH, and are therefore not detectable through pH testing (O'Connor, 1995).

2.5.2 Biochemical Characterisation of Milk

Quality control in the dairy industry relies on the correct identification of spoilage organisms. The use of biochemical tests enables the discrimination of the different strains of the same species. Differences in concrete enzymatic activities may reveal the ecology, physiology or natural habitat of the microorganism. Molecular procedures are ideally used to identity microorganisms because they are faster and more specific, but one should combine these procedures with biochemical characterization if one is interested in knowing more about the ecology/physiology of the microorganism (Von Bocklemann & Von Bocklemann, 1998). Biochemical characterization is often

achieved through the use of preliminary identification, Analytical Profile Index (API) and Biolog Identification System.

2.5.3 Preliminary Identification of the Milk Spoilage Microorganisms

2.5.3.1 Gram Stain

The Gram stain differentiates bacteria into two fundamental categories of cells. Bacteria that retain the initial crystal violet stain (purple) are "Gram-positive", whereas those that are decolorized and stained red with carbol fuchsin (or safranin) are "Gram-negative." This staining response is based on the chemical and structural makeup of the cell walls of bacteria (Beveridge, 2001). The difference between the two groups is as a result of the amount of the peptidoglycan in the bacterial cell wall. Gram positive microorganisms contain a higher amount of peptidoglycan in comparison to the Gram negative microorganisms. During the Gram staining, the iodine and crystal violet precipitate in the thickened cell wall of Gram-positive bacteria and is not eluted by alcohol. However, in Gram-negative microorganisms the iodine and crystal precipitates are eluted by alcohol. As a result, bacteria can be distinguished based on their cell wall properties and morphology due to their staining properties (Lowy, 2009).

2.5.3.2 Catalase Test

Catalase is a proteolytic enzyme capable of destroying hydrogen peroxide. The catalase test is also one of the three main tests used by microbiologists to identify species of bacteria. The presence of catalase in the test isolate is detected using hydrogen peroxide (Rouvinen, 2010). The catalase test is done by placing a drop of hydrogen peroxide on a microscope slide. If the mixture produces bubbles, then the organism is 'catalase-positive'. Vigorous bubbling from the surface of colonies indicates the presence of the enzyme catalase which breaks down the hydrogen peroxide into water + oxygen gas, which is given off as bubbles. If the mixture does not produce bubbles or froth, the organism is 'catalase-negative'. The catalase test is used for rapid differentiation of the genera of gram-positive organisms (Kiiyukia, 2003).

2.5.3.3 Oxidase Test

The oxidase test is carried out to determine whether a bacterium produces cytochrome c oxidases. It uses disks impregnated with a reagent such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) or *N,N*-dimethyl-*p*-phenylenediamine (DMPD), which is also a redox indicator (Azhar *et al*., 2014).

2.5.4 Specific Identification

Following initial identification, which identifies the genera of the microbes, specific identification is done by API and Biolog to determine the species of the microbes.

2.5.4.1 Analytical Profile Index

The API (BioMérieux, Marcy-l'Étoile, France) is a system that was developed for quick identification and classification of bacteria. However, API testing requires prior presumption of the microorganism. This presumption can be achieved by examining the morphological features, Gram stain, catalase and oxidase tests. Based on the tests one can use the appropriate API system selected from the 23 available systems (BioMérieux, n.d.). API test strips consist of micro-tubes containing dehydrated substrates to detect the enzymatic activity or the fermentation of sugars by the inoculated organisms. During incubation, metabolism produces colour changes. The colour changes occur either spontaneously or in reaction to the addition of reagents. Fermentation of carbohydrates changes the pH within the tube and is shown by an indicator (Popp & Bauer, 2015).

To identify an organism, the API web software compares the profiles obtained with the profiles of taxa in the database and assigns a positivity percentage to each test. This system is developed for quick identification of clinically relevant bacteria. Furthermore, discrepancies with respect to conventional methods may be observed. They are due to the different principles of the reactions used in the API technique. In addition, substrate variations that exist also account for percentage differences (BioMérieux n.d.). With regard to the identification of *Enterobacteriaceae*, for example, BioMérieux (n.d.) reports that 5514 collection strains and strains of various origins belonging to

species included in the database were tested. While 92.80 % of the strains were correctly identified, 4.61 % of the strains were not identified, and 2.59 % of the strains were misidentified. Tests on other non-fastidious Gram-negative rods showed similar results.

2.5.4.2 Biolog Microbial ID System

The Biolog Identification System (Biolog, Inc., Hayward, CA) establishes an identification of bacteria based on the relationship between the exchange of electrons generated during respiration and the change in colour of tetrazolium. This system tests the ability of a microorganism to oxidize a panel of 95 different carbon sources (Miller & Rhoden, 1991). The isolate to be identified is grown on agar medium and then suspended in a special "gelling" inoculating fluid (IF) at the recommended cell density. Then the cell suspension is inoculated into the GEN III MicroPlate, 100 µl per well, and the MicroPlate is incubated to allow the phenotypic fingerprint to form. All of the wells start out colourless when inoculated. During incubation there is increased respiration in the wells where cells can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple colour. Negative wells remain colourless (Biolog, 2013). After incubation, the phenotypic fingerprint of purple wells is compared to Biolog's species library. If a match is found, a species level identification of the isolate is made. Biolog uses four protocols, Protocols A, B, C1 and C2. All protocols are performed in the same manner, the only difference being the choice of IF and cell density for inoculation. Protocol A is used for the vast majority of species (Biolog, 2013).

Holmes *et al*. (1994) evaluated Biolog for the identification of 55 gram-negative taxa that could potentially be found in clinical laboratories. They reported that the Biolog system performed best with oxidase-positive fermenters and biochemically active non-fermenters, but did not perform as well with unreactive non-fermenters. It gave significantly better results when the microplates were read manually, rather than by the automated reader.

2.5.5 Molecular Characterization

Some bacteria are difficult to identify with phenotypic identification schemes commonly used outside reference laboratories (Drancourt *et al*., 2000). The 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates (Rouvinen, 2010). Molecular profiling of any given organism requires the extraction of genomic material. There is a wide variety of available methods for DNA extraction. The boiling method is a modification of the bacterial DNA extraction protocol described by Li *et al*. (2003). This method involves placing a small sample of the isolate in a centrifuge tube, and placing it on a heating block for 15 minutes. Afterwards, the sample is centrifuged at 10 000 rpm for 17 minutes. When a pellet is observed at the bottom of the tube, the supernatant is drained and the pellet is reconstituted with DNAse free pure water. DNA is amplified through a process known as Polymerase Chain Reaction (PCR). During a PCR reaction constant regions of DNA sequence to prime the copying of variable regions of DNA sequence are used. PCR typically uses two short pieces of DNA called primers, and these serve as the starting points for the copying of a region of DNA. In PCR, the original DNA is copied, then the copies are copied, those copies are copied, and so forth. For this reason, the main limitation of PCR is contamination, which makes it imperative that the initial DNA is in good condition (Quigley *et al.,* 2011).

Hayes and Boor (2001) caution that characterization of the bacterial population in milk should take cognisance of the limitation inherent in any analytical technique. No one test can detect all bacteria, and even nonselective tests designed to determine total bacterial numbers cannot detect fastidious and slow growing microorganisms, or poor competitors that require selective media to ensure sufficient nutrient access. No one test gives a complete picture of the microbial population; the picture must be pieced together using results from multiple different tests. However, doing all possible tests is not feasible, economically or logistically, and consequently microbial analysis includes deciding which tests will provide the most useful information about the microbial population present based on the purpose of the test. Bosshard *et al.* (2003) propose the use of 16S rDNA sequence analysis if adequate species identification is of concern.

2.6 Conclusion

The composition of milk makes it an ideal growth medium for a wide variety of microbes, of which a large percentage causes spoilage. *Pseudomonas*, *Coliforms, Lactobacillus*, *Micrococcus* and *Bacillus* are the bacteria commonly associated with milk spoilage. These bacteria, found in raw milk, can contaminate UHT milk by either surviving pasteurization, or due to a variety of weaknesses in the UHT and packaging processes.

To identify the organisms responsible for the spoilage of UHT milk, biochemical characterization can be done by initial identification, which includes Gram-stain, catalase and oxidase tests. Specific characterisation, by way of the API and the Biolog Identification Systems, identifies the organisms at the species level. As some bacteria are difficult to identify with phenotypic identification schemes commonly used outside reference laboratories, 16S rDNA sequencing is used for bacterial phylogeny and taxonomy.

CHAPTER 3
MATERIALS AND METHODS

3.1 Location and Sample Selection

Two processors of UHT milk in South Africa were selected for this study. The identity of these processors will not be disclosed due to reasons of confidentiality. These processors will therefore be referred to as Processor 1 and Processor 2. Samples for this study were collected from two processing and packaging plants of each of the two processors. The processing and packaging plants for Processor 1 were located in Queensburgh and Clayville, whereas those for Processor 2 were located in Port Elizabeth and Cape Town. A total of 110 full cream UHT milk samples were collected from each processing and packaging plant during winter in 2014 and in summer in 2015. These samples were randomly chosen from batches that showed evidence of microbial contamination following an onsite quality assurance assessments conducted at the milk processing and packaging plants. Before testing, the samples were incubated at 30ºC for 30 days in a 60L bench incubator at the TetraPak Quality Laboratory, in Isando, Johannesburg.

3.2 Visual Observation of the Milk Packages

After the 30-day incubation period, the samples were visually observed for any signs of spoilage. Visual observation included examining the packaging for its integrity i.e. whether the packs were inflated, and observation of the milk for signs of coagulation, or any other signs of spoilage. The results were recorded.

3.3 pH Analysis of the Milk

The pH value for each of the 400 packs of UHT milk was determined using a Metrohm pH meter (Metromh, Houston, TX). The pH meter was calibrated every 30 minutes with calibration buffer at pH 4.00 and 7.00. Milk with a pH range of 6.40 – 6.68 was considered fresh and suitable for consumption and hence served as a control.

3.4 Microbial Growth in the Milk

Ten microliters of milk from each of the 400 packs of milk was aseptically streaked onto separate sterile agar plates containing standard plate count agar. The plates were then incubated at 30ºC and observed for microbial growth every 24 hours until a maximum incubation period of 72 hrs was reached. Following incubation, pure colonies were picked and aseptically streaked on sterile standard plate count agar. The plates were then incubated at 30ºC for 24 hrs, followed by microbial identification. The purity of the isolates was checked by repetitive streaking on fresh standard plate count agar. Pure colonies on standard agar plates were stored at 4ºC for further applications.

3.5 Biochemical Characterization of the Bacterial Isolates from the Milk

The bacterial isolates were presumptively profiled based on their Gram staining properties, as well as oxidase and catalase biochemical tests.

3.5.1 Gram Stain

The Gram stain test was performed by preparing a slide with an emulsion of a single colony of the purified cultures. The emulsion was then heat fixed and stained. During staining a few drops of crystal violet stain were placed onto the fixed culture. After 30 seconds the crystal violet stain was rinsed off with distilled water and a few drops of Grams iodine (Lugol solution) was added for 30 seconds. Thereafter Gram's iodine was gently washed off with distilled water; Merck decolourizer (containing ethanol and acetone) was then added to decolourize the stained culture. The slide was then rinsed with distilled water and a few drops of safranin were added. After 20 seconds the solution was rinsed off with distilled water. The slides were then observed under a light microscope and the results were recorded. Bacteria that retained the initial crystal violet stain (purple) were identified as Gram-positive. Those that were decolorized and stained red with safranin were identified as Gram-negative.

3.5.2 Catalase Test

A bacterial colony was placed on a slide and then a drop of 10% (v/v) H_2O_2 was added to the surface of the colony. Vigorous bubbling from the surface of colony was an indicator of the ability for the bacterial isolate to produce catalase. The lack of bubbling was an indicator of the inability for the microorganism to produce catalase.

