

**The impact of the combined lactoperoxidase and pasteurisation  
treatment on the safety of goat milk and cottage cheese.**

By

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## **DECLARATION**

I declare that the dissertation submitted for the degree Masters in Institutional Agriculture (Food production and processing) at the University of Pretoria has not been previously been submitted by me for a degree at any other university or institution of higher education.

## **DEDICATION**

This thesis is dedicated to my younger brothers (Thatayaone, Keeme, Maatla) and sister (Kealeboga) Mariba, as I would like to see them doing well in their studies.

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## ABSTRACT

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### **The impact of the combined lactoperoxidase and pasteurisation treatment on the safety of goat milk and cottage cheese.**

By

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Supervisor: Prof E. M. Buys

Department: Food Science

Degree: Masters in Institutional Agriculture (Food production and processing)

This study investigated the effect of the Lactoperoxidase system (LPS) alone and in combination with pasteurisation, on the growth of *Listeria monocytogenes* (LM) ATTC 7644 in goat milk and goat milk cottage cheese during a shelf life of 10 days at 4 °C.

Goat milk was inoculated with LM ATTC 7644 and divided into two samples, one the control and Lactoperoxidase (LP) was activated in the other sample. Both the control and LP activated samples were kept at ambient temperature for 6h. After 6h the control and LP activated samples were again divided into two and one of the respective samples was pasteurised at 72 °C for 15 s. All the four samples were analysed for LM ATTC 7644 immediately after LP activation at 0h, after 6h of LP activation and after pasteurisation. Goat milk cottage cheese was made with all four samples, i.e. control raw, control pasteurised, LP activated raw and LP activated pasteurised goat milk and analysed for LM ATTC 7644 on days 1, 2, 5, and 10.

Six hours after LP activation the mean LM ATTC 7644 count for the LP activated milk decreased by log 0.5 cfu/ml where as the LM ATTC 7644 for the control increased by log 0.5 cfu/ml. The reduction of LM ATTC 7644 count in LP activated milk when

compared to the control shows that goat milk lactoperoxidase is capable of reducing *L. monocytogenes* when stored at ambient temperatures.

Furthermore, LM ATTC 7644 count in LP activated pasteurised goat milk decreased by log 1.1 cfu/ml more, compared to the control pasteurised goat milk. Therefore, pasteurisation together with LP activation may be more effective than pasteurisation alone in controlling the growth of *L. monocytogenes* in goat milk.

For the control raw goat milk cottage cheese on day 10, the LM ATTC 7644 count was 90 % less than on first day of storage. The LP activated raw goat milk cottage cheese count followed a similar trend to the control raw goat milk cottage cheese, and reached levels of log 2.9 cfu/g on the last day of storage. The control pasteurised goat milk cottage cheese LM ATTC 7644 count on day 10 was 92 % lower compared to day 1 where as the LP activated pasteurised goat milk cottage cheese LM ATTC 7644 count was 98 % less than on day 1.

The results of this study indicate that the activation of the LPS significantly ( $p \leq 0.05$ ) decreased the LM ATTC 7644 count in goat milk, during a period of 6h. Combined pasteurisation and LP activation had a synergistic effect on the LM ATTC 7644 count in goat milk. The LM ATTC 7644 count declined in cottage cheese made from both control and LP activated goat milk. A greater decrease was observed in LP activated pasteurised goat milk cottage cheese over the storage period of 10 days at 4 °C. This combination may be used to reduce the multiplication of LM ATTC 7644 for production of safer products like goat milk and goat milk cottage cheese.

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## CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

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### 1.1 INTRODUCTION

Milk in developing countries such as Ethiopia and Kenya is transported to collection centres (Lambert, 2001). From the collection centres it is then sent to processing plants by unrefrigerated trucks (Barabas, 1995). The delay between milking and processing can exceed six hours and the lack of modern storage facilities and refrigerated transport can contribute to difficulties in preserving milk in developing countries (Lambert, 2001; Barabas, 1995). In countries with high ambient temperatures (around 30 °C), bacterial growth is accelerated at these temperatures, affecting the bacteriological quality of milk, causing deterioration and affecting the shelf life and safety (Barabas, 1995).

Alternative processes are therefore required to address this problem. One such process that can be applied to delay milk deterioration is the Lactoperoxidase system (LPS) (Pruitt and Reiter, 1985; Barabas, 1995; Zapico, Medina, Gaya, and Nunez, 1998). The LPS consists of three components, the lactoperoxidase enzyme, a substrate ( $\text{SCN}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ , or  $\text{Cl}^-$ ) and hydrogen peroxide (Davidson and Brannen, 1993). The lactoperoxidase is an enzyme in milk saliva and tears (Pruitt and Kamau, 1991; Pruitt and Reiter, 1985). The lactoperoxidase enzyme catalyses the oxidation of thiocyanate a substrate (e.g.  $\text{SCN}^-$ ) to hypothiocyanous acid ( $\text{HOSCN}^-$ ) and hypothiocyanate ( $\text{OSCN}^-$ ) (Davidson and Brannen, 1993). Hypothiocyanate and hypothiocynous acid are highly reactive oxidising agents and react with sulfhydryl ( $\text{SH}^-$ ) groups, of bacterial cells (Pruitt and Reiter, 1985; Kamau, Doores and Pruitt, 1990a).

*Listeria monocytogenes* has been implicated as a causative agent in several large outbreaks of listeriosis around the world, caused by consumption of milk and milk products (Lunden, Tolvane and Korkeala, 2004). Listeriosis outbreaks have been associated with dairy products manufactured from raw milk products, especially soft cheeses. Pasteurised milk and milk products have also been implicated in outbreaks due to suspected post pasteurisation contamination of products by *L. monocytogenes* or inadequate pasteurisation. *L. monocytogenes* has caused outbreaks of listeriosis in the USA, Canada and Europe (Lunden *et al.*, 2004). The organism is a psychotropic

foodborne pathogen widely distributed in nature (Farber and Peterkin, 1991). It can survive for many years in the cold and in naturally infected sources (Farber and Peterkin, 1991). *L. monocytogenes* is a gram positive, motile short rod, capable of growth at 4 °C (Frazier and Westhoff, 1988). It is an opportunistic pathogen occurring sporadically and primarily infecting immune compromised individuals (Farber and Peterkin, 1991).

According to Seifu, Buys, Donkin and Petzer, (2004a) LPS activation has a bactericidal effect against *L. monocytogenes* in goat milk at 30 °C. Kamau *et al.*, (1990a) indicated that activation of the LPS before thermal treatment can enhance thermal destruction of *L. monocytogenes* in milk during pasteurisation. Therefore LPS in combination with pasteurisation may be used to address the growth of *L. monocytogenes* in goat milk and goat milk cottage cheese. The aim of this study was to determine the effect of the LPS alone and in combination with pasteurisation on the growth of *L. monocytogenes* in goat milk and goat milk cottage cheese.

## **1.2 LITERATURE REVIEW**

### **1.2.1 Goat milk production and its importance in developing countries**

World milk production has increased since 1999 from 562 million tonnes to 630 million tonnes in 2005 (FAO, 2006). In 2005 world goat milk production was 13 million tonnes, with Africa alone producing about 2 800 000 million tonnes. Asia produced around 6 700 000 million tonnes of goat milk and South America produced an average of 180 000 million tonnes in 2005 (FAO, 2006). South Africa produced about 2 180 tonnes of goat milk in 2004 (FAO, 2004).

Promotion of goat milk is important in developing countries as the populations grow rapidly thus increasing demand for additional animal protein food (Devendra and McLeroy, 1982). Goat milk is a good source of quality animal protein and can assist in reduction of malnutrition especially in children (Chamberlain, 1989). For people with cow milk allergies, cow lactalbumin and gastro-intestinal ailments, goat milk is an ideal substitution (Haenlein, 2004). Goat milk also has other health benefits such as reduction

of cholesterol levels and has higher levels of essential amino acids compared to cow milk, which if consumed, meet or even exceed the daily dietary requirements (Haenlein, 2004). The milk is also easily digestible since it has smaller fat globules (Devendra and McLeroy, 1982). In developing countries about 20 % of milk produced is lost or undervalued because of poor microbial quality (Lambert, 2001) because of this, techniques that can decrease microbial load of milk and extend shelf life are required.

### 1.2.1.1 Goat milk safety

Milk drawn from a healthy udder is not sterile but has low microbial count (Varnam and Sunderland, 1994). Contamination of freshly drawn milk may come from the environment, equipment, dust, personnel, transport or the water source (Varnam and Sunderland, 1994). De-Buyser, Dialasser, Hummel and Bergdoll, (1987) found that 34 % of goat milk samples were positive for staphylococci but had no evidence of clinical mastitis. *Pseudomonas* spp. usually represents not more than 10 % of the microbial population of freshly drawn milk (Sorhaung and Stepaniak, 1997). According to Haenlein, (1996) goats do not or rarely suffer from tuberculosis and they may have much safer milk with lower microbial counts than cow milk.

Bacteria in freshly drawn milk may increase due to mastitis as bacteria may enter through the teat duct and may be shed into the milk. Bacteria that most commonly cause mastitis include the following *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus* spp. These organisms can be pathogenic to humans (Varnam and Sunderland, 1994). Other human pathogens that occasionally cause mastitis include *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Nocardia* spp, *Actinomyces* spp. as well as enteric pathogens *Salmonella*, *Bacillus cereus*, *Clostridium perfringens* and *L. monocytogenes* (Varnam and Sunderland, 1994).

De-Buyser *et al.*, (1987) found that *Staphylococcus aureus* was the predominant Staphylococcal species isolated from goats with clinical symptoms of mastitis. *Staphylococcus aureus* is capable of producing a toxin that may lead to food poisoning if the milk contaminated by the toxin in high concentrations is consumed (White and Hinckley, 1999). White and Hinckley, (1999) continue to say that the most common organism isolated from goat milk is the non-haemolytic *Staphylococcus* spp. Apart from

being a threat to milk safety, staphylococcal infections may become chronic and may lead to udder soreness, elevated somatic cell count and decreased production of milk. White and Hinckley, (1999) also isolated *Streptococcus* spp., *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli* and *Pseudomonas* spp. from the infected goat milk. Schneider, (1994) says a number of healthy goats are subclinical carriers of *L. monocytogenes* and the animals may excrete the organism in their faeces and milk when stressed.