3.5.3 Oxidase Test

A bacterial colony was placed on a strip with a disk impregnated with N,N,N',N'-Tetramethyl-p-phenylenediamine·hydrochloride and the colour change was recorded. If the colour of the disk changed to a dark blue or maroon colour, then the bacterial isolate was oxidase positive. However, if there was no colour change then the bacterial isolate was oxidase negative.

3.6 Specific Characterisation

3.6.1 Analytical Profile Index

Following presumptive organism identification using morphological features, Gram-stain, catalase and oxidase, the appropriate API system was selected using Table 2.

Table 2: Relevant API systems to be used for the identification of organisms

Presumptive Organism ID	Relevant API system to use
Gram negative *Bacillus* • Oxidase positive • Non-fastidious • Non-*Enterobacteriaceae*	API 20 NE
Lactobacillus	API 50CHL
Bacillus cereus	API 50CHB
Micrococcus	API STAPH
Gram negative *Bacillus* • Oxidase negative • *Enterobacteriaceae* & other non-fastidious GNB	API 20 E

The API systems were inoculated in accordance with the manufacturer's instructions. All systems followed the same procedure for inoculation. A large colony (2-3 mm diameter) of a pure bacterial isolate was inoculated into 5 ml saline (0.85% NaCl) solution and vortexed. The bacterial inoculum was then dispensed into each well on the API strip taking care to avoid bubbles. Each well was filled, except those that required anaerobic conditions in which case sterile mineral oil was used. After inoculation, all identification systems were incubated at 36ºC for 24 - 48 hrs. Each system was read after 24 hrs incubation, with a final reading made after 48 hrs. The reactions were read according to the Reading Table and the identification of the organisms were obtained by using the identification software.

3.6.2 Biolog

The purified bacterial isolates were cultured on blood agar at 30°C for 16 - 24 hours prior to the Biolog assay. Using a cotton-tipped inoculator swab, a 3 mm diameter area of bacterial cell growth was picked from the surface of the agar plate and used to prepare an inoculum in the range of 90-98% T. Then GEN III Microplates (Biolog Identification Systems, Hayward, CA) were inoculated with 100 µl of the cell suspension and incubated at 30°C for 8 - 36 hours. The incubation period was selected based on the growth rate of the micro-organisms. The turbidimeter was calibrated at the start of the assay and thereafter at 30 minute intervals, using a turbidity standard of 65% T. After incubation the GEN III Microplates were placed into the OmniLog incubator/reader, and the organism was identified by the Biolog Microbial Identification software.

3.7 Molecular Profiling

3.7.1 DNA Extraction

The bacterial isolates were inoculated into Tryptic Soy Broth (Oxoid, Basingstoke, England) and incubated at 37°C overnight. Genomic DNA was extracted from these cultures using the boiling method (Li *et al*., 2003). About 1.5 ml of each of the cultures (isolates 1 – 5) were place in a centrifuge tube and placed on a heating block at 100ºC for 15 minutes. It was then centrifuged at 10 000 rpm for 17 minutes. Using the bench

centrifuge the supernatant was drained and the pellet reconstituted with 100 µl of DNAse-free pure water.

3.7.2 Qualitative and Quantitative Assessment of DNA

The concentration of the extracted genomic DNA was measured using a Nano Drop-spectrophotometer (NanoDrop Technologies, Wilmington, DE). The purity of the extracted genomic DNA was determined based on the A260/A280 ratio. DNA samples that had a ratio that was greater than 1.0 were considered pure. The DNA of the isolates used in all reactions was diluted and adjusted to 50 ng/µl.

3.7.3 DNA Electrophoresis

To determine the quality of the genomic DNA, an amount of 2 µl of the isolated DNA was mixed with 2 µl of 6X loading dye and allowed to separate at 90V for an hour on 1% (w/v) agarose gel (Sigma-Aldrich, St Louis, MO). A 1000 bp plus DNA ladder (Sigma-Aldrich) was used as a molecular weight standard.

3.7.4 Polymerase Chain Reactions (PCR)

Fragments of the 16S rRNA genes were amplified using the eubacterial universal primers 27F (5'GAGTTTGATCCTGGCTCAG-3') and 907R (5'CCGTCAATTCCTTTGAGTTT-3') (Lane *et al.*, 1985). A PCR reaction contained 50 ng of the extracted DNA, 25 mM $MgCl_2$, 0.25 µM of each of primers 27F and 907R, and 2X KAPA Robust Hotstart Readymix (Kapa Biosystems, Boston, MA) in a total volume of 25 µl was prepared. The PCR cycling conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 45 seconds and primer extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The amplification reactions were performed in a Bio-Rad C1000 Thermocycler (Hercules, CA). The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel (Sigma-Aldrich) containing ethidium bromide (3 µl). About 2 µl of each PCR product and 2 µl of 6X loading dye (Sigma-Aldrich) were loaded into agarose gel wells and run in 1X TBE buffer. A 1000 bp plus DNA ladder was used as a molecular weight standard. The

PCR products were separated by gel electrophoresis at a constant voltage of 90 V for 60 minutes. Then the DNA bands were visualized by ultraviolet (UV) illumination at 300 nm wave-lengths on a UV trans illuminator system (Herolab, Wiesloch, Germany).

3.7.5 Nucleotide Sequencing and Analysis

Post amplification of the 16S rDNA gene amplicons were sequenced in both directions with the primers used in PCR at Inqaba Biotech (Pretoria, South Africa). The resulting sequence chromatograms were visualised and analysed using Chromas Lite 2.4 (Technelysium Pty, South Brisbane, Australia) and BioEdit v. 7.2.5. The sequences were then compared to those in GenBank (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN).

3.8 Statistical Analysis

After the pH values for each of the one hundred packs (sample batch) from each plant were recorded, the mean pH for each sample batch was calculated. The population standard deviation of the mean was then calculated using the formula:

$$\sigma = \sqrt{\frac{\sum(X-\mu)^2}{n}}$$

where,

σ = population standard deviation

$\sum$ = sum of...

μ = population mean

n = number of scores in sample (Huysamen, 1976).

CHAPTER 4
RESULTS

This study set out to identify the dominant bacteria associated with the spoilage of UHT full cream milk packaged by four different processing and packaging plants of two processors of UHT milk in South Africa (these processors will be referred to as Processor 1 and Processor 2). This chapter highlights the results of the study.

4.1 Visual Observation

The visual signs of microbial spoilage of the UHT milk are summarised in Table 3.

Table 3: Table showing seasons and visual signs of microbial spoilage

Processor and location	Season	Visual signs of microbial spoilage			
		Bulging	Coagulation	Fluorescence under UV	Cream separation
Processor 1 Queensburgh KZN	Winter	+	+	-	-
	Summer	+	+	-	-
Processor 1 Clayville, GP	Winter	-	+	-	-
	Summer	-	+	-	-
Processor 2 PE, EC	Winter	-	+	-	-
	Summer	-	-	-	-
Processor 2, Cape Town, WC	Winter	-	-	+	-
	Summer	+	-	+	-

+ indicates positive for the test, - Indicates negative for the test

Both the summer and winter samples from Queensburgh, KwaZulu-Natal (KZN) showed signs of bulging. When opened these milk packs were coagulated. On the other hand, the milk packs obtained from Clayville, Gauteng Province (GP) for both seasons were only coagulated, with no other signs of spoilage (Table 3).

The milk packs obtained during the winter from Port Elizabeth (PE) were coagulated, with no other signs of spoilage. On the other hand, the milk samples taken during

winter and summer from Cape Town (CT) showed varied signs of spoilage. The milk for the summer showed signs of bulging and the milk showed fluorescence with a greenish tint under ultra-violet (UV) light. The milk samples taken during the winter showed no signs of spoilage on initial observation; however, under ultra-violet (UV) light the milk was fluorescent with a greenish tint.

4.2 pH of the Milk

In this study the pH of all the milk samples collected was determined.

Processor 1

The average pH of all the UHT milk packs collected from Queensburgh during the winter and summer seasons was 4.2 ± 0.3 and 3.8 ± 0.3, respectively (Fig. 3). The difference in the mean pH observed in the samples for both seasons was not significant (p = 0.460).

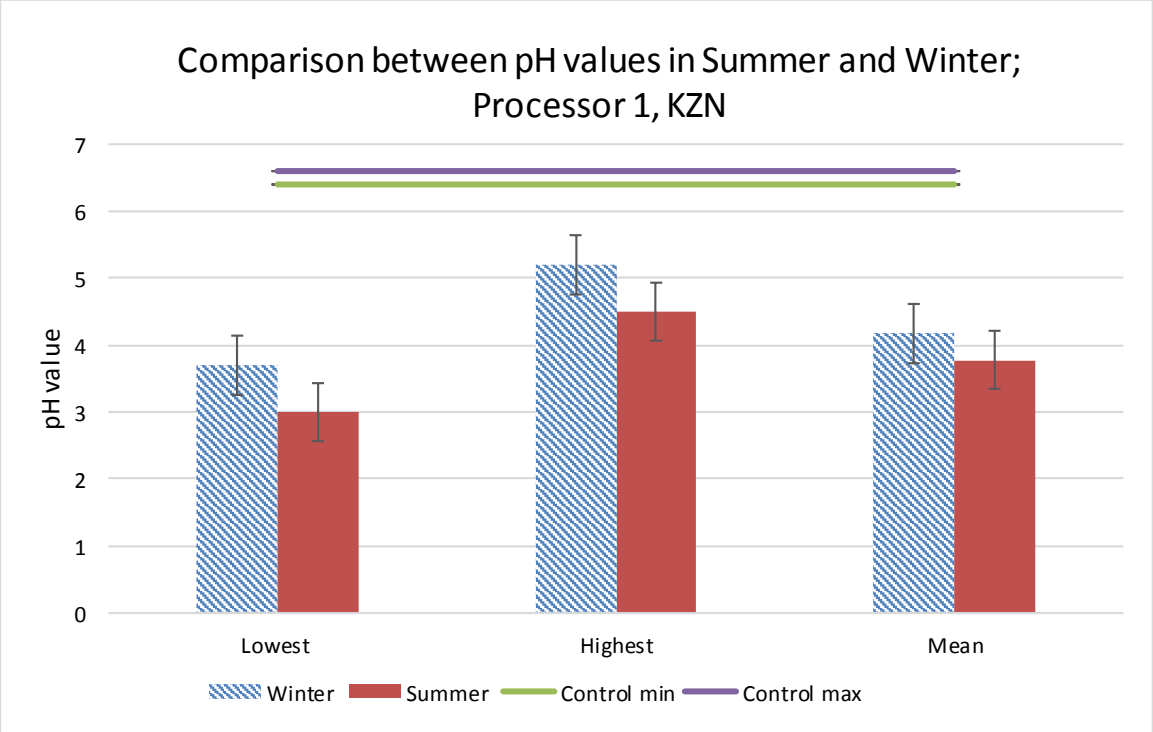

Figure 3: Comparison between the pH results of 100 samples of UHT milk processed during Winter and Summer by Processor 1, Queensburgh.

The highest pH values recorded from the samples taken from the Queensburgh plant during the winter and summer were 5.2 and 4.5, respectively, whereas the lowest pH values recorded during winter and summer were 3.7 and 3, respectively (Fig. 3).

The average pH of all the milk packs collected from Clayville during the winter and summer was 4.9 ± 0.3 and 6.4 ± 0.3, respectively (Fig. 4). The highest pH values from the Clayville samples during the winter and summer were 5.6 and 6.7, respectively, whereas the lowest pH values recorded during the winter and summer were 3.8 and 6, respectively (Fig. 4). The difference in the mean pH observed in samples taken during the summer and winter was highly significant (p = 0.00001).