#### **1.2.1.2 Effect of pasteurisation on microbial quality of goat milk.**

Pasteurisation of milk is used to reduce the amount of spoilage microorganisms and inhibit growth of pathogenic vegetative cells. Spore formers and thermophilic gram positive bacteria may survive pasteurisation. Spore formers can either be unaffected by pasteurisation or activated (Jay, 2000; Giffel, Beumer, Hoekstra and Rombouts, 1995). Among the bacteria that may survive pasteurisation *Bacillus* spp. dominates and also dominates among psychrotrophs. *Bacillus cereus* produces an enterotoxin which may cause illness in humans (Varnam and Evans, 1996). Other thermophilic psychrotrophs that may survive pasteurisation are the *Arthrobacter* spp, *Microbacterium* spp, *Streptococcus* spp, *Corynebacterium* spp and *Clostridium* spp. Although *Pseudomonas* spp may not survive pasteurisation temperatures, they may release enzymes (proteinases and lipases) in the raw milk that may survive pasteurisation and produce off-flavours during storage of pasteurised milk (Ray, 2004; Sorhaug and Stepaniak, 1997).

#### **1.2.2 Cottage cheese**

According to Scott, (1998) cottage cheese is a soft unripened white cheese with 80 % maximum moisture and 4 % minimum fat in dry matter. Cream and salt can be added (Robinson, 1995). The cheese has been commonly made from skim milk with the addition of herbs or fruits to give the product flavour, but full fat or whole milk cottage cheeses are also available (Robinson, 1995).

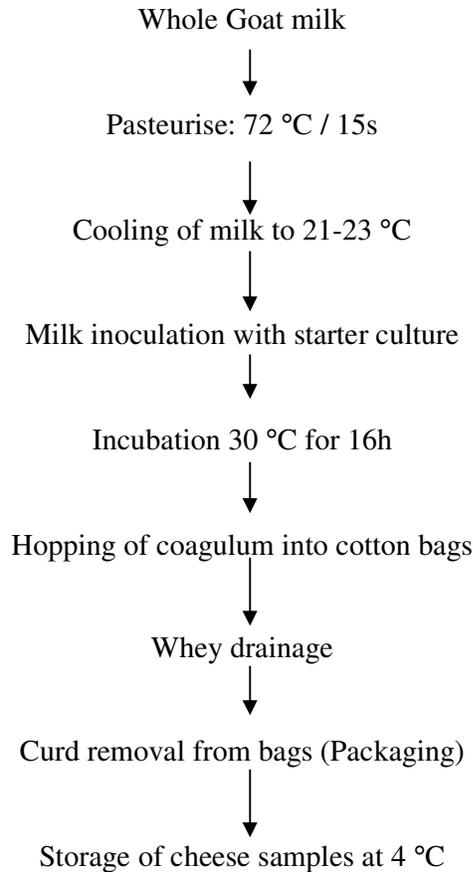
### **1.2.2.1 Manufacturing of cottage cheese**

Cottage cheese can be manufactured with either the long set or the short set method (Robinson and Wilbey, 1998). During the long set method 0.5 % of a mesophilic starter culture is used and the coagulation of milk is usually done overnight (14-16h). The short set method coagulation takes (5-6h) and the starter inoculation is 5.0 %. First the milk is heated at 72 °C 15 s and then cooled to temperatures around 20-23 °C (Robinson and Wilbey, 1998). The milk is then inoculated with starter cultures usually *Lactococcus lactis* sub-spp *lactis* and *cremoris* (Robinson and Wilbey, 1998). For the short set method low level of rennet may be added to assist coagulation but not usually used for the long set method (Robinson and Wilbey, 1998).

The milk is incubated at 21 to 22 °C for the long set method and 30-32 °C for the short set method (Brocklehurst and Lund, 1985). The curd is cut into cubes and the pH is allowed to reach 4.5 to 4.6. After cutting the whey is drained, flavour added and stored. Manufacturing methods may vary according to whether the cheese will be scalded or washed. Scalding is usually done by raising the temperature to 54 °C over a period of 2h. Washing removes excess lactose and prevents late acidification and lowers the cheese temperature (Figure 1.1) (Robinson, 1995).

### **1.2.2.2 Shelf life of cottage cheese**

The shelf life of cottage cheese is usually 14-21 days stored at 3-4 °C without the addition of chemical preservatives (Mannheim and Soffer, 1995). Addition of preservatives like potassium sorbate may prolong shelf life of cottage cheese under refrigerated storage for three more weeks, but preservatives may cause undesirable off-flavours (Ray, 2004).



**Figure 1.1 Flow diagram to show cottage cheese manufacturing (compiled from Kosikowki and Mistry, 1999)**

### 1.2.2.3 Microbial quality of cottage cheese

The high moisture content (80 %) of cottage cheese may support survival of a variety of spoilage and pathogenic microorganisms. Psychrotrophic bacteria such as *Pseudomonas* spp. *Proteus*, *Aeromonas* and *Alcaligenes* spp. may grow and that may result in off-flavours, pigment formation or a slimy curd (Brocklehurst and Lund, 1985). Yeasts and moulds such as *Geotrichum*, *Penicilium*, *Mucor* and *Alternaria* can grow during storage (Ray, 2004). The moulds may cause spoilage and the cheese may have change of flavour, texture, and appearance. Gonfa, Foster and Holzapfel, (2001) examined traditionally fermented milk products in Ethiopia. Ayib is a highly acidic and high moisture content Ethiopian cottage cheese. Gonfa *et al.*, (2001) found that there were high numbers of lactose-assimilating yeasts in the traditional cottage cheese and

proteolytic yeasts made up 46.9 % of the total isolates. The presence of proteolytic yeasts is said to have a major effect on the flavour and the keeping quality of the cheese.

Brocklehurst and Lund, (1985) examined the microbial changes of cottage cheese stored at 7 °C and found that the main contaminating bacteria in creamed cottage cheese were *Bacillus* spp., micrococci, *Pseudomonas fluorescens* and *Pseudomonas maltophilia*. *Pseudomonas fluorescens* multiplied rapidly and was evident 14 days after manufacturing. *Enterobacter agglomerans*, was also isolated and the counts were 10<sup>8</sup> cfu/g after 16 days of storage at 7 °C. The yeasts isolated from the cheese were *Cryptococcus laurentii* and *Candida famata* and were detected during late stages of storage.

*L. monocytogenes* is capable of growing at refrigeration temperatures and has been involved in several outbreaks attributed to soft or soft ripened cheeses. Rogga, Samelis, Kakouri, Katsiari, Savvaidis and Kontominas, (2005) found that *L. monocytogenes* survived in commercially manufactured Galotyri cheese throughout storage for 28 days at 4 °C and for 14 days at 12 °C. These researchers found that *L. monocytogenes* declined most, in all samples from days 0 to 3, which was followed by smaller decline from days 3 to 28 at 4 °C and to day 14 at 12 °C. Morgan, Bonnin, Mallereau and Perrin, (2001) noted that *L. monocytogenes* decreased from 3.3 log cfu/g after draining to 1.5 log cfu/g on day 28 day. *Salmonella*, *L. monocytogenes* and enteropathogenic *Escherichia coli* have been categorised as high risk organisms associated with the cheese industry (Lunden *et al.*, 2004). The presence of all these microorganisms in cheese could be a health risk to consumers or decrease the shelf life of the product.

#### **1.2.2.4 Survival of *Listeria monocytogenes* in cottage cheese and other soft cheeses**

*L. monocytogenes* have been found to survive in unpasteurised milk and unpasteurised milk products. Morgan *et al.*, (2001) found that *L. monocytogenes* is able to survive in soft lactic acid cheeses made from raw goat milk. Tham, (1988) also studied the survival of *L. monocytogenes* in semi-soft cheese made of unpasteurised goat milk. The raw goat milk was inoculated with 10<sup>5</sup>-10<sup>6</sup> cfu/ml, *L. monocytogenes* was observed to have survived throughout the curing period. In developing countries, some milk is sold

raw or may be used to make products which do not need any refrigeration (Chamberlain, 1989). In Ethiopia Ergo, a traditionally fermented Ethiopian milk product, is made with raw milk and is usually consumed after 24h. Ashenafi, (1994) studied the growth of three different strains of *L. monocytogenes* during the souring of Ergo and found that *L. monocytogenes* grew rapidly within 24h and reached counts of  $>10^7$  cfu/g from initial level of  $10^2$  to  $10^4$  cfu/g.

Cottage cheese is a soft cheese and soft cheeses have been associated with outbreaks of listeriosis in countries such as Europe and United States of America (Pitt, Harden and Hull, 1999). In food processing plants the conditions can allow for *L. monocytogenes* to survive and grow as the organism can survive low temperatures, (Kozak, Balmer, Byrne and Fisher, 1996). Chen and Hotchkiss, (1993) studied the safety of cottage cheese and other soft cheeses and observed rapid growth of *L. monocytogenes* in cottage cheese at pH 5.14 at 7 °C as the numbers increased 1000 fold within 16 days. The rapid growth observed in cottage cheese suggests that cottage cheese could sustain growth of *L. monocytogenes*. Lunden *et al.*, (2004) says the prevalence of *L. monocytogenes* in soft and semi soft cheeses may be due to the high water activity of these cheeses. In a study by Hicks and Lund, (1991) *L. monocytogenes* survived during storage at temperatures of 4, 8 and 12 °C for 14 days but decreased in all the cottage cheese samples studied.

Back Langford and Kroll, (1993) found that *L. monocytogenes* survived in Camembert cheese and did not decrease in numbers under all conditions tested but declined in numbers at the centre of the cheese after 40 days of storage at 3 °C. In other cheeses Back *et al.*, (1993) observed no or little growth of *L. monocytogenes* on blue or white Stilton at 5 °C. No growth was also observed on blue or white Lymeswold Brie, Chaume or soft cheese with garlic and herbs stored at 3 °C or mycella stored at 5 °C but the organism still survived. Storage of cheeses at 3°C severely restricted growth of *L. monocytogenes* but survived and in some cases increased numbers slightly (Back *et al.*, 1993).

Buazzi, Johnson and Marth, (1991) studied survival of *L. monocytogenes* during the manufacture of mozzarella cheese and found that populations of *L. monocytogenes* changed at different rates during various phases of making the cheese. The organism

was unable to grow during the manufacturing and during cooking of the curd but it was reduced by 38 % and during cheddaring and completely eliminated when the cheese was stretched in hot water at (77 °C).