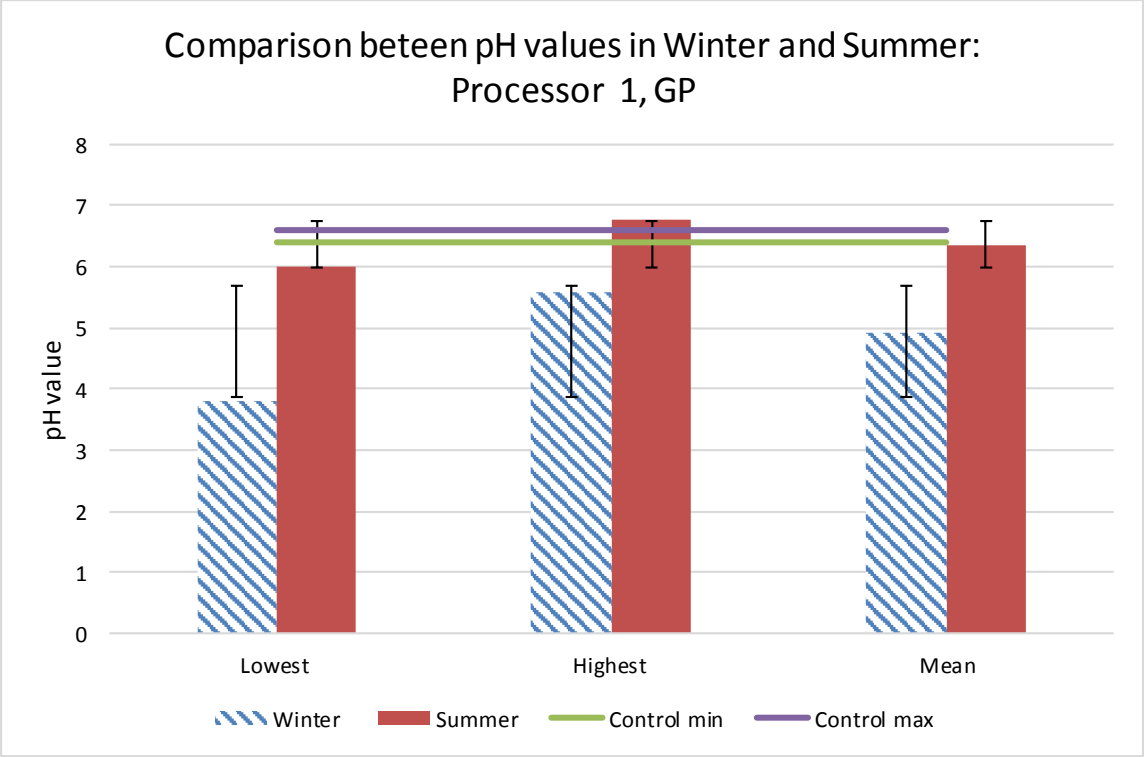

Figure 4: Comparison between the pH results of 100 samples of UHT milk process during Winter and Summer by Processor 1, Clayville

Processor 2

The average pH of all the UHT milk packs collected from the processing plant in PE during the winter and summer was 6.6 ± 0.013 and 5.4 ± 0.140, respectively (Fig. 5). The highest pH value recorded from this site during winter and summer were 6.6 and 5.7, respectively (Fig. 5), whereas the lowest pH values recorded during winter and

summer were 6.6 and 4.4, respectively (Fig. 5). The difference in the mean pH observed in samples taken during the summer and winter was highly significant (p = 0.00001).

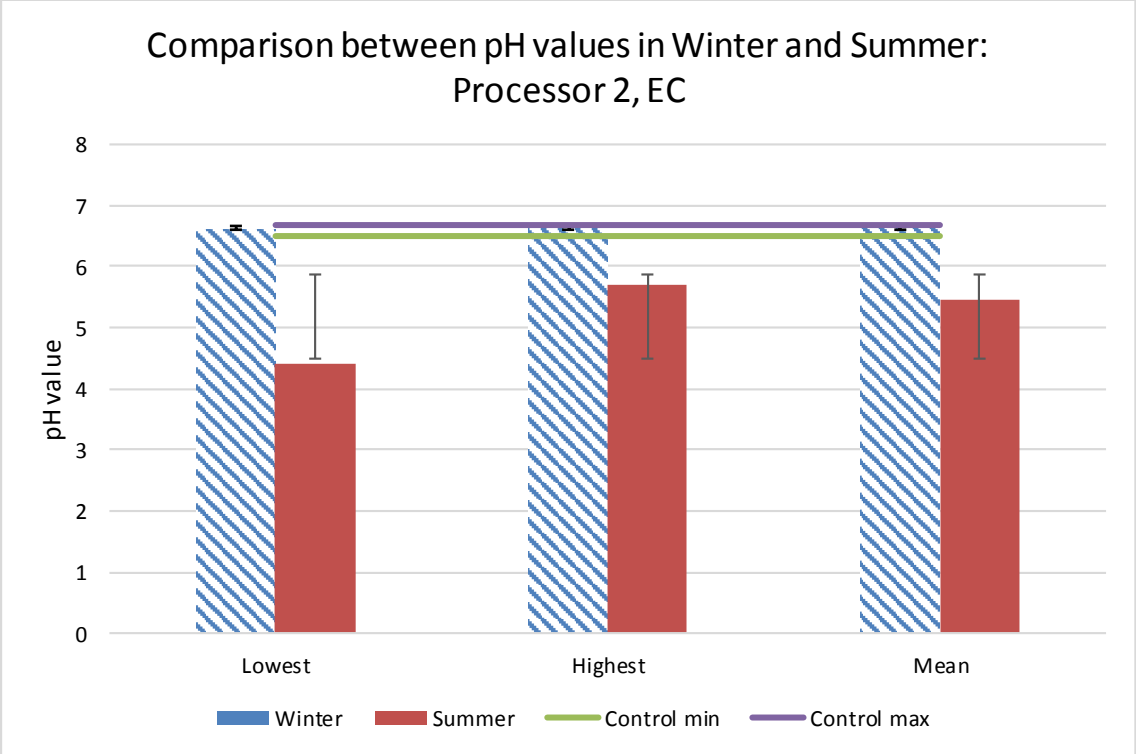

Figure 5: Comparison between the pH results of 100 samples of UHT milk process during Winter and Summer by Processor 2

The average pH of all the milk packs from the Western Cape (WC) during the winter and summer was 6.6 ± 0.073 and 3.9 ± 0.205, respectively (Fig. 6). The highest pH values recorded from the samples during the winter and summer were 6.7 and 4.5, respectively, whereas the lowest pH values recorded during the winter and summer were 6.0 and 3.0 respectively (Fig. 6). The difference in the mean pH observed in samples taken during the summer and winter was highly significant (p = 0.00001).

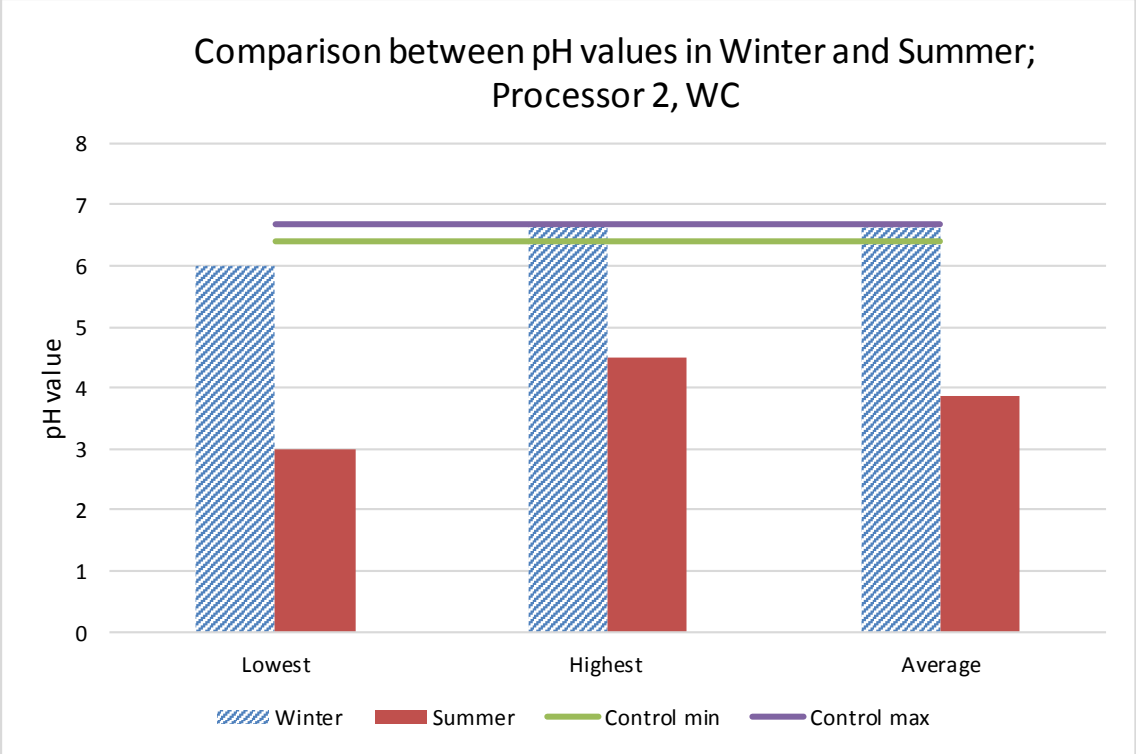

Figure 6: Comparison between the pH results of 100 samples of UHT milk process during Winter and Summer by Processor 2, Western Cape

4.3 Biochemical Characterisation

Preliminary biochemical characterisation was done to establish the genera of the organisms and specific identification to establish the species.

4.3.1 Preliminary Identification

The winter samples of processor 1 from Queensburgh produced two distinct colonies. The first colony (Colony A) consisted of motile rod-shaped organisms that tested negative for Gram-stain, positive for catalase and negative for oxidase (Table 4). The second colony (Colony B) consisted of non-motile rods that were Gram-positive, catalase negative and oxidase negative. However, the summer sample produced only one colony (Colony C).

Table 4: Processor, season, colony ID, morphological features, Gram reaction, catalase and oxidase test results of the colonies identified in the full cream UHT milk samples

Processor and location	Season	Colony ID	Gram reaction	Catalase	Oxidase
Processor 1 Queensburgh, KZN	Winter	Colony A	- motile rods	+	-
		Colony B	+ non-motile rods	-	-
	Summer	Colony C	- motile rods	+	-
Processor 1 Clayville, GP	Winter	Colony D	+ cocci that were arranged in pairs, irregular clusters and tetrads. growing in circular, entire, convex and had a creamy yellow pigmentation	+	-
	Summer	Colony E	+ non-motile rods	+	-
		Colony F	+ cocci that were arranged in pairs, irregular clusters and tetrads, growing in circular, entire, convex and had a creamy yellow pigmentation.	+	-
Processor 2 PE, EC	Winter	Colony G	+ straight motile rods with rounded ends, arranged singly or in chains	+	-
		Colony H	straight motile rods with spores and rounded ends, arranged singly or in chains	-	-
	Summer	Colony I	+ cocci that were arranged in pairs, irregular clusters and tetrads, growing in circular, entire, convex and had a creamy yellow pigmentation	+	-
Processor 2 Cape Town, WC	Winter	Colony J	- rods that were greenish and glowed under UV light	+	+
	Summer	Colony K	- rods that were fluorescent with a greenish hue under UV light.	+	-
				+	+
		Colony L	- motile rods	-	+

+ indicates that the organism tested positive, - Indicates that the organism tested negative

The isolated microorganism was motile, rod-shaped, Gram-stain negative, catalase positive and oxidase negative (Table 4). Distinct colonies were obtained from the winter sample from Clayville. The first colony (Colony D) consisted of cocci that were

arranged in pairs, irregular clusters and tetrads. It produced circular, convex and creamy yellow pigmented colonies. Colony D was Gram positive, catalase positive and oxidase negative. The second colony (Colony E) consisted of non-motile rods that were Gram-positive, catalase negative and oxidase negative. Two distinct colonies were obtained in the summer samples. The first (Colony F) consisted of cocci that were arranged in pairs, irregular clusters and tetrads. Colony F grew in circular, entire, convex and had a creamy yellow pigmentation. Colony F was Gram-positive, catalase positive and oxidase negative. The second colony (Colony G) consisted of straight motile rods with rounded ends, arranged singly or in chains. They were Gram-positive, catalase positive and oxidase negative.