### **1.2.3 *Listeria monocytogenes***

*L. monocytogenes* is a gram-positive facultative anaerobic, non-spore forming bacilli or cocco-bacilli (Eley, 1992). It has an ubiquitous nature, Herald and Zottola, (1988) and it may survive for long periods in the environment (Eley, 1992). Pitt *et al.*, (1999) found that *L. monocytogenes* has been isolated from surface and spring water, fresh and processed foods, raw and treated sewage, vegetation including dead and decaying plant matter and poor quality and spoiled silage. *L. monocytogenes* has also been found in cultivated and uncultivated soils and mud. *L. monocytogenes* has the ability to attach to stainless steel under ambient temperatures at pH range of 5-9 and can also attach to glass, polypropylene and rubber after short contact times (Herald and Zottola, 1988). Due to these characteristics associated with the organism, there is a possibility of proliferation and colonisation of the surfaces at ambient and cold storage temperatures (Mafu, Roy, Goulet and Magny, 1990). This is likely to present a problem of safety in the dairy plant.

*L. monocytogenes* is a soil organism, which has evolved the ability to invade and mobilise within eukaryotic cells (McLauchlin, Mitchel, Smerdon and Jewell, 2004). The bacterium is not host adapted to man and is an opportunistic pathogen. The organism is highly pathogenic to man. People at risk are immuno-compromised individuals such as pregnant women, young children, those on drug therapy, on chemotherapy, and those with diseases such as AIDS, are susceptible to infection. In healthy individuals the organism produces influenza-like symptoms or even no symptoms at all (Griffiths, 1989). Mortality rate in systemic listeriosis is said to be between 20 and 40 % (Farber and Peterkin, 1991).

The organism has been isolated from a variety of food (Eley, 1992). Ham, pork, beef, poultry products, seafood, vegetables raw and cooked eggs (Pitt *et al.*, 1999). Dairy products have a stronger association with listeriosis than other types of food, with both

raw, pasteurised milk and milk products having been identified as sources of food listeriosis outbreaks (Varnam and Evans, 1996; Lunden *et al.*, 2004). Moura, Destoro and Franco, (1993) isolated *Listeria* spp. in 220 raw milk samples and pasteurised products in Brazil, and 9.5 % of the samples contained *L. monocytogenes* and *Listeria ivanovii*. *L. monocytogenes* is a common contaminant of soft cheeses and numbers may be as high as  $10^6$  cfu/g. In another study done by Beckers, Soentoro and Delfgou-Van Asch, (1987) *L. monocytogenes* was detected in 65 % of the cheeses made with raw milk where as no *L. monocytogenes* was detected in cheese made of pasteurised milk. According to Tham, (1988) *L. monocytogenes* has the ability to survive in semi-soft cheeses made from unpasteurised goat milk cheese during the ripening period.

### **1.2.3.1 Pathogenicity**

Infection with *L. monocytogenes* in humans is sporadic with meningeal involvement (Pitt *et al.*, 1999). Exposure to the organism does not always cause a disease. The likelihood of systematic infection may depend on the host susceptibility, the virulence of the organism and the infectious dose. Farber and Peterkin, (1991) suggested that the infectious dose ranged from  $2.7 \times 10^6$  to  $3.4 \times 10^9$  cells of *L. monocytogenes* per gram or millilitre of food.

Pathogenesis of *L. monocytogenes* is mostly caused by the production of the toxin  $\beta$ -haemolysin, the haemolysin is called listeriolysin. *L. monocytogenes* also has cytolysin and phosphatase activity. These toxins have the ability to lyse blood cells and tissue (Pearson and Marth, 1990; Jay, 2000). Other virulence factors include intracellular invasion, in this case *L. monocytogenes* enters the susceptible cells before replication and replicates within the cells. It also has a monocytosis producing activity (Jay, 2000).

### **1.2.4 Effect of pH, $a_w$ , sodium chloride and storage temperature on growth of *Listeria monocytogenes***

#### **1.2.4.1 pH**

Ashenafi, (1994) studied the growth of *L. monocytogenes* in traditional Ethiopian fermented milk and found that *L. monocytogenes* strains were able to reach  $10^7$  cfu/ml

after 24h in the control. Ashenafi, (1994) also noted that the strains used in the study were able to proliferate while the pH decreased to 5.0 in the first 24h. When the pH fell to 4.0 the count slightly declined. The strains studied in the traditional Ethiopian fermented milk could withstand pH as low as 3.9 for more than 24 h at 25 °C (Ashenafi, 1994). Datta and Benjamin, (1997) studied factors controlling acid tolerance of *L. monocytogenes* and found that the strains LS2 and LS111 of *L. monocytogenes* were more sensitive to pH ranging from 2.0 to 5.0. *L. monocytogenes* has been found to be less acid tolerant than food borne pathogens like *Escherichia coli* and *Shigella* (Datta and Benjamin, 1997).

Other researchers studied survival of *L. monocytogenes* in acidic foods and during milk fermentation. According to Gahan, O'Driscoll and Hill, (1996) the acid tolerant and the acid adapted strains of *L. monocytogenes* showed enhanced long term survival in yoghurt and cottage cheese compared to the non adapted type. Gahan *et al.*, (1996) indicated that commercial cottage cheese dressed with non acidic cream may not provide a sufficiently acidic environment to allow a selective advantage for acid resistant cultures. Adaptation of *L. monocytogenes* to acid, ethanol, heat, hydrogen peroxide, sodium chloride and ethanol increased the organism's susceptibility to hydrogen peroxide (Gahan *et al.*, 1996).

Hill, Cotter, Sleator and Gahan, (2002) showed that a primary consequence of acid adaptation is the increase in intracellular pH of the microorganism as acid adapted and acid mutant cells survived more at an elevated intracellular pH relative to wild type at low external pH. Other mechanisms that play a role in *L. monocytogenes* response to acid are the Glutamate decarboxylase and the F<sub>0</sub>F<sub>1</sub>-ATPase enzyme. El-Shawy and Marth, (1990) studied the behaviour of *L. monocytogenes* in cottage cheese prepared from pasteurised skim milk that was inoculated with 10<sup>6</sup> cfu/g *L. monocytogenes* and coagulated for 3h using hydrochloric acid gluconic acid or bovine rennet. The temperature of milk was increased from 2-32 °C and the *L. monocytogenes* populations decreased ~ 4.5 and >6.0 log cfu/g in fully cooked curd. El-Shawy and Marth, (1990) found that *L. monocytogenes* was inhibited and suggested that the increase in temperature and acidic conditions had a bactericidal effect on the organism.

Phan-Thanh, Mahouin and Alige, (2000) studied the acid tolerance of *L. monocytogenes* and indicated that the lowest pH that the microorganism can survive depends on medium composition, the strain and its physiological state. Phan-Thanh *et al.*, (2000) also noted that acid adapted *L. monocytogenes* had increased resistance against heat shock (52 °C), osmotic shock, 25-30 % NaCl and alcohol stress of 15 %. Heat adapted *L. monocytogenes* also showed resistance against acid shock (Phan-Thanh *et al.*, 2000). Hicks and Lund, (1991) also found that *L. monocytogenes* grown in acidic conditions at pH 5.5 prior to inoculation into the cottage cheese survived better than *L. monocytogenes* which has been cultured in tryptose phosphate broth at pH of 7.3 (Phan-Thanh *et al.*, 2000).

Rogga *et al.*, (2005) examined the survival of *L. monocytogenes* in a traditional Greek soft acid curd cheese stored aerobically at 4 °C and 12 °C. They found that the higher the pH of a soft cheese the greater the survival of the pathogen. These researchers indicated that a pH of 5.5 and above in soft ripened cheeses, allows for survival and growth of *L. monocytogenes* where as soft ripened cheeses with pH 4.5 and salt content of about 2 % may not support growth but may allow survival at 4 °C for extended periods of storage. Kroll and Patchet, (1992) studied induced tolerance of *L. monocytogenes* and found that incubation of the organism at pH 5.0 increased survival of *L. monocytogenes* over 40-fold compared with the survival of cells previously grown at pH 7.0. Faleiro, Andrew and Power, (2003) observed that strains isolated from cheese with different pH values had similar pH tolerance. Vasseur, Baverel, Hebraud and Labadie, (1999) exposed *L. monocytogenes* to three different acids, lactic, acetic and hydrochloric acid. The sensitivity of the five strains of *L. monocytogenes* studied varied. Acetic acid was found to be more antilisterial than lactic acid and hydrochloric acid. Acetic acid also had longer lag and generation periods compared to the other two acids.

#### **1.2.4.2 Temperature**

*L. monocytogenes* does not grow at temperatures below -1.5 °C but can readily survive much lower temperatures. Temperatures below 0 °C freezes the culture or food and moderately inactivate the pathogen and may cause limited reduction in viable population of the organism (Lou and Yousef, 1999). The organism can grow at

temperature range of -1.5 °C to 45 °C (Hudson, Mott and Penney, 1994). The ability of the organism to initiate growth at low refrigeration temperatures is of particular concern and importance as all dairy plant environments provides an ideal condition for growth and survival of the pathogen (Kozak *et al.*, 1996). The microorganism has an optimum growth temperature of 30-37 °C. Although the organism can survive drying and freezing, it is not tolerant to temperatures achieved during food processing such as cooking and pasteurisation (Kells and Gilmore, 2004).

#### **1.2.4.3 Water activity ( $a_w$ ) and sodium chloride**

Moisture requirement of microorganisms can be best expressed in terms of  $a_w$ . Salt and  $a_w$  are interrelated and the inhibition of bacteria by salt reflects the effect of salt in reducing  $a_w$  (Beresford, Fitzsimons, Brennan and Cogan, 2001). Low or no salt content and lack of ripening may allow survival and growth of *L. monocytogenes* in soft unripened cheeses (Faleiro *et al.*, 2003). The optimum  $a_w$  for *L. monocytogenes* is ~ 0.97 but this organism has a unique ability to survive and multiply at  $a_w$  as low as 0.90 (Petran and Zottola, 1989). Griffiths, (1989) reported that *L. monocytogenes* survived  $a_w$  of 0.932 with glycol as humectant and 0.942 with sucrose or sodium chloride.