One distinct colony (Colony H) was obtained in the sample from Port Elizabeth. Colony H consisted of straight motile rods with spores and rounded ends, arranged singly or in chains. They were Gram-positive, catalase positive and oxidase negative. One colony (Colony I) was obtained from summer sample. Colony I consisted of cocci arranged in pairs, irregular clusters and tetrads that grew in circular, entire, convex and creamy yellow pigmented colonies. They were Gram-positive catalase positive and oxidase negative.

One distinct colony (Colony J) was obtained from milk samples obtained in winter from Processor 2, Cape Town. This colony (J) consisted of rods that were straight or slightly curved and were fluorescent with a greenish hue under UV light. They were Gram-negative, catalase positive and oxidase positive. Two distinct colonies were obtained from the milk samples taken in summer. The first colony (Colony K) consisted of Gram-negative straight or slightly curved rods that were fluorescent with a greenish hue under UV light. They were catalase positive and oxidase positive. The second colony (Colony L) consisted of motile rods that were Gram-negative, catalase negative and oxidase positive.

Based on the results obtained from the Gram-stain, catalase and oxidase tests, a preliminary identification was done according to the Rough ID chart (Fig. 7) to determine the genera of the organisms.

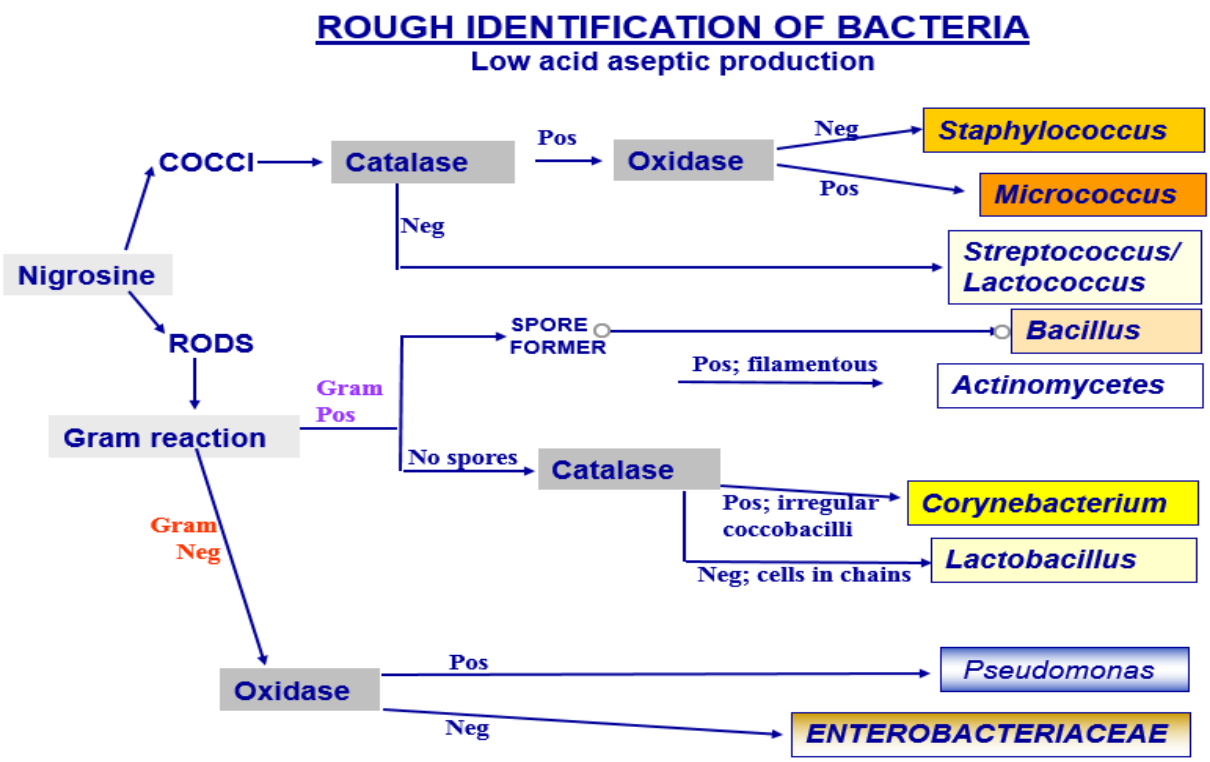

Figure 7: Chart used for preliminary identification of bacteria (TetraPak, 2013)

The consolidated results of the Gram-stain, catalase and oxidase tests and the preliminary identification of the genera of the bacteria was done according to the rough identification chart (Fig. 7) as shown in Table 5 below.

Table 5: Consolidated results of preliminary identification

Processor	Location of Plant	Season	Colony ID	Identificaton
Processor 1	Queensburgh	Winter	Colony A	*Enterobacter*
			Colony B	*Lactobacillus*
		Summer	Colony C	*Enterobacter*
	Clayville	Winter	Colony D	*Micrococcus*
			Colony E	*Lactobacillus*
		Summer	Colony F	*Micrococcus*
			Colony G	*Bacillus*
Processor 2	Port Elizabeth	Winter	Colony H	*Bacillus*
		Summer	Colony I	*Micrococcus*
	Cape Town	Winter	Colony J	*Pseudomonas*
		Summer	Colony K	*Pseudomonas*
			Colony L	*Enterobacter*

Following the results of the biochemical characterisation, five isolates were cultured from composites of colonies with identical morphology, and presumptively identified as being the same organism during the preliminary identification. The isolate identification numbers are shown in Table 6.

Table 6: Identification number of isolates cultured from composite cultures

Organism	Isolate no.
Pseudomonas	1
Lactobacillus	2
Bacillus	3
Micrococcus	4
Enterobacter	5

4.4 Specific Identification

4.4.1 Analytical Profile Index

The API is used for quick identification and classification of bacteria based on prior presumption of the microorganism achieved by examining the morphological features, Gram stain, catalase and oxidase tests. Based on the tests the appropriate API system is selected from the 15 available systems. API test strips consist of micro-tubes containing dehydrated substrates to detect the enzymatic activity or the fermentation of sugars by the inoculated organisms.

4.4.1.1 Isolate 1

Based on the results of the preliminary identification, isolate 1 was presumptively identified as *Pseudomonas*, which required the use of the API 20NE system. API 20NE should only be used with non-fastidious Gram-negative rods which do not belong to the *Enterobacteriaceae* family. The API 20NE strip consists of 20 microtubes containing dehydrated substrates. During incubation, metabolism produced colour changes that are either spontaneous or revealed by the addition of reagents. The colour changes indicated that the organisms tested positive for nitrate, esculin,

gelatinase and milate. The results were recorded on the results sheet, and the API software identified the organism as *Shewanella putrefaciens group*, with a 93.7% probability (Table 7).

Table 7: API identification results

Isolate	Preliminary identification	API identification	% Probability
1	*Pseudomonas*	*Shewanella putrefaciens group*	93.7
2	*Lactobacillus*	*Lactobacillus fermentum 1*	98.5
3	*Bacillus*	*Bacillus cereus 1*	95.0
4	*Micrococcus*	*Kochuria varians*	99.9
5	*Enterobacter*	*Enterobacter aerogenes*	98.9

4.4.1.2 Isolate 2

Isolate 2 was presumptively identified as *Lactobacillus*, which required the use of the API 50 CHL system, which tests carbohydrate fermentation. After incubation the colour changes were recorded on the results sheet. The API software identified the organism as *Lactobacillus fermentum 1* with a 98.5% probability (Table 7).

4.4.1.3 Isolate 3

Isolate 3 was presumptively identified as *Bacillus*, which required the use of the API 50 CHB system. The colour changes in the wells of the API strip were observed and the results recorded on the results sheet. The API software identified the organisms as *Bacillus cereus 1* with a 95% probability (Table 7).

4.4.1.4 Isolate 4

Isolate 4 was presumptively identified as *Micrococcus*, which required the use of the API STAPH system. The colour changes in the wells were recorded on the results sheet, and the API software identified the organism as *Kochuria varians* with a 99.9% probability (Table 7).

4.4.1.5 Isolate 5

Isolate 5 was presumptively identified as *Enterobacter*, which required the use of the API 20E. This is a standardized identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative rods. The colour changes in the wells were recorded on the results sheet. The API software identified the organisms as *Enterobacter aerogenes* with a 98.9% probability (Table 7).

4.4.2 Biolog

After incubation, the GEN III Microplates were placed into the OmniLog incubator/reader, and the organisms were identified by the Biolog Microbial Identification software. No visual observation of the results was required. The results are shown in Table 8.

Table 8: Biolog Identification System results

Isolate no.	Organism	Probability
1	*Pseudomonas aeruginosa*	0.952
2	*Lactobacillus corniformis* sp. *torquens*	0.787
3	*Bacillus cereus / thuringiensis*	0.596
4	*Micrococcus luteus*	0.589
5	*Enterobacter asburiae*	0.896

4.5 Molecular Profiling of the Isolates

4.5.1 Qualitative and Quantitative Assessment of DNA

The concentration and purity of the DNA solutions were measured using Computerized Nano Drop-spectrophotometer, and the A260/280 ratio was recorded. This is shown in Table 9.

Table 9: Concentration of undiluted and diluted DNA

Isolate #	A260/280 Ratio (undiluted)	DNA concentration undiluted (ng/µl)	DNA concentration diluted (ng/µl)
1	1.69	1296	62
2	1.74	1430	59
3	1.66	1102	52
4	1.61	3566	58
5	1.12	651	54

4.5.2 DNA Electrophoresis

The extracted 16s DNA was electrophoresed in a A 1 % (w/v) agarose gel (Sigma-Aldrich, St Louis, MO) with a 1000 bp plus DNA ladder (Sigma-Aldrich, St Louis, MO) was used as a molecular weight standard to compare the intensity of the five DNA isolates, as reported in Table 7, is shown in Fig. 7. The DNA concentration was too high, and was therefore diluted to be within the range of 50 to 65 ng/µl. Isolates 1 - 5 were diluted from 1296 to 62 (ng/µl), from 1430 to 59 (ng/µl), from 1102 to 52 (ng/µl), from 3566 to 50 (ng/µl) and from 51 to 54 (ng/µl), respectively. Both the undiluted and the diluted gel suspensions were submitted to DNA electrophoresis. The bands, as shown in Fig. 8, were observed with the DNA clearly having migrated through the gel. Isolates 1-5 had ratios >1.0 and concentrations ->50 ng/µl which were considered to be pure and detectable.

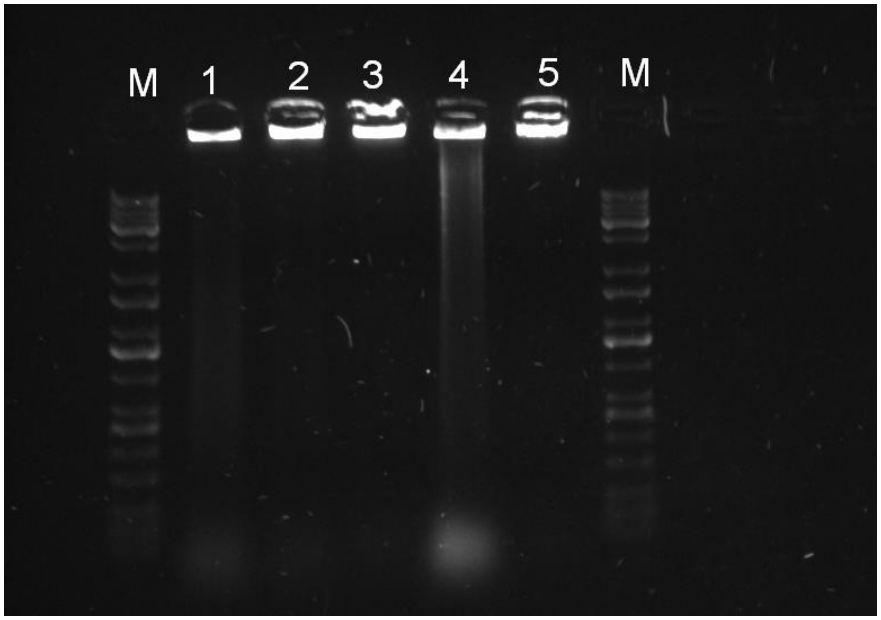

Figure 8: Electrophoresis of the genomic DNA

Figure 9 indicates the presence of DNA for both the diluted and undiluted DNA. However, as can be seen from Fig. 9, the difference between the results for the undiluted and diluted DNA was not significant. Therefore, only the undiluted DNA was sent to Inqaba Technologies for sequencing.

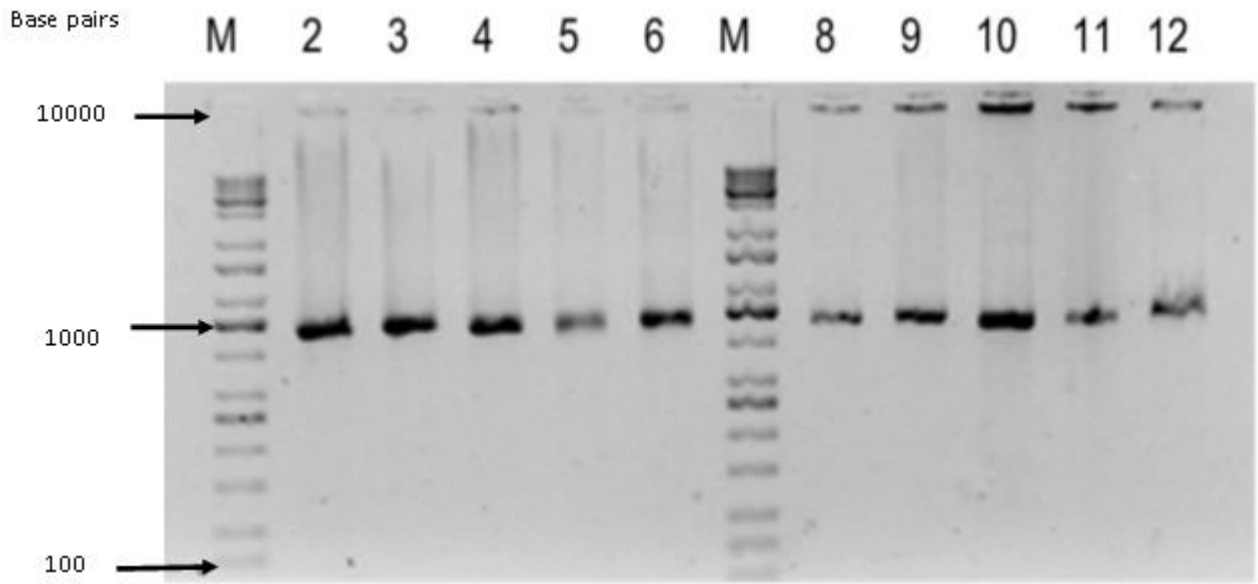

Figure 9: Electrophoresis of the 16S rRNA PCR fragments

4.5.3 Polymerase Chain Reaction (PCR)

The results of the PCR showed that the DNA had successfully migrated through the gel and all the isolates were 1000 bp long (Fig. 9).

4.5.4 Nucleotide Sequencing and Sequence Analysis

The 16S rRNA gene amplicons were sequenced in both directions at Inqaba Biotech (Pretoria, South Africa). The resulting sequence chromatograms were visualised and analysed using Chromas Lite 2.4 (Technelysium Pty, South Brisbane, Australia) and BioEdit v. 7.2.5. Sequencing of isolate 4 failed, as shown in Fig. 10.

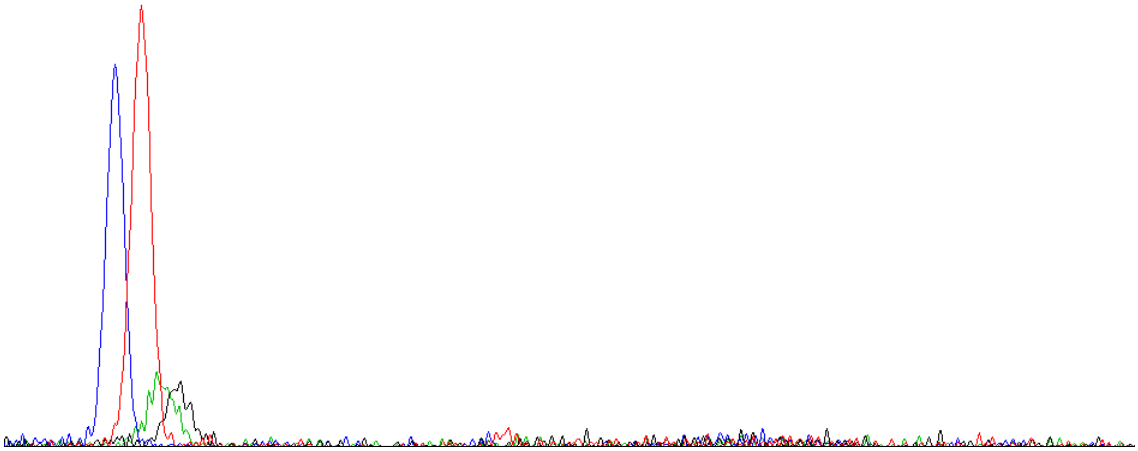

Figure 10: Chromatogram of isolate 4

The sequences were then compared to those in the GenBank, and the BLASTN results of all 5 isolates are summarized in Table 10.

Table 10: BLASTN results for isolates 1 - 5

Isolate no.	Description	Max score	Total score	Query cover %	E-value	Identity %	Accession no
1	*Pseudomonas aeruginosa*	1223	12233	100	0	99	KU570307.1
2	*Lactobacillus fermentum*	1171	1171	100	0	99	KT159934.1
3	*Bacillus cereus*	1125	1125	100	0	99	KU258289.1
4	*Uncultured bacterium*	ND	ND	ND	ND	ND	ND
5	*Enterococcus faecalis*	1013	1013	100	0	99	KU644346.1

ND: Not Determined

The results of the preliminary identification, specific identification and molecular profiling were consolidated and shown in Table 11 below.

Table 11: Consolidated results of organisms

ID	Preliminary Identification	Isolate no.	Specific identification		Molecular Profile
			API	BIOLOG	
Prc. 1 Queensburgh KZN. Winter	*Enterobacter*	5	*Enterobacter aerogenes*	*Enterobacter asburiae*	*Enterococcus faecalis*
	Lactobacillus	2	*Lactobacillus fermentum 1*	*Lactobacillus corniformis ss torquens*	*Lactobacillus fermentum*
Prc. 1 Queensburgh, KZN Summer	*Enterobacter*	5	*Enterobacter aerogenes*	*Enterobacter asburiae*	*Enterococcus faecalis*
Prc. 1 Clayville, GP Winter	*Micrococcus*	4	*Kochuria varians*	*Micrococcus luteus*	*Uncultured bacterium*
	Lactobacillus	2	*Lactobacillus fermentum 1*	*Lactobacillus corniformis ss torquens*	*Lactobacillus fermentum*
Prc.1 Clayville, GP Summer	*Bacillus*	3	*Bacillus cereus 1*	*Bacillus cereus / thuringiensis*	*Bacillus cereus*
	Micrococcus	4	*Kochuria varians*	*Micrococcus luteus*	*Uncultured bacterium*
Prc. 2 PE, EC Winter	*Bacillus*	3	*Bacillus cereus 1*	*Bacillus cereus / thuringiensis*	*Bacillus cereus*
Prc. 2 PE, EC Summer	*Micrococcus*	4	*Kochuria varians*	*Micrococcus luteus*	*Uncultured bacterium*
Prc. 2 Cape Town, WC Winter	*Pseudomonas*	1	*Shewanella putrefaciens group*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
Prc. 2 Cape Town, WC Summer	*Enterobacter*	5	*Enterobacter aerogenes*	*Enterobacter asburiae*	*Enterococcus faecalis*
	Pseudomonas	1	*Shewanella putrefaciens group*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1 Visual Observation of Microbial Spoilage

With the exception of cream separation, the different milk samples showed various visual signs of microbial activity (Table 3). Bulging of the pack is the most recognisable indication of milk spoilage because it is visible without the need to open the pack. Bulging of the packs is caused by gas formation as a result of fermentation. According to Cogan & Accolas (1990) lactic acid bacteria (LAB), such as *Lactobacillus* and *Streptococcus* ferment lactose in milk to galactose, glucose and lactic acid. Glucose can be further fermented not only to lactic acid, but also to lactic acid, ethanol and CO_2 (gas) which causes bulging. Bulging of the milk packs may also be due to coliforms such as *Enterobacter* that are able to ferment lactose with the production of acid and gas (Gilmour & Rowe, 1990), specifically hydrogen (Tanisho, 1998).

Coagulation of milk is a visual indicator of the presence of lactose fermenting microorganisms such as *Enterococcus, Lactobacillus, Lactococcus, Micrococcus* and *Streptococcus*. When lactose is fermented lactic acid is formed and the increased acidity causes the milk proteins (mainly casein) to tangle into solid masses (Goff, 2014). At normal pH levels (6.5 – 6.7), the main protein in milk, casein, remains evenly dispersed. At pH lower than 4.6, the protein coagulates due to production of acid from fermentation (Lu *et al*., 2013).

Further analysis of some of the milk samples showed slight coagulation which is also referred to as sweet curdling. Curdling without pronounced acid production is sweet curdling. This is prevalent in heat-treated milk, and is caused by thermoduric sporulant bacteria, especially *B. cereus/subtilis* that produce rennet-like enzymes that coagulate milk without souring. This is due to the production of an extracellular enzyme similar to rennin by bacteria that precipitate casein before the development of sufficient acidity (Goff, 2014). In this study sweet curdling was found only in the winter samples from Clayville and Port Elizabeth.

The milk samples from Cape Town were the only ones that showed fluorescence with a greenish tint. The greenish tint is caused by pyoveridine, a siderophore produced by

the bacteria. The pyoveridine, like any other siderophore, is employed by bacteria to bind iron in the environment, which is then delivered to the cell and used for respiration (Lindsey-Lynch, 2003).

The monitoring of visual signs of microbial spoilage is important because it is the first step in quality control for detecting potential spoilage in a batch of UHT milk. However, visual observation is not conclusive as spoilage may not necessarily result in visually observable signs, such as in the summer samples from Port Elizabeth. Other tests, such as pH, are used in conjunction with the results from the visual observation to determine the agents of spoilage.