In some cheeses, salt is added to inhibit bacterial growth (Guinee and Fox, 1987). Sodium chloride is a component that regulates  $a_w$  of several foods (Faleiro *et al.*, 2003). *L. monocytogenes* has been found to have survived in tryptose soy broth containing 25.5 % NaCl for 4 days at 37 °C and the period of survival increased to more than 132 days at 4 °C (Faleiro *et al.*, 2003). *L. monocytogenes* is a psychrotrophic and salt tolerant organism (Lou and Yousef, 1999). Some strains of *L. monocytogenes* have been found to survive 25.5 % w/v sodium chloride for at least 132 days when stored at 4 °C (Griffiths, 1989). The organism can also grow in salt concentrations of up 10-14 % (Farber, Coates and Daley, 1992). Ryser and Marth, (1987) studied the behaviour of *L. monocytogenes* during the ripening of brick cheese and indicated that *L. monocytogenes* could survive in 22 % salt brine.

Galediero, D'Isanto and Alibertion, (1997) studied the effect of saline concentration, pH and growth temperature on the invasive capacity of *L. monocytogenes* and found that

sodium chloride concentrations higher than 10 % decreased the invasion ability of *L. monocytogenes* at 30 °C. *L. monocytogenes* grown at 4 °C with a sodium chloride between 3 and 12 % still retained some invasion ability (Galeiro, D'isanto and Aliberti, 1997). Faleiro *et al.*, (2003) noted that at 8 °C concentrations higher than 2 % were inhibitory to most of the *L. monocytogenes* strains studied. The major antimicrobial effect of sodium chloride is said to be plasmolysis, other effects include dehydration, interference with the enzyme, removal of oxygen and the toxicity of chloride and sodium ions when sodium chloride is ionised (Lin and Chou, 2004). Vasseur *et al.*, (1999) studied the effect of osmotic, alkaline, acid and thermal stresses on the growth and inhibition of *L. monocytogenes*, these researchers found that the increase in sodium chloride concentration in the growth medium increased the lag phase in all five strains studied.

#### **1.2.4.4 Effect of heat on *Listeria monocytogenes***

Pasteurisation has been found to be a highly effective method of preservation that destroy spoilage as well as pathogenic bacteria in milk hence making the product commercially safe for human consumption (Lou and Yousef, 1999; Fleming, Cochi, Macdonald, Brondum, Hayes, Plikaytis, Holmes, Audurier, Broome and Reingold, 1985). *L. monocytogenes* survives freezing and drying but it does not survive milk pasteurisation at 72 °C at 15 s (Beckers *et al.*, 1987; Bradshaw, Peeler and Tweet, 1991). This shows that pasteurisation of milk used for processing can prevent growth of *L. monocytogenes* in milk products. Isolation of *L. monocytogenes* in pasteurised milk and milk products can therefore be due to post contamination (Beckers *et al.*, 1987) or inadequate pasteurisation (Bradshaw *et al.*, 1991).

Some listeriosis outbreaks have been linked to pasteurised milk products like in Massachusetts in 1985 and this led to studies investigating whether *L. monocytogenes* survives pasteurising (Fleming *et al.*, 1985). Farber, (1989) indicated that naturally contaminated milk with *L. monocytogenes* serotype 1 at around  $10^4$  cfu/ml was run through high time short time pasteurizer at different temperatures for a minimum time of 16.2 s. Farber, (1989) reported that *L. monocytogenes* survived temperatures ranging between 60-65 °C, but at 69 °C *L. monocytogenes* was not detected. Bradshaw *et al.*,

(1991) studied thermal resistance of six *Listeria* strains suspended in raw milk farm bulk tank. Three *Listeria* strains were found to be less resistant than *L. monocytogenes* strains. The time-temperature combinations during pasteurisation eliminated the numbers of pathogens that might be naturally present in raw milk (Brashaw *et al.*, 1991).

In a study to find out if naturally acquired listerial mastitis that exists as facultative intracellular bacterium within phagocytic leucocytes (neutrophils and macrophages) will survive pasteurisation Bradshaw *et al.*, (1991) found that the intracellular bacterium did not protect *L. monocytogenes* from thermal destruction. Lovett, Wesley, Vandermaaten, Bradshaw, Francis, Crawford, Donney and Wesser, (1990) studied the survival of suspended and intracellular *L. monocytogenes* during high temperature short time pasteurisation and no *L. monocytogenes* was detected after exposure to pasteurisation.

Ryser, Marth and Doyle, (1985) inoculated cottage cheese made from pasteurised milk containing  $10^4$  and  $10^5$  cells per millilitre with two strains of *L. monocytogenes*. These researchers found that after cooking of the curd at 57.2 °C for 30 minutes the numbers of the organism remained unchanged. After storage at 3 °C for 28 days about 52.7 % of the samples were found to be positive for the organism. Pasteurisation affects microorganisms by distribution of heat uniformly in a cell, cellular targets for heat damage are said to be the ribosomes, nucleic acids, enzymes and proteins, causing damage to most sensitive molecules of the cell (Abee and Wouters, 1999).

Optimal heat-shock temperatures for maximal thermotolerance in mesophilic organisms are usually between 45-50 °C, which is about 10 to 15 °C above the microbes optimal temperature. *L. monocytogenes* has heat-shock temperatures in this range (Lou and Yousef, 1999). Hill *et al.*, (2002) says growth of bacteria at mildly elevated temperatures induces a heat shock response that permits prolonged survival at normally lethal temperatures. Heat shock response has the potential to enhance bacterial survival in foods. *L. monocytogenes* is said to be able to elicit shock response and also clearly induce synthesis of the conserved heat shock proteins following a sub-lethal heat shock (Hill *et al.*, 2002). Heat shock proteins are said to act as molecular chaperonins, protecting essential bacterial proteins from heat denaturation. The heat shock proteins

are also induced when exposed to other environmental stresses such as low pH, elevated salt and ethanol (Hill *et al.*, 2002).

Lin and Chou, (2004) studied the effect of heat shock on thermal tolerance and susceptibility of *L. monocytogenes* to other environmental stresses. These researchers found that the 45 °C-1 h heat shocked cells of *L. monocytogenes* Scott A showed and increased thermal tolerance at 55 °C where as the *L. monocytogenes* V7 heat shocked at 45 °C was comparable to the control. These results showed that the effect of heat shock on the thermal tolerance of *L. monocytogenes* may vary with the conditions of heat shock treatment and strains of *L. monocytogenes*.

Yura, Kanemori and Morita, (2000) says in a temperature change from 30 to 42 °C heat shock protein synthesis increases almost immediately and reaches a maximum induction (10 to 15 fold) within 5 minutes. The rapid induction is followed by a gradual decrease, during the adaptation phase to attain a new steady-state level. In heat shock involving extreme temperatures above 50 °C, synthesis of most proteins ceases, where as the heat shock proteins continues. The rapid change in transcription of the heat shock genes permits the cell to attain new steady-state heat shock protein levels (Yura *et al.*, 2000).

### **1.2.5 Incidence of *Listeria monocytogenes* in dairy products**

The first scientifically proven listeriosis outbreak due to consumption of contaminated food is reported to have occurred in 1981 (Schlech, Lavigne, Bortolussi, Allen, Haldane, Wort, Hightower, Johnson, King, Nicholls and Brome, 1983). A major occurrence of listeriosis in Halle Germany in 1949 to 1957 was believed to have been due to raw milk (Seeliger, 1961 cited in Lunden *et al.*, 2004). In Switzerland, from 1983 to 1987, cheese made from raw milk was determined to be the cause of listeriosis outbreaks. In Austria, in 1986 the consumption of raw milk and organic vegetables caused about 39 cases of listeriosis (Lunden *et al.*, 2004). In Denmark *L. monocytogenes* was isolated in 12.3 % of raw meat samples with 3.6 % of samples containing more than 100 cfu/g, where as on the other hand preserved samples contained a higher (23. 5 %) of *L. monocytogenes*. Even though levels were high in preserved samples, only 0.6 % contained more than 100 cfu/g (Norrung, Anderson and Schlundt, 1999).

In France a soft cheese made of raw milk caused outbreak of listeriosis in 1997. In Sweden in 2001 consumption of fresh cheese made of raw milk caused febrile gastroenteritis due to contamination with *L. monocytogenes* (Table 1.1) (Lunden *et al.*, 2004). In Canada in 1973, an infant died 33h after delivery after the mother drank raw milk and cream (Ryser, 1999). In Japan, in 2001 routine monitoring for *Listeria* was done on domestic cheeses. *L. monocytogenes* was isolated from cheese produced at a contaminated plant, out of the 19 cheese samples, 15 were positive of *L. monocytogenes* (Makino, Kawamoto, Takeshi, Okada, Yamasaki, Yamamoto and Igimi, 2005). In Finland there was an outbreak of listeriosis caused by butter made of pasteurised milk in 1998 to 1999. A total of 25 cases were reported and 6 resulted in death. In Austria there were 39 cases of listeriosis in 1989, caused by raw milk and vegetables (Lunden *et al.*, 2004).

Studies in various countries have been carried out to find out how frequent does *L. monocytogenes* occur in dairy products. In Chile, Cordano and Rocourt, (2001) found different strains of *L. monocytogenes* in Chilean food and 3.6 % of the samples tested positive. In dairy products serotype 4b, 1/2a and 12b were found. These serotypes have been frequently isolated in food and have been associated with human listeriosis (Farber and Peterkin, 1991). In England and Wales, Greenwood, Roberts and Burden, (1991) found *L. monocytogenes* and other listeria species in 17 % of the contaminated cheese samples. Although levels were low the ability of the organism to multiply at refrigeration temperatures and above indicate that it may cause problems in dairy plants. In Brazil Moura *et al.*, (1993) studied incidences of *Listeria* species in raw and pasteurised milk in Brazil and found 28 % of the samples positive for *Listeria* species in raw milk where as in pasteurised milk only 0.9 % of the samples tested were positive.

Fleming *et al.*, (1985) did a study in Massachusetts after an outbreak of listeriosis after consumption of *L. monocytogenes* contaminated pasteurised milk. All of the 42 patients infected were immunocompromised, 14 of them passed away (Table 1.1). In Sweden, Loncarevic, Danielsson-Tham and Tham, (1995) found that 6 % of cheese samples out of the 333 tested positive for *L. monocytogenes*. Fourty two percent of the *L. monocytogenes* positive cheeses were made from raw milk compared to 2 % for the pasteurized milk cheeses. In France, Salvat, Toquin, Michel and Colin, (1995) indicated

that out of the 270 surface, air, meat and delicatessen samples, 35 % were positive for *L. monocytogenes*, 68 % of the samples were positive for *L. monocytogenes* in raw products areas, and 33 % positive in the finished product area. The consumption of these products can lead to listeriosis.