5.2 pH as an Indicator of Spoilage

Fresh milk has a pH ranging between 6.5 and 6.7 and is therefore slightly acidic. Values higher than 6.7 denote mastitic milk and values below 6.5 indicate bacterial deterioration of milk specifically due to lactic acid producing microorganisms (TetraPak, 2013). This study showed, generally, that when the pH of the milk dropped below normal, the samples were coagulated (Fig. 3, 4, 5, 6 and Table 3). This was evident from the winter and summer samples obtained from Queensburgh, the winter samples from Clayville, and the summer samples from Port Elizabeth with the exception of Cape Town. The study highlights the relationship between pH and coagulation of milk. Coagulation is generally due to the presence of lactic acid bacteria. A slight drop in pH of the samples (6 – 6.7) may indicate early bacterial contamination, as in the summer samples from Clayville. Early contamination of the milk as observed in the winter samples from Port Elizabeth may not reduce the pH but still cause sweet coagulation. No seasonal correlations could be made with regards to the pH of the milk samples. The monitoring of pH is the second step in the quality control of UHT milk. However, since milk is a buffer solution, significant bacterial contamination may take place without a significant change in the pH, suggesting that pH cannot provide any accurate information regarding spoilage.

5.3 Identification of Spoilage Organisms

5.3.1 Preliminary Identification

The dominant bacteria presumptively identified in the milk samples were *Lactobacillus, Micrococcus, Bacillus, Pseudomonas* and *Enterobacter*. All these genera are psychrotrophs commonly associated with the spoilage of UHT milk (Adams *et al*., 1975; IDF Bulletin, 1976; Garg, 1990; Eneroth *et al.*, 1998; Frank & Hassan, 2003; Lafarge *et al*., 2004; Barbano *et al*., 2006; Mane *&* Ghandi 2010; De Jonghe *et al.*, 2011; Samaržija *et al*., 2012; De Oliveira *et al*., 2015). The genera identified were consistent with the pH values and visual signs of spoilage, as these genera are associated with these indicators of spoilage in UHT milk**.**

The preliminary identification did not reveal any seasonal predominance of specific bacteria, but rather that the occurrences of these bacteria were plant-specific, specifically at the Queensburgh, Clayville and Cape Town plants.

5.3.2 Specific Identification

The API and Biolog identification of the 5 isolates (Table 4) was consistent to the level of genera for isolates 2, 3 and 5 (Tables 5, 6). However, there were discrepancies in the identification of isolate 1 (*Pseudomonas*) and isolate 5 (*Enterobacter*) by the two systems. Isolate 1 was identified as *Shewanella* with the API system and *Pseudomonas* by the Biolog system. The Biolog system was in agreement with the initial classification using biochemical tests. The API system classified isolate 4 as *Kochuria.* There has been some discrepancy in the classification of Kochuria/*Micrococcus luteus. Micrococcus luteus* was re-classified as *Kochuria varians* by Stackebrand *et al*. (1995). The anomaly in identification by the different systems could therefore be considered as one of nomenclature within the identification systems, and not a contradictory identification.

5.3.3 Molecular Identification

Table 11 shows the results of the DNA identification of the 5 isolates from Table 4 to the species level. Isolate 4 was not identifiable. Although the DNA was successfully extracted and amplified by PCR, the sample could not be sequenced. The molecular identification was consistent with API and Biolog to the level of the genera for isolates 1 – 4. However, whereas API and Biolog identified isolate 5 as *Enterobacter*, it was identified by molecular profiling as *Enterococcus*. As species-specific PCR could be regarded as 100% accurate (Moraes *et al*., 2013), the discussion of the microbial spoilage of milk is based on the identification of the organisms at species level by BLASTN.

5.4 Microbial Spoilage of UHT Milk

The impact of *Enterococcus faecalis* on milk is similar to that of *Enterobacter*. Although *Enterococcus faecalis* is not a classic coliform (Jay, 2000), unlike *Enterobacter*, it is a common indicator organism; its presence in foods indicates faecal contamination, improper sanitation, and possible presence of pathogens (Gilmour & Rowe, 1990). As a LAB, they ferment glucose primarily to lactic acid, or to lactic acid, CO_2 and ethanol, thereby causing bulging of the packs. *Enterococcus* can cause a drop in the pH to below 4.6, resulting in coagulation through the precipitation of casein proteins. Although mesophilic, some can grow below 5ºC and some as high as 45 ºC (Jay, 2000).

Enterococcus does not survive pasteurization (Banks & Dalgleish, 2011). The presence of *Enterococcus* is therefore due to post-processing contamination. As an indicator organism, its presence could be attributed to poor hygienic practices, including insufficient cleaning-in-place (CIP). The Queensburgh plant has a high intake of short-term contract workers during the summer months, and the presence of *Enterococcus* could be indicative of poor training and supervision. However, *Enterococcus* was also found in the winter samples, and its presence could therefore not be ascribed to seasonal operational weaknesses.

Lactobacillus is used as a starter culture in the manufacturing of cheese and yoghurt in all the processing plants that were part of this study, and cross-contamination from raw materials in these processing plant is therefore one possible explanation for its presence. *Lactobacillus* is also indicative of poor CIP, and the presence of both *Enterococcus* (Winter and Summer) and *Lactobacillus* (Winter) at the Queensburgh plant could therefore be an indication of post-processing contamination or that the plant suffers from poor hygienic practices.

Lactobacillus and *Enterococcus* cause bulging of milk packs due to gas formation as a result of fermentation (Cogan & Accolas, 1990), as well as coagulation of milk due to lactose fermentation (Goff, 2014). The presences of these organisms are therefore consistent with the pH values and coagulation found in the milk samples from Queensburgh, Clayville and Cape Town. Where *Kochuria varians* occurred together with *Lactobacillus* in the winter samples from Clayville, their identification is consistent with a reduction in pH. However, although *Lactobacillus* is capable of causing bulging of the packs due to gas formation, the packs were not bulging. The pH distribution was also consistent with the coagulation and identification of *Kochuria varians* and *Lactobacillus fermentum,* as both species cause a reduction in pH, due to acid formation as a result of fermentation to below 4.6, at which point milk coagulates.

Despite reducing the pH to a range of 4.4 – 5.7, *Kochuria varians* does not cause observable signs of spoilage, as was observed in the summer samples from the Port Elizabeth. However, where *Micrococcus* co-occurred with *Bacillus*, there was only a slight reduction in the pH (6 – 6.6), and the milk was only slightly coagulated (sweet curdling), which is maybe due to contamination by *Bacillus cereus*. Where *Bacillus* was the only organism present, the pH stayed within the range of normal milk, and sweet curdling occurred. In this case, *Bacillus* appears to be the main causative agent of the observable signs of spoilage, and seemed to have mitigated the impact of *Kochuria varians* on the pH. One explanation for the predominance of the impact of *Bacillus* on the visual signs of spoilage and the pH in these samples is competitive exclusion (Li & Tian, 2012; Skandamis & Nychas, 2012). This finding confirms that pH is not a reliable indicator of milk spoilage.

The Clayville plant made major equipment purchases in the winter months and this led to major adjustments to the layout and design of the processing plant. It was found that some of the major pipes were not properly welded and these caused integrity breaches which could explain the entry of *K. varians* via air or water. The operational procedures during winter, however, do not explain the presence of *K. varians* in summer.

In the samples where *Pseudomonas* was present, the milk was fluoresced with a greenish tint under UV light. The greenish tint is consistent with the identification of *Pseudomonas*, which uses pyoveridine to bind iron in the environment, which is then delivered to the cell and used for respiration (Lindsey-Lynch, 2003). However, the pH was not indicative of microbial activity. Where *Pseudomonas* co-occurred with *Enterococcus* bulging of milk packs and fluorescence was probably due to the presence of *Enterobacter* and *Pseudomonas,* respectively, while the low pH was due to the presence of *Enterococcus. Pseudomonas aeruginosa* is found in soil and water and its presence in milk indicates poor cleaning practices. Similarly, the co-occurrence of *E. faecalis* could indicate inadequate cleaning practices in the plant.

Despite the discrepancies in the identification of the isolates, all the identified bacteria are psychrotrophs. Spoilage of pasteurized milk is due to one of two reasons; the growth of organisms that have survived the heat treatment, or post-pasteurization contamination. The presence of Gram-positive organisms suggests that spore-forming bacteria have survived heat treatment, whereas Gram-negative rods indicate post-pasteurization contamination (Banks & Dalgleish, 1990). A discussion of the bacteria that were found in the tested milk samples and isolates will consequently focus on the possible causes for the presence of the bacteria in the milk.

Psychrotrophs play a role in milk spoilage due to several key characteristics that enable them to grow in cold environments. Psychrotrophs are able to grow at 0ºC regardless of their optimal growth temperature (IDF Bulletin, 1976). Psychrophiles appear to have evolved genotypic and/or phenotypic features to overcome barriers inherent to permanently cold environments (D'Amico *et al*., 2006).

An extensive body of research exists on the role of psychrotrophs in the spoilage of raw and pasteurised milk. Due to the fact that psychrotrophs, especially *Pseudomonas*, are commonly associated with milk spoilage, and the fact that refrigeration is used for the storage of raw milk (IDF Bulletin, 1976 & Beales, 2004), contamination of milk by psychrotrophs is usually ascribed to the adaptation of psychrotrophs to a cold environment (D'Amico *et al*., 2006). However, UHT processing destroys psychrotrophs, and UHT milk is not refrigerated after thermal treatment. Refrigeration and cold adaptation of psychrotrophs can therefore not explain the presence of the identified bacteria, with the exception of *Bacillus cereus*, in the milk samples. *Bacillus cereus* is a spore-former and it could be argued that the spores survived the UHT process.

UHT processing is designed to kill the bacteria in raw milk. The presence of *Bacillus* in UHT milk could be due to spores that survived the UHT process. This explanation, however, does not account for the presence of the other bacteria, all of which are non-sporulating. Furthermore, the co-occurrence of *Bacillus* and *Micrococcus* in the summer samples from Clayville invalidates the explanation that spores, having survived the UHT process, were responsible for the presence of *Bacillus*. The presence of the non-sporeforming bacteria found in this study therefore requires further examination. The scope of this study was the identification of the dominant bacteria in the milk from selected manufacturers, and did not extend to the identification of their point of entry. However, a study of the dominant bacteria found in UHT inevitably raises the question regarding the source of the contamination, since the parameters of UHT processing are designed to effect commercial sterility by eliminating the organisms found in raw milk. Several studies provide evidence that the biggest source of post-pasteurization contamination is ineffective CIP procedures of the interior of processing equipment. This creates milk residues and biofilms that allow bacteria to multiply and contaminate subsequent milk flow (Austin & Bergeron, 1995; Allison *et al*., 1998; Mittelman, 1998, Ralyea *et al*., 1998; Adams & Moss, 2008; Raaijmakers *et al.*, 2010, Marchand *et al*., 2012; Samaržija, 2012; Toyofuku, *et al*., 2012; Teh *et al*., 2014; De Oliveira *et al*., 2015). These studies also reveal that the five genera of bacteria found in the milk samples in this study, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Lactobacillus* and *Enterococcus*, are commonly associated with the formation of biofilms in the interior of the post-UHT processing equipment, due to

ineffective CIP procedures (Austin & Bergeron, 1995; Ralyea *et al*., 1998; Fernandes, 2009). In addition, the biofilms act as a shelter for spore formers, especially *B. cereus* spores (Mosteller & Bishop, 1993; Eneroth *et al*., 1998; Sillankorva *et al*., 2008; Simões *et al*., 2010) and these spores are protected against the disinfectants commonly used in the dairy industry (Ryu & Beuchat, 2005; Bremer *et al.*, 2006; Adams & Moss, 2008).

De Oliveira *et al.* (2015) state that the limited experimental data generated to date indicates that the traditional approach of studying milk spoilage organisms as planktonic monocultures has most likely led to a distorted conceptualisation of spoilage processes *in situ*, and future studies would benefit from experimental approaches designed to mimic the biofilms found in bulk storage and processing systems.

5.5 Conclusion

There was some discrepancy in identifying some of the bacterial isolates which are pure isolated cultured colonies using the different identification systems being tools used in the identification. However, molecular characterisation of the main isolates found in the UHT milk showed that five genera of bacteria were present, namely *Pseudomonas*, *Micrococcus*, *Enterococcus*, *Lactobacillus* and *Bacillus*.

These psychrotrophic bacteria are destroyed by UHT processing, and their presence in milk could therefore only be explained with reference to post-processing contamination, specifically biofilm formation in the interior of the equipment, and inadequate CIP. Without further study, it is not possible to conclude whether the contamination is due to inadequate CIP procedures, unhygienic conditions inside the plants, or the level of resistance of biofilm to CIP procedures and agents.

This study showed that the geographical location and the two months of sampling had no influence on the organisms found in the milk samples. The same set of organisms was identified in the different locations and in two different seasons. The samples show a more distinct manufacturer-specific prevalence of bacteria. The identification

of organisms during both seasons at three of the plants indicates persistent contamination from a persistent source.

This study showed that UHT processing is not adequate in ensuring commercial sterility of UHT milk. Commercial sterility of the finished UHT processed product depends on excluding microbial contamination from two sources, namely:

- Raw milk which may harbour heat-resistant spores that could survive the UHT process
- Post-process contamination due to unsterile packaging and/or a failure in the integrity of the aseptic filling system (Gilmour & Rowe, 1990).

It emerged from this study that current UHT practices do not take cognisance of factors other than sufficient heat, such as biofilm and post-processing contamination, and cleaning practices. 'Other appropriate treatments' are also not sufficiently circumscribed, and are therefore inadequate in ensuring commercial sterility of milk.

De Oliveira *et al*., (2015) states that despite the existence of an extensive body of research, numerous gaps exist in our understanding of the biology of the psychrotrophic bacteria of importance for the dairy industry.

5.6 Recommendations

Stringent CIP protocols should be developed and adhered to.

Psychrotrophs require further studies, not as monocultures, but in biofilms in the post-processing equipment, to gain an understanding of the complex interaction between the organisms and their environment, and among the various species.

Further studies should be conducted to determine the source of contamination and point of entry of the organisms commonly associated with the spoilage of UHT milk.

A systemic approach to the realization of commercial sterility is necessary, one which encapsulates both the thermal treatment of raw milk, post-processing procedures and equipment, and CIP processes and materials.

Discrepancies with respect to conventional methods for identification of bacteria have been observed. Future studies should include the use of culture-independent genetic fingerprinting techniques due to the failure of some organisms to grow, culture-dependent methods may overestimate the microbial diversity. Moreover, culture-dependent methods may underestimate microbial diversity due to non-selectivity of the culture media.

REFERENCES

Adams, D.M., Barach, J.T. & Speck, M.L. (1975) Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. *Journal of Dairy Science,* 58: 828–834.

Adams, M.R. & Moss, M.O. (2008) *Food Microbiology.* (3rd Ed). RSC Publishing. Guildford, UK.

Afzal, A., Mahmood, M.S., Hussain, I. *et al.* (2011) Adulteration and microbiological quality of milk: a review. *Pakistan Journal of Nutrition,*10(12):1195-1202.

Allison, D.G., Ruiz, B., SanJose, C. *et al*., (1998) Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiology Letters*, 167: 179-184

Austin, J.W. & Bergeron, G. (1995) Development of bacterial biofilms in dairy processing lines. *Journal of Dairy Research,* 62: 509-19.

Azhar, M., Uniyal, V., Chauhan, N. *et al*. (2014) Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India. *International Journal of Current Microbiology and Applied Sciences,* 3: 753-760.

Bai, A.J. & Rai, V.R. (2011) Bacterial quorum sensing and food industry. *Comprehensive Reviews in Food Science and Food Safety,* 10: 183–193.

Banks, W. & Dalgleish, D.G. (1990) Milk and milk processing. In: Robinson, R.K. (ed.). *Dairy Microbiology*. Volume 1. (2nd Ed). London: Elsevier Applied Science.

Barbano, D.M., Ma, Y. & Santos, M.V. (2006) Influence of raw milk quality on fluid milk shelf life. *Journal of Dairy Science*. 89: E15–19.

Beales, N. (2004) Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Comprehensive Reviews in Food Science and Food Safety,* 3: 1–20.

Beveridge, T.J. (2001) Use of the Gram stain in microbiology. *Biotechnic & Histochemistry,* 76(3): 111-118.

BIOLOG. (2013) [Online] Available from: ww.biolog.com/products-static/microbial _identification_overview.php. [Accessed: 23/01/205]

Bosshard, P.P., Abels, S., Zbinden, R. *et al.* (2003) Ribosomal DNA sequencing for identification of aerobic, Gram-positive rods in the clinical laboratory. *Clinical Microbiology*, 41: 4134-4140.

Bramley, A.J. & McKinnon, C.H. (1990) The microbiology of raw milk. In: Robinson, R.K. (ed). *Dairy Microbiology,* Volume 1. London: Elsevier Applied Science: 163-208.

Bremer, P., Fillery, S. & Mcquillan, A.J. (2006) Laboratory Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology*, 106: 254-62.

Bylund, G. (1995) *Dairy Processing Handbook*. Sweden: Tetra Pak Processing Systems.

Caffarelli, C., Baldi, F., Bendandi, B. *et al.* (2010) Cow's milk protein allergy in children: a practical guide. Italian Journal of Pediatrics, 36: 5.

Chen, H. & Hoover, D.G. (2003) Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety*, 2: 82-100.

Chen, L., Daniel, R.M. & Coolbear, T. (2003) Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal*, 13: 255–275.

Chirlaque, R.A. (2011) Factors influencing raw milk quality and dairy products. Universidad Politecnica de Valencia, Gandia.

Cleto, S., Matos, S., Kluskens, L. *et al.* (2012) Characterization of contaminants from a sanitized milk processing plant, *PLoS ONE*. 7: 1–8.

Cogan, T.M. & Accolas, J.P. (1990) Starter cultures: types, metabolism and bacteriophage. In: Robinson, R.K. (ed). *Dairy Microbiology*. Volume 1. London: Elsevier Applied Science.

D'Almico, S., Collins, T., Marx, J.C. *et al*. (2006) *Psychrophilic microorganisms: challenges for life. EMBO*, 7(4): 385-90.

D'Aoust, J., Barnes, R., Carter, M. *et al*. (2007) *Food Safety Handbook.* bioMérieux France.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.P. & Raoult, D. (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of Clinical Microbiology*, 38(10): 3623–3630.

Datta, N. & Deeth, H.C. (2001) Age gelation of UHT milk - a review. *Food and Bioproducts Processing,* 79: 197–210.

Datta, N. & Deeth, H.C. (2007) *Advances in thermal and non-thermal food preservation.* Ames, Iowa: Blackwell Publishing.

Deegan, L.H., Cotter, P.D., Hill, C. & Ross, P. (2006) Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, 16: 1058-1071.

De Jonghe, V., Coorevits, A., Van Hoorde, K. *et al.* (2011) Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Applied Environmental Microbiology,* 77: 460-470.

De Oliveira, G.B., Favarin, L., Luchese, R.H. *et al*. (2015) Psychrotrophic bacteria in milk: how much do we really know? *Brazilian Journal of Microbiology,* 46(2): 313–321.

De Vos, P., Bucko, M., Gemeiner, P. *et al.* (2009) Multiscale requirements for encapsulation in medicine and biotechnology. *Biomaterials*, 30: 2559-2570.

Dunstall, G., Rowe, M.T., Wisdom, G.B. *et al.* (2005) Effect of quorum sensing agents on the growth kinetics of *Pseudomonas* spp. of raw milk origin. *Journal of Dairy Research,* 72: 276–280.

Dutta, A. & Chaudhuri, K. (2010) Analysis of tRNA composition and folding in psychrophilic, mesophilic and thermophilic genomes: indications for thermal adaptation. *FEMS Microbiology Letters,* 305(2): 100–108.

Eneroth, A., Christiansson, A., Brendehang, J. *et al.* (1998) Critical contamination site in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. *International Dairy Journal,* 8: 829-834.

Epstein, E. (2015) *Disposal and management of solid waste: pathogens and diseases.* New York: CRC Press.

Ercolini, D. (2003) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods,* 56: 297–314.

Erkmen, O. & Bozoglu, T.F. (2016) *Food microbiology: principles into practice*. NY: Wiley.

European Economic Council (1992) *Council Directive 92/46/EEC of 16 June 1992.* [Online] Available at: http://data.europa.eu/eli/dir/1992/46/oj. [Accessed: 14/03/2014]

EC (2005) *European Commission Regulation No 2074/2005 of 05 December 2005.* [Online] Available from: http://www.food.gov.uk/sites/default/files/multimedia/pdfs. [Accessed: 14/03/2014]

Fernandes, R. (2009) *Microbiology handbook: dairy products*. (3rd Ed.) Leatherhead Food International Microbiology Handbooks. [Online] Available from: http://ww.leatherheadfood.com. [Accessed: 12/02/2016]

Food and Agriculture Organization of the United Nations. (2012) *Food Outlook: Biannual Report on Global Food Markets*. [Online] Available from: http://www.fao.org/docrep/019 /i3473e/ i3473e.pdf. [Accessed: 14/07/2015]

Frank, J.F. & Hassan, A.N. (2003). Microorganisms associated with milk. In: Roginski, H., Fuquay, J.W. & Fox, P.F. (eds.*). Encyclopedia of dairy sciences*. London, UK. Academic Press, Elsevier Science.

Franzetti, L., & Scarpellini, M. (2007). Characterization of *Pseudomonas* spp. isolated from foods. *Annals of Microbiology*, 57: 39-47

Garg, S. K. (1990). Psychrotrophs in milk. *Indian Journal of Dairy Science,* 43(3): 433-440.

GBIF. (2012) GBIF Backbone Taxonomy. [Online] Available from: http://w.gbif.org/species/5427834. [Accessed: 16/09/2015]

Gerosa, S. & Skoet, J. (2012) *Milk availability: trends in production and demand and medium-term outlook*. [Online] Available at: www.fao.org/economic/esa. [Accessed: 16/07/2014]

Gilmour, A. & Rowe, M.T. (1990). In: Robinson, R.K. (ed.). *Dairy Microbiology.* Volume 1. (2nd Ed). London: Elsevier Applied Science Publishers.

Goff, H.D. (2014) *Dairy Science and Technology Education Series*. [Online] Available from: https://www.uoguelph.ca/foodscience/book-page/dairy-science-and-technology-ebook. [Accessed: 24/09/2014]

Griffiths, M.W. & Phillips, J.D. (1981) Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. *Journal of Applied Bacteriology,* 50(2): 289 - 303.

Griffiths, M. W., Phillips, J. D., & Muir, D. D. (1987) Effect of low temperature storage on the bacteriological quality of raw milk. *Food Microbiology,* 45: 285–291.

Griffiths, M.W. & Phillips, J.D. (1990) Incidence, source and some properties of psychrotrophic *Bacillus* spp found in raw and pasteurized milk. *International Journal of Dairy Technology,* 43 (3): 62-66.

Hassan, A.N. & Frank, J.F. (2001) Starter cultures and their use. In: Marth, E. & Steele (eds). *Applied dairy microbiology*. (2nd Ed). Basil, Switzerland: Marcel Dekker AG.

Haug, A., Høstmark, A.T. & Harstad, O.M. (2007) Bovine milk in human nutrition – a review. *Lipids in Health and Disease*, 6: 25.

Hayes, M.C. & Boor, K. (2001) Raw milk and fluid milk products. In: Marth, E. & Steele (eds). *Applied Dairy Microbiology*. 2nd Ed. Basil, Switzerland: Marcel Dekker AG.

Hayes M.C., Ralyea, R.D., Murphy, S.C. *et al*. (2001) Identification and characterization of elevated microbial counts in bulk tank raw milk. *Journal for Dairy Sciences,* 84: 292-8.

Holmes, B., Costas, M., Ganner, M. *et al*. (1994) Evaluation of Biolog system for identification of some gram-negative bacteria of clinical importance. *Journal of Clinical Microbiology,* 32: 1970–1975.