**Table 1.1 Incidences of Listeriosis in dairy products**

Dairy Product	Place	Year	Cases	Deaths	Reference
Pasteurised milk	Massachusetts	1985	42	14	Fleming <i>et al.</i> , 1985
Raw milk & soft cheese	Switzerland	1983-1987	122	unknown	Lunden <i>et al.</i> , 2004
Raw milk	Austria	1986	39	unknown	
Blue mould cheese	Denmark	1989-1990	69	unknown	
Raw milk & brie cheese	France	1995	37	unknown	
Raw milk & soft cheese	France	1997	14	unknown	
Raw milk fresh cheese	Sweden	2001	48	unknown	
Butter	Finland	1998-1999	25	6	

Compiled from (Fleming *et al.*, 1985 and Lunden *et al.*, 2004)

### 1.2.6 The Lactoperoxidase system

Lactoperoxidase system is an antimicrobial naturally found in raw milk and it consists of three components, which are the lactoperoxidase enzyme, an oxidisable substrate and hydrogen peroxide (Davidson and Brannen, 1993). The peroxidation reaction is important because it has antimicrobial activity and prevents accumulation of hydrogen peroxide excreted by micro organisms and the host cells, which may be highly toxic if it accumulates (Naidu, 2000; Seifu, Buys and Donkin, 2005). In mammals the lactoperoxidase system is said to be a major utilizer of hydrogen peroxide that is constantly produced in mammary secretions (Silanikove, Shapiro, Shamay and Leitner, 2005). The biological significance of this system is related to prevention of intestinal infection in the neonate (Bjorck, Claesson and Schulthess, 1979).

Bjork *et al.*, (1979) found that the effect of the LPS on bacteria present in raw milk is inversely related to temperature. At ambient temperatures the LPS is said to be able to decrease bacteria levels for 7-8h where as at 15-20 °C the bacteriostatic effect is prolonged. Kamau, Doores and Pruitt, (1990b) indicated that *L. monocytogenes* was inhibited for 8h by the LPS at 35 °C where as at 10 °C *L. monocytogenes* was found to have increased steadily from log 4.5 cfu/ml but did not reach log 5 cfu/ml.

### 1.2.6.1 The lactoperoxidase enzyme

Lactoperoxidase belongs to a group of enzymes, which catalyses the oxidation of numerous organic and inorganic substrates by hydrogen peroxide (Kohler and Jenzer, 1989). Peroxidases exist in various oxidation states. There are five known enzyme intermediates which are ferrous enzyme, ferric or native enzyme, compound I, compound II and compound III peroxide (Kohler and Jenzer, 1989). Lactoperoxidase enzyme is found in mammary, salivary and lachrymal glands of mammals and their secretions like milk, saliva and tears (Wolfson and Sumner, 1993).

Lactoperoxidase is the most abundant enzyme in bovine milk and is influenced by feeding practices (Reiter, 1985). Reiter, (1985) reported a concentration of around 30 mg/l in bovine milk. This concentration is substantially greater than the 0.5-10 ug/ml necessary for the system to function efficiently (Bjork, 1978; Naidu, 2000). Fonteh, Gradison and Lewis, (2002) indicated that the mean lactoperoxidase activity in cow milk ranged from 1.5-2.7 U/ml with an overall mean of 2.3 U/ml. Lactoperoxidase activity has been found to be much lower in goat milk than in cow's milk. Fonteh, *et al.*, (2002) studied variations of lactoperoxidase activity and thiocyanate content in cows and goats milk throughout lactation and reported the highest enzyme activity in samples collected during mid lactation.

Zapico, Gaya, Medina and Nunez, (1990) reported lactoperoxidase activity ranging from 0.05-3.55 U/ml in goat milk and these concentrations are different from those by Saad de Schoos, Oliver and Fernandez, (1999) who found concentrations ranging from 0.48 to 9.28 Um/l where as Fonteh *et al.*, (2002) reported concentrations of 0.04 to 0.16

U m/l. In a more recent study by Seifu *et al.*, (2004a), lactoperoxidase activity of 0.26 U/ml in Indigenous goat milk was reported and Saanen goat milk had 0.79 U/ml.

The lactoperoxidase enzyme is relatively heat resistant and is only partially inactivated by short time pasteurisation at 74 °C for 10 s or 72 °C for 15 s. It has been used as an index of pasteurisation efficiency in milk. (Wolfson and Sumner, 1993). This leaves enough to catalyse the reaction between thiocyanate and hydrogen peroxide. Marks, Gradison and Lewis, (2001) reported that after pasteurisation of cow milk at 72 °C for 15 s, an active LPS enhanced the keeping quality of milk inoculated with *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus thermophilus*. The lactoperoxidase catalyses the oxidation of thiocyanate (SCN) by hydrogen peroxide to hypocyanous acid (HOSCN) and hypothiocyanate anion (OSCN) (Earnshaw, Banks, Defrise and Francotte, 1989).

#### **1.2.6.2 Thiocyanate (SCN)**

Is widely distributed in animal tissues, secretions and is a constituent of the intracellular fluid (Wolfson and Sumner, 1993). It is said that 15 ppm thiocyanate is sufficient to obtain a significant improvement in the quality of stored raw milk (Bjock *et al.*, 1979). The thiocyanate content of milk may vary with the diet (Reiter, 1985)

Fonteh *et al.*, (2002), found that thiocyanate content in goat milk varies widely throughout the lactation period. Fonteh, *et al.*, (2002) reported large variations in thiocyanate content within goats sampled throughout the sampling period. Thiocyanate is an ubiquitous anion in animal systems and is found in animal systems and is in sufficient quantities in bovine milk for maximal LPS activity (Davidson and Brannen, 1993). In milk thiocyanate is the primary substrate and generally enhances the bacterial spectrum of the milk.

Major dietary sources of thiocyanate are the glucosinolates and cyanogenic glucosides found in vegetables (Wolfson and Sumner, 1993). Glucosinolates are found in vegetables such as cabbage cauliflower, brussel and sprouts which upon hydrolysis yield thiocyanate together with other products. Cyanogenic glucosides are found in

cassava, potatoes, maize, peas, beans, millet sugar cane which are hydrolysed to give cyanide and other reaction products (Oerlemans, Barrett, Suades, Verkerk and Dekker, 2006). Glucosinolates are sulphur containing anions, the decomposition products are produced when plant cells are ruptured. Glucosinolates present in vacuoles are hydrolysed and the hydrolysis products include thiocyanates, isothiocyanates, nitriles and other reaction products (Vaughn and Berhow, 2005).

### **1.2.6.3 Hydrogen Peroxide**

Hydrogen peroxide is one of the inorganic peroxide compounds and a strong oxidising agent and exhibit varying degrees of antimicrobial activity. Hydrogen peroxide is the other major component of the LPS. Milk does not normally contain hydrogen peroxide (Davidson and Brannen, 1993), but under aerobic conditions sufficient hydrogen peroxides may be produced by lactobacilli, lactococci and streptococci, which may be sufficient to activate the LPS (Wolfson and Sumner, 1993). Manipulation of the LPS by addition of hydrogen peroxide or other hydrogen peroxide generating systems like glucose oxidase and xanthine oxidase, the bacterial spectrum is enhanced (Pruitt and Kamau, 1991).

Hydrogen peroxide generating system is said to be more effective than hydrogen peroxide as a component of antimicrobial system (Wolfson and Sumner, 1993). Hydrogen peroxide has been found to be highly toxic for mammalian cells. At low levels of 100  $\mu\text{M}$  concentrations in the presence of LP and  $\text{SCN}^-$  mammalian cells are protected from this toxicity (Pruitt and Kamau, 1991).

### **1.2.6.4 The antimicrobial spectrum of the Lactoperoxidase system.**

The LPS can inhibit growth and metabolism of different species of microorganisms. It is capable of inhibiting viruses, gram-positive bacteria, gram-negative bacteria, fungi, mycoplasmas and parasites (Pruitt and Reiter, 1985). Susceptibility of microorganisms to LPS depends on the state of their microbial growth (Naidu, (2000). Cells in a stationary phase are more susceptible to killing or inhibition compared to metabolically active cells, the LPS is also more effective at low cell densities than at high densities (Pruitt and Reiter, 1985).

The cytoplasmic membrane or the cytoplasm are the major targets of the LPS antimicrobial products (Naidu, 2000). The structural damage of microbial cytoplasmic membranes by the oxidation of sulphhydryl groups results in leakage of potassium ion, amino acids and polypeptides into the medium. Subsequently the uptake of glucose, amino acids, purines, pyrimidines in the cell and the synthesis of proteins DNA and RNA is also inhibited (Reiter and Harnulv, 1984). The cell wall and the membrane may partially limit accessibility of the LPS products into the cell but does not exclude it completely (Naidu, 2000). The hypothiocyanate produced by the LPS is found to be bactericidal to enteric pathogens including multiple antibiotic resistant *Escherichia coli* strains (Naidu, 2000). Zapico, Medina, Gaya and Nunez, (1995) found that activation of the LPS in goat milk lowered *Escherichia coli* counts compared to the control during storage of goat milk at 8 °C for 5 days. These researchers also found that the LPS was bactericidal against *Pseudomonas fluorescens*.

Marshall, Cole and Bramley, (1986) studied the effect of LPS on *Streptococcus uberis* and found that in the absence of the LPS, *Streptococcus uberis* in autoclaved milk grew well, but growth was inhibited when lactoperoxidase was added, and growth was prolonged when SCN<sup>-</sup> and a hydrogen generating source were added. Siragusa and Johnsson, (1989) also reported that the LPS extended the lag phase of *L. monocytogenes* (from 9 to 12-36h) in skim milk at 20 °C. According to Zapico, Gaya Medina and Nunez, (1993) the LPS in goat milk is bactericidal against different strains of *L. monocytogenes* for 3-9 days at 4 °C and 1-7 days at 8 °C. Marshall and Reiter, (1980) found that the hypothiocyanate anion had a bactericidal effect on *Escherichia coli* and bacteriostatic effect on *Streptococcus lactis*. Three strains of *Streptococcus uberis* in autoclaved milk were inhibited by the LPS (Marshall *et al.*, 1986).

Shin, Yamauchi, Teraguchi, Hayasawa and Imoto, (2002) reported that *Helicobacter pylori* strains were inhibited by the LPS in BHI-serum broth at different levels. Clinical isolate no 32 was the most susceptible to the LPS as the organism was reduced by log<sub>10</sub> 4 compared to the control. Strain ATCC 43629 was the most resistant as was reduced by less than log<sub>10</sub> 1.