Holsinger, V.H., Rajkowski, K.T. & Stabel, J.R. (1997) Milk pasteurisation and safety: a brief history and update. *Scientific and Technical Review of the Office International des Epizooties,* 16: 441-451.

Huysamen, G.K. (1976) *Inferensiële statistiek en navorsingsontwerp*. H&R Acemica: Pretoria.

IDF Bulletin (1976) *Psychrotrophs in milk and milk products*. International Dairy Federation, Brussels, E-Doc 68.

O'Connor, C.B. (1995) Rural dairy technology*. ILRI Training Manual.* ILRI: Addis Ababa.

Jay, J.M. (2000) *Modern food microbiology*. 6th Ed. Maryland: Aspen Publishers, Inc.

Jaspe, A., Oviedo, P., Fernandez, L. *et al.* (1995) Cooling raw milk: change in the spoilage potential of contaminating *Pseudomonas. Journal of Food Protection,* 58: 915–921.

Kiiyukia, C. (2003). *Laboratory manual of food microbiology for Ethiopian Health and Nutrition Research Institute.* Unido Project Ya/ETH/03/436/.

Klein, C., Olsson, L. & Nielsen, J. (1998) Glucose control in Saccaromyces cerevisiae: the role of Mig1 in metabolic functions, *Microbiology*, 144: 13-24.

Kozdroj, J. & Van Elsas, J.D. (2000) Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. *Soil Biology and Biochemistry*, 32(10): 1405-1417.

Lafarge, V., Ogier, J.L., Girard, V. *et al.* (2004) Raw cow milk bacterial population shifts attributable to refrigeration. *Applied and Environmental Microbiology,* 70: 5644-5650.

Lane, D. J., Pace, B., Olsen, G. J. *et al*. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America,* 82: 6955-6959.

Ledenbach, L.H. & Marshall, R.T. (2010) Microbiological spoilage of dairy products. In: Sperber, W.H. & Doyle, M.P. (eds.) *Compendium of the Microbiological Spoilage of Foods and Beverages*, NY: Springer Science and Business Media: 41- 67.

Li, M., Gong, J., Cottrill, M. *et al.* (2003) Evaluation of QIAamp DNA Stool Mini Kit for ecological studies of gut microbiota. *Journal of Microbiological Methods,* 54: 3–20.

Li, Y-H. & Tian, X. (2012) Quorum sensing and bacterial social interactions in biofilms. *Sensors*, 12: 2519-2538.

Lindsey-Lynch, R. (2003) *Science in the real world*. [Online] Available from: http://www.umsl.edu/~microbes/pdf/Something's%20Glowing.pdf. [Accessed: 17/02/2015]

Liu, X., Krause, W.C. & Davis, R.L. (2007) GABA (A) receptor RDL inhibits Drosophila olfactory associative learning. *Neuron* 56 (6): 1090- 1102.

Lu, M., Shiau, Y., Wong, J. *et al.* (2013) Milk spoilage: methods and practices of detecting milk quality. *Food and Nutrition Sciences*, 2013: 113-123.

Lowy, F. (2009) *Bacterial classification, structure and function*. [Online] Available from: www.columbia.edu/itc/hs/medical/pathophys/id/2009/intorNotes. [Accessed: 14/4/2014]

Mane, N.V. & Gandhi, M.B. (2010) Studies on proteolytic thermoduric psychrotrophic bacteria in milk and fermented milk products. *Journal of Environmental Research and Development,* 5: 348 – 355.

Mann, E.E. & Wozniak, D.J. (2012) Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiology Review*, 36(4): 893–916.

Mantere-Alhonen, S. (1995) Propionibacteria used as probiotics: a review, *Le Lait*. 75: 447-452.

Marchand, S., De Block, J., De Jonghe, V. *et al*. (2012) Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety*, 11: 133–147.

McPhee, J.D. & Griffiths, M.W. (2011) Psychrotrophic bacteria *Pseudomonas* spp. In: John, W.F. (ed.) *Encyclopedia of Dairy Sciences*. 2nd. ed. Academic Press: San Diego.

Merck Manual. (2010). Whitehouse Station, 10th Ed. N.J: Merck & Co.

Miller, J.M. & Rhoden, D.L. (1991) Preliminary evaluation of Biolog: a carbon source utilization method for bacterial identification. *Journal of. Clinical. Microbiology*, 29: 1143-1147.

Mittelman, M.W. (1998) Structure and functional characteristics of bacterial biofilm in fluid processing operations. *Journal of Dairy Science,* 81: 2760-2764.

Mosteller, T.M., Bishop, J.R. (1993) Sanitizer efficacy against attached bacteria in milk biofilm. *Journal of Food Protection,* 56: 34-41.

Moraes, P.M., Perin, L.M. Silva Júnior, A. *et al.* (2013) Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Brazilian Journal of Microbiology,* 44: 109-112.

Muir, D.D. (1990) The shelf-life of dairy products: factors influencing raw milk and fresh products. *Journal of the Society of Dairy Technology,* 49 : 24-32.

Muyzer, G. (1999) DGGE/TGGE: A method for identifying genes from natural ecosystems. *Current Opinion in Microbiology,* 2: 317-322.

Nabrdalik, M., Grata, K. & Latala, A. (2010) Proteolytic activity of *Bacillus cereus* strains. *Proceedings of ECOpole, 4:* 273-276.

Němečková I., Solichova, K., Roubal, P. *et al*. (2011) Methods for detection of *Bacillus* sp., *B. cereus*, and *B. lichenformis* in raw milk*. Czech Journal of Food Sciences.* 29 (Special Issue): S55-S60.

O'Brien, B. & Guinee, T.P. (2011) Milk: seasonal effects on processing properties of cows' milk. In: Fuquay, J.W. (ed.) *Encyclopedia of Dairy Sciences.* 2nd Ed. Academic Press; San Diego: 598–606.

Onilude, A.A., Fagade, O.E., Bello, M.M. & Fadahunsi, I.F. (2005) Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *African Journal of Biotechnoly,* 41: 21-26.

Overman, E.M. (1998) *Aseptic packaging and shelf stability of foods*. M.S. Report. Kansas State University.

Popp, J. & Bauer, M. (2015) *Modern techniques for pathogen detection*. Weinheim, Germany: Wiley Blackwell.

Potter, N.N. & Hotchkiss, J.H. (2013) Food Science. 5th Ed. ML: Aspen.

Quigley, L., O'Sullivan, O., Beresford, T.P. *et al.* (2011) Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology,* 150: 81-94.

Raaijmakers, J.M., De Bruijn, I., Nybroe, O. *et al.* (2010): Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiology Reviews*, 34*:* 1037-1062.

Raats, D., Offek, M. & Minz, D. (2011) Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. *Food Microbiology,* 28: 465–471.

Ralyea, R.D., Wiedmann, M. & Boor K.J. (1998) Bacterial tracking in a dairy production system using phenotypic and ribotyping methods*. Journal of Food Protection,* 61: 1336-1340.

Reese, B.K., Koester, J.A., Kirkpatrick, J. *et al.* (2014) How extreme is extreme? *Eco-DAS X Symposium Proceedings*: 69-87.

Riley, M.A. & Wertz, J.E. (2002) Bacteriocins: evolution, ecology and application. *Annual Review of Microbiology*, 56: 117-137.

Rouvinen, V. (2010) *Changes in bacterial population and antimicrobial resistance during cold storage of conventional and organic raw milk*. A thesis submitted in partial fulfilment of the requirements of the University of Helsinki for the Degree of Magister Technologiae. [Online]. Available at: http://hdl.handle.net/10138/17240. [Accessed: 14/08/2014]

Ryu, J. H. & Beuchat, L. R. (2005) Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *Journal of Food Protection,* 68: 2614–2622.

Ryser, E.T. (2001) Public health concerns. In: Marth, E. & Steele. *Applied dairy microbiology*. 2nd Ed. Basil, Switzerland: Marcel Dekker AG.

Samaržija, D., Zamberlin, S. & Pogačić, T. (2012) Psychrotrophic bacteria and milk quality. *Mljekarstvo,* 62: 77-95.

SAMPRO. (2014) *Trends of retail sales of nine dairy products and eight other food products based on information up to December 2012.* [Online]. Available from: ww.milksa.co.za/category/all-reports/industry-information-reports/trends-milk-production. [Accessed: 20/11/2014]

Scott, D.L. (2008) *UHT processing and aseptic filling of dairy foods.* A report submitted in partial fulfilment of the requirements for the degree Master of Science of Kansas State University.

Simões, M., Simões, L.C. & Vieira, M.J. (2008). Physiology and behavior of *Pseudomonas fluorescens* single and dual strain biofilms under diverse hydrodynamics stresses. *International Journal for Food Microbiology*, 128: 309–316.

Sillankorva, S., Neubauer, P. & Azeredo, J. (2008) *Pseudomonas fluorescens* biofilms subjected to phage philBB-PF7A. *BMC Biotechnology*, 78*:* 1-12.

Singh, H., McCarthy, O.J., & Lucey, J.A. (1997) Physico-chemical properties of milk. In: Fox, P.F. (ed.) *Advanced Dairy Chemistry.* Volume. 3. London: Elsevier Applied Science.

Skandamis, P.N. & Nychas, G.J. (2012) Quorum sensing in the context of food microbiology. *Applied Environmental Microbiology,* 78: 5473-5482

Sørhaug, T. & Stepaniak, L. (1997) Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends in Food Science & Technology,* 8: 35–41.

Sperber, W. & Doyle, M.P. (2010) *Compendium of the microbiological spoilage of foods and beverages*. New York: Springer.

Stackebrandt, E., Koch, C., Gvozdiak, O. *et al.* (1995) Taxonomic Dissection of the Genus *Microcossus: Kocuria* Gen. nov., *Nesterenkonia* Gen. nov., Kytococcus Gen. nov., *Dermacoccus* Gen., nov., and *Micrococcus cohn* 1872 Gen. Emend. *International Journal of Systematic Bacteriology*, 45: 682-692.

Tabit, F.T. & Buys, E. (2011) Incidence and survival of *Bacillus Sporothermodurans* during processing of UHT milk. *British Food Journal*, 113: 505-518.

Teh, K.H., Flint, S. & Palmer, J. (2014) Biofilm - an unrecognised source of spoilage enzymes in dairy products? *International Dairy Journal*, 34: 32–40.

TetraPak. (2008) *An introduction to commercial sterility*. [Online] Available from: http://www.tetrapak. com/ documentbank/Sterility_63601.pdf. [Accessed: 08/06/2014]

TetraPak. (2013) *Internal manufacturer trends*. [Online] Available from: http://www.intranet@tetrapak.com /records/trends. [Accessed: 03/12/2014]

Todar, K. (2012) *Todar's online textbook of bacteriology*. [Online] Available from: http://www.textbookofbacteriology.net. [Accessed: 17/05/2016]

Toyofuku, M. Roschitzki, B. Riedel, K. *et al*. (2012) Identification of proteins associated with the *Pseudomonas aeruginosa* biofilm extracellular matrix. *Journal of Proteome Research,* 11: 4906-4915.

Vanetti, M. (2009) Spoilage detection. In: Nollet, L.M. & Toldra, F. (eds) *Handbook of Dairy Foods Analysis.* Boca Raton: CRC Press.

Von Bocklemann, B. & Von Bocklemann, I. (1998) *Long-life products: heat-treated, aseptically packed: a quality guide*. Akarp, Sweden: Dr Bernhard von Bocklemann.

[Online] Available from: https://ingalimentos.files.wordpress.com/2007 /10/long-life-products.pdf. [Accessed: 20/06/2014]

Zall, R.R. (1990) Control and destruction of microorganisms. In: Robinson, R.K. (ed.). *Dairy Microbiology*. Volume 1. (2nd Ed). London: Elsevier Applied Science.