Jacob, Anthony, Sreekumar and Harida, (2000) studied the antifungal and the antibacterial properties of lactoperoxidase in goat milk. Jacob *et al.*, (2000) found that most bacteria he studied showed very large inhibition zones. Some of the bacteria which were found to be inhibited by the LPS include *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* *Salmonella* Enteritidis, *Salmonella* Typhi, *Vibrio cholerae* and *Staphylococcus aureus*. Jacob *et al.*, (2000) found that goat milk inhibited the growth and proliferation of most fungal colonies they studied except *Candida albicans* and *Pythium spp.* Different species were inhibited at different concentrations of goat milk lactoperoxidase, for example *Aspergillus niger* required 475 µg/ml minimum, where as *Claviseps spp.* required 62 µg/ml for total inhibition. Zapico *et al.*, (1998) studied the effect of LPS and nisin on *L. monocytogenes* at stationary phase and on actively growing cells of *L. monocytogenes*. A synergistic effect against *L. monocytogenes* Scott A in UHT skim milk resulted in decrease in counts up to 5.6 log units lower than the control (Zapico *et al.*, 1998).

Kamau, Doores and Pruitt, (1991) studied the effect of LPS prior to pasteurisation on extension of milk and found that activation of the LPS greatly enhanced thermal destruction of *L. monocytogenes* in milk during pasteurisation. Growth of *L. monocytogenes* in LPS treated milk started after 12 days where as in the untreated and hydrogen peroxide treated started after 4 days. The counts in untreated and hydrogen peroxide treated milk reached  $10^6$ - $10^7$  cells/ml compared to  $10^3$  cells/ml in LPS treated milk.

Seifu *et al.*, (2004a) studied the antibacterial activity of the LPS against foodborne pathogens in Saanen and South African Indigenous goat milk. Seifu *et al.*, (2004a) also found that LPS exhibited bactericidal effect against *L. monocytogenes* in both Saanen and indigenous goat milks. The LPS in indigenous milk reduced the rate of growth of *Staphylococcus aureus* by 18 % compared to the control. In the Saanen goat milk the researchers found that *Staphylococcus aureus* was reduced by 0.1 log units in the LP activated milk where as it in the control it increased by 0.14 log units.

In the indigenous goat milk the *L. monocytogenes* count decreased by 0.12 log units in the LP treated milk after 6h where as it increased by 0.92 log units in the control. In the

Saanen goat milk *L. monocytogenes* was found to have decreased by 0.24 log units in the LP treated milk and increased by 0.21 log units in the control after 6h (Seifu *et al.*, 2004a). The level of *L. monocytogenes* in lactoperoxidase activated indigenous goat milk was 91 % less than the control after 6h incubation. In Saanen goat milk, *L. monocytogenes* was lower in the lactoperoxidase activated goat milk by 65 % compared to the control (Seifu *et al.*, 2004a). In another study, Kamau *et al.*, (1990a) found that the LPS was both bactericidal against *L. monocytogenes* and bacteriostatic against *Staphylococcus aureus* in milk.

Seifu *et al.*, (2004a) found that activation of the LPS decreased *Brucella melitensis* in LP activated indigenous goat milk whereas in Saanen goat milk the organism increased by 1.32 log units in the control and decreased by 0.36 log units in the LP activated milk. Touch, Hayakawa, Yamada and Kaneko, (2004) found that the LPS inhibited the growth of *Salmonella* Enteritidis in UHT whole milk and skim milk for about 4h. Dennis and Ramet, (1989) noted that LPS in UHT milk resulted in inhibition of growth of *L. monocytogenes* with viable population which remained constant for several days followed by complete inactivation of the bacterial cells.

In another study Seifu, Buys and Donkin, (2004b) studied the quality of goat milk Gouda cheese made with lactoperoxidase activated milk. These researchers found the aerobic and lactic acid bacteria in the LP activated cheese decreased slightly during ripening period of 90 days. Seifu *et al.*, (2004b) also found that coliforms and the coagulase-positive staphylococci were significantly lower in the LP activated cheese compared to the control. Activation of the LPS was found to have no significant differences between the control and LP activated cheese in mould counts. Seifu *et al.*, (2004b) noted that activation of LPS lowered the coliform counts significantly compared to the control cheese throughout the ripening of the Gouda cheese.

Earnshaw *et al.*, (1989) indicated that the activation of lactoperoxidase effectively reduced the numbers of pseudomonas, *Escherichia coli* and *Salmonella* Typhimurium in cottage cheese. Earnshaw *et al.*, (1989) observed that lactoperoxidase activated cottage cheese had no detectable levels of pseudomonas throughout the 21 day storage period whereas the control the count reached  $10^8$  cfu/g within 14 days of storage. *Salmonella* Typhimurium was stable in the control throughout storage whereas in the LPS activated

cottage cheese there was no detectable levels of the organism. *Escherichia coli* levels in the control cottage cheese declined from  $10^2$  to undetectable levels during storage whereas in lactoperoxidase activated cottage cheese the organism was not detected throughout storage (Earnshaw *et al.*, 1989). Dennis and Ramet, (1989) noted that *L. monocytogenes* demonstrated susceptibility to the LPS. It was found that the addition of the lactoperoxidase system on the surface of French soft cheese previously inoculated to  $10$  to  $10^6$  cfu/g led to elimination of *L. monocytogenes* cells whereas in the control the organism was detected (Dennis and Ramet, 1989).

Boussouel, Mathieu, Revol-juelles and Milliere, (2000) found that activation of the LPS and addition of nisin had a synergistic bacterial effect on *L. monocytogenes* ATCC 15313 in skim milk. Kennedy, O'Rourke, McLay and Simmonds, (2000) examined the effect of the LPS and monolaurin in ground beef on *Escherichia coli*, *L. monocytogenes* and *Staphylococcus aureus* in red meat. They found that the combination of the LPS and monolaurin inhibited growth of *Escherichia coli* 0157, *L. monocytogenes* and *Staphylococcus aureus* (Kennedy *et al.*, 2000). Borch, Wallentin, Rosen and Bjorck, (1988) studied the effect of the lactoperoxidase against strains of *Campylobacter* isolated from poultry and found that the LPS had a bactericidal effect against strains of *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry. An additional bactericidal effect was observed at pH 6.6 at 37 °C while at pH 6.0 and pH 5.5 the effect decreased considerably. These researchers found that the number of viable cells for all strains tested was reduced from  $10^6$  cfu/ml to an undetectable level in the presence of the LPS.

#### **1.2.6.5 The chemical reactions of the Lactoperoxidase system**

The lactoperoxidase catalyses the oxidation of thiocyanate ( $\text{SCN}^-$ ) by hydrogen peroxide to yield thiocyanogen ( $\text{SCN}_2$ ) which is then hydrolysed to hypocyanous acid (HOSCN) or hypothiocyanate (OSCN) (equation 1, 2 and 3). (Aune and Thomas, 1977; Earnshaw *et al.*, 1989). The OSCN is the major intermediate oxidation product of the LP catalysed oxidation of SCN (Aune and Thomas, 1977). Other short lived intermediates which can be found in varying amounts are the thiocyanogen ( $\text{SCN}_2$ ), cyanogen thiocyanate (NC-SCN), cyanosulphurous acid ( $\text{HO}_2\text{SCN}$ ) and cyanosulphuric

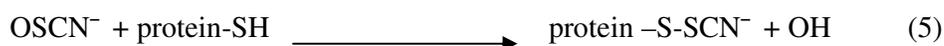
acid (HO<sub>3</sub>SCN) (Pruitt and Kamau, 1991). The hypocyanous acid and hypothiocyanate are highly reactive oxidising agents. They react with the sulfhydryl groups and reduced nicotinamide nucleotides of microbial cells (Davidson and Brannen, 1993). The oxidation of these cellular components, cytoplasmic membranes, carbohydrate and amino acid transport systems and glycolytic pathways are impaired (Davidson and Brannen, 1993).



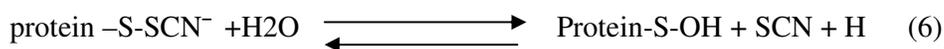
Thiocyanate can also be directly oxidised to hypothiocyanate (see equation 4).

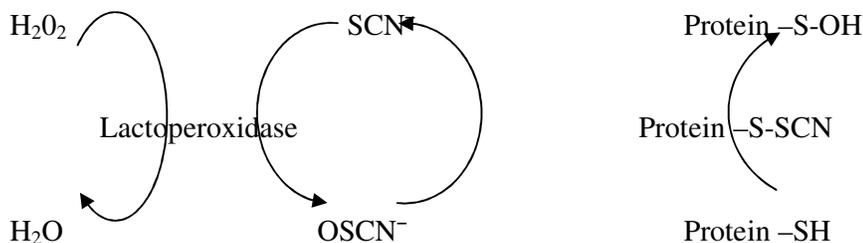


The oxidation of SCN<sup>-</sup> is followed by the oxidation of albumin sulfhydryls by hypothiocyanate anion and hypothiocyanous acid to yield sulfenyl thiocyanate –S-SCN<sup>-</sup> (equation 5). The release of sulfenyl thiocyanate is favoured at low SCN<sup>-</sup> concentrations (Aune and Thomas, 1978). After formation of sulfenyl thiocyanate, disulfides-S-S-are formed.



The sulfenyl thiocyanate –S-SCN derivatives are also be modified to yield sulfenic acid -S-OH- (equation 6) (Aune and Thomas, 1978).

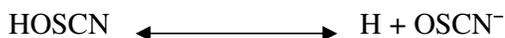




**Figure 1.2 Oxidation of the  $SCN^-$  (Aune and Thomas, 1978)**

Thiocyanate is released from sulfenyl thiocyanate and the thiocyanate can be reoxidised and participate in oxidation of another sulfhydryl (Fig 1.2) (Aune and Thomas, 1978). The oxidation of sulfhydryls to sulfenic acid does not consume  $SCN^-$  so the amount of sulfhydryls oxidised does not depend on the amount of  $SCN^-$  (Fig 1.2) (Wolfson and Sumner, 1993). Exposure of susceptible cells to LPS results in rapid (less than 1 minute) leading to inhibition of metabolism and leakage of amino acids, potassium, carbohydrates transport, utilization and oxygen uptake, amino acid and purine transport, hence nucleic and protein synthesis and growth is inhibited (Pruitt and Reiter, 1985). The presence of reducing compounds can block inhibition of microbial cells (Pruitt and Reiter, 1985). According to Davidson and Brannen, (1993) the presence of catalase and enzymes horseradish peroxidase can reduce the inhibitory activity of the LPS by competing for the hydrogen peroxide with LP.

At neutral pH the hypothiocyanous acid  $HOSCN$   $pka$  5.3 and hypothiocyanate ( $SCN^-$ ) exist in equilibrium (Thomas, 1985).



Both  $HOSCN$  and  $OSCN^-$  exert bactericidal activity but there is uncharged  $HOSCN$  is more bactericidal. The stability of  $OSCN^-$  is affected by different factors, such as pH, light, metals, glycerol and ammonium sulphate as well as by the presence and removal of LP (Thomas, 1985).

### 1.3 Hypothesis

- 1.3.1 Activation of the LPS in goat milk will make *L. monocytogenes* present in goat milk more susceptible to thermal destruction, due to the synergistic effect of the antibacterial compounds hypothiocyanate, hypothiocynous acid and pasteurisation on *L. monocytogenes*.
- 1.3.2 *L. monocytogenes* present in goat milk cottage cheese produced from pasteurised LP activated goat milk will be inhibited during storage due to the synergistic affect of the antibacterial compounds hypothiocyanate, hypothiocynous acid, pasteurisation and the decrease in pH on *L. monocytogenes*.

### 1.4 Objectives

- 1.4.1 To determine of the effect of the LPS alone and in combination with pasteurisation on the growth of *L. monocytogenes* in goat milk.
- 1.4.2. To determine the effect of the LPS alone and in combination with pasteurisation on the growth of *L. monocytogenes* in goat milk cottage cheese, produced from pasteurised LP activated goat milk, during a shelf life of 10 days at 4 °C.

## CHAPTER 2 RESEARCH

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### **2.1 The impact of the combined lactoperoxidase and pasteurisation treatment on the safety of goat milk and goat milk cottage cheese.**

Submitted to the *Journal of Food safety*

#### ***Abstract***

The effect of the LPS alone and in combination with pasteurisation, on the growth of *L. monocytogenes* (LM) ATTC 7644 in goat milk and goat milk cottage cheese during shelf life of 10 days at 4 °C was studied. Goat milk was inoculated with LM ATTC 7644. The milk was divided into two samples, one sample was used as the control and in the other sample LP was activated. Both the control and LP activated goat milk were kept at ambient temperature for 6h. After 6h both the control and LP activated samples were divided into two samples and one of each respective sample was pasteurised at 72 °C for 15 seconds. All the goat milk samples were analysed for LM ATTC 7644 immediately after LP activation, after 6h of LP activation and after pasteurisation. Goat milk cottage cheese was made with all four samples, i.e. control raw, control pasteurised, LP activated raw and LP activated pasteurised goat milk. The goat milk cottage cheese was analysed for LM ATTC 7644 after storage at 4 °C on days 1, 2, 5, and 10. After 6h of LP activation LM ATTC 7644 significantly ( $p \leq 0.05$ ) decreased by log 0.5 cfu/g in LP activated goat milk, where as the level in the control increased by log 0.5 cfu/g. After pasteurisation the LM ATTC 7644 count in LP activated goat milk decreased further by log 4.7 cfu/ml, compared to a log 3.6 cfu/ml decrease in the control. The final count after pasteurisation was log 1.2 cfu/g and log 2.9 cfu/g in the LP activated and pasteurised goat milk and the control raw respectively. In the LP activated pasteurised goat milk cottage cheese and the control pasteurised goat milk cottage cheese samples the LM ATTC 7644 count decreased constantly over a period of 10 days of storage at 4 °C. In the cheese made from either control or LP activated milk the LM ATTC 7644 count was slightly higher than in the cottage cheese made from pasteurised, LP activated or control, milk at the end of storage. Finally in LP activated pasteurised goat milk cottage cheese count decreased more during storage by log 1.69 cfu/g compared to log 1.1 cfu/g decrease in the control pasteurised goat milk cottage cheese and all other treatments. This indicates that there was a

synergistic effect by LP activation and pasteurisation on the LM ATTC 7644 count in both the goat milk and goat milk cottage cheese. Therefore LP activation can be used to control growth of LM ATTC 7644 in raw or pasteurised goat milk and goat milk cottage cheese.

**Keywords:** Lactoperoxidase, goat milk, cottage cheese, *Listeria monocytogenes*.

### 2.1.1 Introduction

Milk in developing countries such as Ethiopia and Kenya is transported to collection centres (Lambert, 2001). From the collection centres it is then sent to processing plants by unrefrigerated trucks (Barabas, 1995). The delay between milking and processing can exceed six hours and the lack of modern storage facilities and refrigerated transport can contribute to difficulties in preserving milk in developing countries (Lambert, 2001; Barabas, 1995). In countries with high ambient temperatures (around 30 °C), bacterial growth is accelerated at these temperatures, affecting the bacteriological quality of milk, causing deterioration and affecting the shelf life and safety (Barabas, 1995).

Alternative processes are therefore required to address this problem. One such process that can be applied to delay milk deterioration is the LPS (Pruitt and Reiter, 1985; Barabas, 1995; Zapico *et al.*, 1998). The LPS consists of three components, the lactoperoxidase enzyme, a substrate ( $\text{SCN}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ , or  $\text{Cl}^-$ ) and hydrogen peroxide (Davidson and Brannen, 1993). The lactoperoxidase is an enzyme in milk saliva and tears (Pruitt and Kamau, 1991; Pruitt and Reiter, 1985). The lactoperoxidase enzyme catalyses the oxidation of thiocyanate ( $\text{SCN}^-$ ) to hypothiocyanous acid ( $\text{HOSCN}^-$ ) and hypothiocyanate ( $\text{OSCN}^-$ ) (Davidson and Brannen, 1993). Hypothiocyanate and hypothiocyanous acid are highly reactive oxidising agents and react with sulfhydryl ( $\text{SH}^-$ ) groups, of bacterial cells (Pruitt and Reiter, 1985; Kamau *et al.*, 1990a).

*L. monocytogenes* has been implicated as a causative agent in several large outbreaks of listeriosis around the world, caused by consumption of milk and milk products (Lunden *et al.*, 2004). Listeriosis outbreaks have been associated with dairy products manufactured from raw milk products, especially soft cheeses. Pasteurised milk and milk products have also been implicated in listeriosis outbreaks due to suspected post pasteurisation contamination of products by *L. monocytogenes* or inadequate pasteurisation. *L. monocytogenes* has caused outbreaks of listeriosis in the USA, Canada and Europe (Lunden *et al.*, 2004). The organism is a psychrotrophic foodborne pathogen widely distributed in nature (Farber and Peterkin, 1991). It can survive for many years in the cold and in naturally infected sources (Farber and Peterkin, 1991). *L. monocytogenes* is a gram positive, motile short rod, capable of growth at 4 °C (Frazier and Westhoff, 1988). It is an

opportunistic pathogen occurring sporadically and primarily infecting immune compromised individuals (Farber and Peterkin, 1991).

According to Seifu *et al.*, (2004a) LPS activation has a bactericidal effect against *L. monocytogenes* in goat milk at 30 °C. Kamau *et al.*, (1990a) indicated that activation of the LPS before thermal treatment can enhance thermal destruction of *L. monocytogenes* in milk during pasteurisation. Therefore LPS in combination with pasteurisation may be used to address the growth of *L. monocytogenes* in goat milk and goat milk cottage cheese. The aim of this study was to determine the effect of the LPS alone and in combination with a pasteurisation on the growth of *L. monocytogenes* in goat milk and goat milk cottage cheese.

## **2.2 Materials and methods**

### **2.2.1 Raw goat milk**

Raw milk was obtained from a herd of Saanen goats from the University of Pretoria (UP) Pretoria, South Africa. The goats were milked with a milking machine following standard procedures. Five litre batches of goat milk were collected and transported to the Department of Food Science (UP) immediately after milking. On arrival the milk was pasteurised at 63 °C for 30 minutes and cooled to 25 °C.

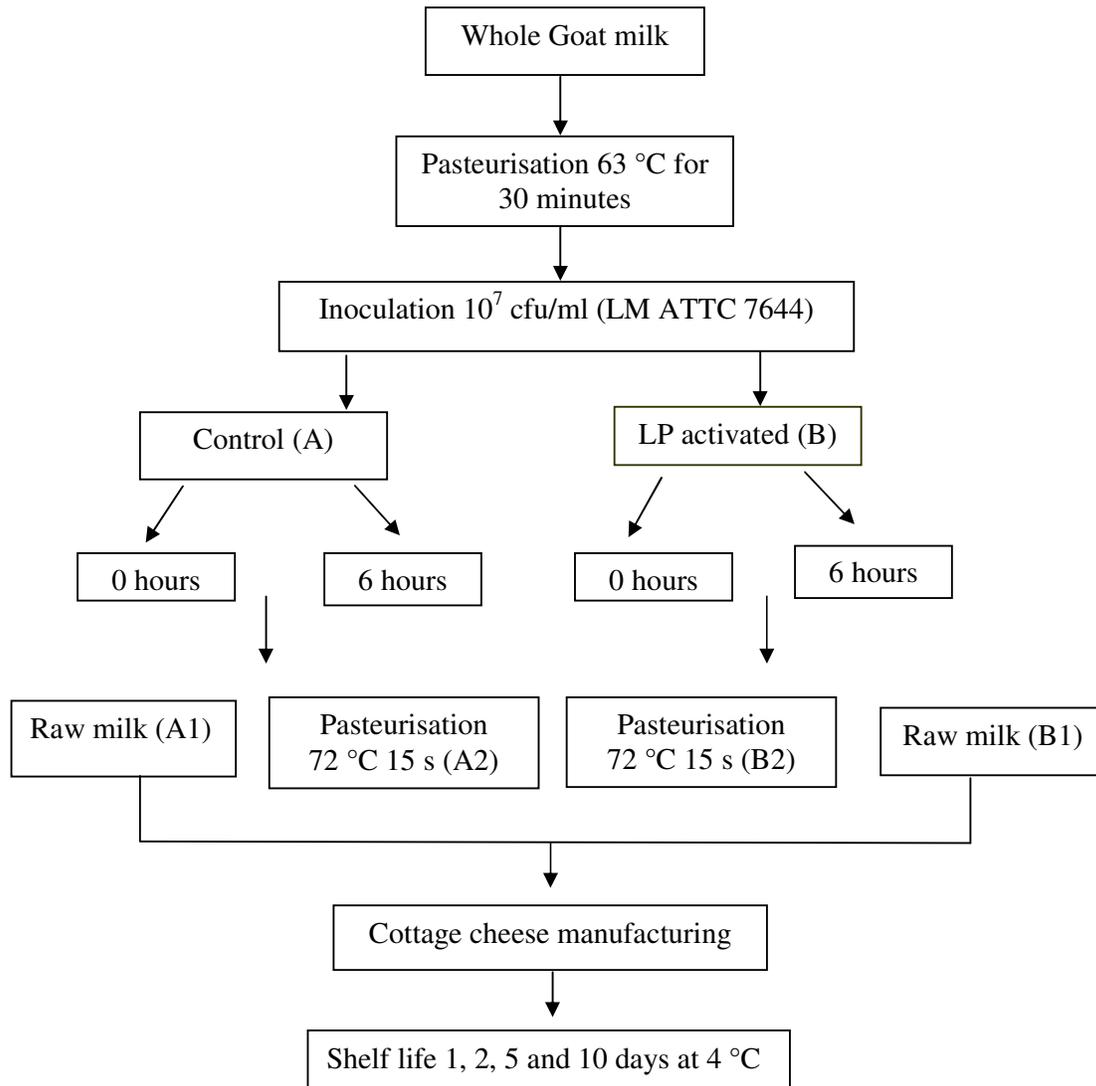
### **2.2.2 Inoculation of milk with *Listeria monocytogenes* ATCC 7644**

One litre of goat milk was used for chemical analysis. Four litres of goat milk was inoculated with 40 ml LM ATCC 7644 suspension. The milk was then divided into two samples, A and B. Sample A was the control and the lactoperoxidase was activated in sample B. Both the control A and the LP activated samples B were kept at ambient temperature (30 °C) for 6 hours (Figure 2.1).

### **2.2.3 Preparation of *Listeria monocytogenes* ATCC 7644 culture**

LM ATCC 7644 was obtained from the Agricultural Research Council (ARC), Irene (South Africa). Stock cultures of LM ATCC 7644 were grown by transferring a loopful

from the original culture onto slants of Tryptone soy agar (TSA) (Oxoid Pty, Ltd, Basingstoke, England) supplemented with 0.6 % yeast extract (Oxoid) and incubated at 37 °C for 24 h. The slants were kept at 4 °C until required. Working cultures of LM ATCC 7644 were prepared by streaking a loopful from the slants on to plates of Blood tryptose agar (BTA) (Oxoid) and incubated at 37 °C for 24 h.



**Figure 2.1 Experimental design for the manufacture of goat milk cottage cheese after lactoperoxidase activation and storage at 4 °C for 10 days**

#### **2.2.4 Preparation of *Listeria monocytogenes* ATCC 7644 suspensions**

Physiological saline was used for preparation of the LM ATCC 7644 suspensions. Suspensions were prepared by transferring colonies of LM ATCC 7644 from Blood Tryptose Agar plates into 40 ml of sterile physiological saline solution. The suspensions were inoculated to reach  $10^7$ /ml LM ATCC 7644 in the milk. The turbidity of the suspensions was checked using the McFarland standard (Balows, Hausler, Herrman, Isenberg and Shadomy, 1991).

#### **2.2.5 Activation of Lactoperoxidase system**

The natural thiocyanate ( $\text{SCN}^-$ ) content and the lactoperoxidase (LP) activity in goat milk samples were first determined (IDF, 1988). The milk was then divided into two samples (Fig 2.1). One sample served as a control and the other was subjected to the activation of LP by addition of 14 mg/l sodium thiocyanate (Saarchem Pty Ltd, Midrand, South Africa) as a source of  $\text{SCN}^-$  to increase the  $\text{SCN}^-$  level. After 1 minute of thorough mixing 30 mg/l sodium per carbonate (Sigma-Aldrich Chemical Co., Midrand, South Africa) was added as a source of hydrogen peroxide (IDF, 1988).

#### **2.2.6 Pasteurisation of milk**

Both the control A and LP activated B samples were divided into two samples each A1 and A2 and B1 and B2 (Fig 2.1). Samples A2 and B2 were subjected to pasteurisation at 72 °C for 15 s. Samples A1 and B1 were not pasteurised.

#### **2.2.7 Manufacturing of goat milk cottage cheese**

Goat milk cottage cheese was manufactured using all four samples (A1, A2, B1 and B2) according to the method suggested by (Kosikowki and Mistry, 1999). The goat milk was pasteurised at 72 °C for 15 s. The milk was then cooled to 23 °C and inoculated with a lactic acid producing starter culture (*Lactococcus lactis* subsp. *Lactis*) and incubated at 30 °C over night. The whey was drained, the curd removed from cotton bags, packed (about 85 g each) and stored at 4 °C until required for microbial analysis.

### **2.2.8 Shelf life of cottage cheese**

The cottage cheese from all the treatments was stored at 4 °C for 10 days. The cheese was analysed for LM ATCC 7644 on days 1, 2, 5 and 10.

### **2.2.9 Enumeration of *Listeria monocytogens* ATCC 7644**

Ten-Fold serial dilutions of cheese samples were made by aseptically transferring 1g sample from 85 g of packaged cheese into 9 ml of sterile quarter Ringer's solutions (Oxoid). Samples were then homogenised for 2 minutes using a stomacher. Further ten-fold serial dilutions of up to  $10^{-8}$  were made by transferring 1 ml of successive serial dilutions into 9 ml of sterile quarter Ringers solution. LM ATCC 7644 was enumerated on Listeria selective agar base (Oxoid) supplemented with Listeria selective agar supplement (Oxoid) after incubation at 37 °C for 24 h.

Calculation of percentage reduction in LM ATCC 7644 count was done as follows:

$100 \times (\text{count in the control milk at 6h} - \text{count in LP activated milk at 6h}) / \text{count in the control milk at 6h}$

### **2.3 Lactoperoxidase activity of goat milk**

The lactoperoxidase activity of milk was determined according to the method by (Kumar and Bhatia, 1999). Three millilitres of ABTS solution and 0.1 ml milk sample dissolved in phosphate buffer. The reaction was initiated by the addition of 0.1 ml of hydrogen peroxide solution and absorbance measured at 412 nm for 2 minutes at 10 seconds intervals.

#### **2.3.1 Thiocyanate content in goat milk**

Thiocyanate content of milk was determined by mixing 4.0 ml of goat milk with 20 % (w/v) trichloroacetic acid. The mixture was allowed to stand for 30 minutes and then filtered with Whatman NO 40 filter paper 1.5 ml of the filtrate was mixed with 1.5 ml of ferric nitrate reagent and absorbance measured at 460 nm within 10 minutes of addition of ferric nitrate. Thiocyanate concentration was measured against a standard curve (IDF, 1988).

### 2.3.2 Titratable acidity (T.A)

Nine grams of soft cottage cheese curd was measured into a small beaker using a top balance. Phenolphthalein (0.5 ml) indicator was added and titrated with N/10 NaOH while stirring constantly, until a definite pink colour lasting 30 seconds was attained. The burette reading was taken (IDF 86, 1981)

$$\% \text{ TA as lactic acid} = \frac{\text{ml NaOH} \times \text{normality of NaOH} \times 0.09 \times 100}{\text{Weight of sample}}$$

### 2.3.3 pH

The pH of cheese was measured with a penetration electrode meter (Sentron Integrated Sensor Technology, Sentron Inc., USA). Only pasteurised milk samples were taken to avoid contamination of the pH meter with *L. monocytogenes*.

### 2.3.4 Statistical analysis

The experiment was repeated three times and duplicate samples were analysed during each repetition. Analysis of variance (ANOVA) was used to determine whether LP activation, pasteurisation and time (0 and 6 hours) significantly influenced the LM ATTC 1644 count in goat milk. ANOVA was also used to determine whether LP activation, pasteurisation and time during day 1, 2, 5 and 10, significantly ( $p \leq 0.05$ ) influenced the LM ATTC 7644 count in goat milk cottage cheese during storage for a period of 10 days at 4 °C Statistica release 7 software (Tulsa, Oklahoma USA, 2003).

## 2.4 Results

### 2.4.1 Effect of the Lactoperoxidase system (LPS) on the growth of *Listeria monocytogenes* ATTC 7644 in raw and pasteurised goat milk

**Table 2.1 Lactoperoxidase activity and thiocyanate content of raw goat milk (n =30)**

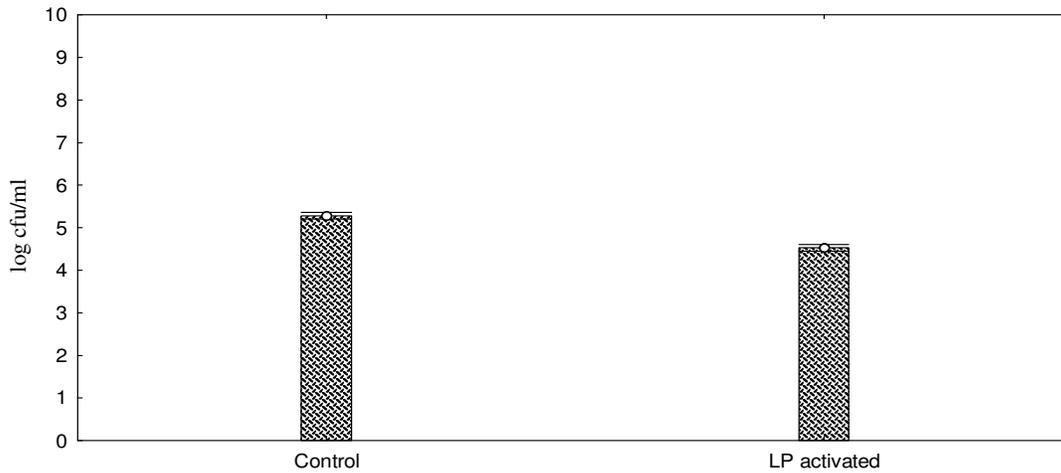
Repetitions (n=10)	Lactoperoxidase (U/ml)	Thiocyanate content (ppm)
Trial 1	0.22	4.37
Trial 2	0.49	5.69
Trial 3	0.33	4.88
Average (n = 30)	0.34 ( $\pm$ 0.08)	4.98 ( $\pm$ 0.25)

Std =  $\pm$ Standard deviation in parenthesis

The average lactoperoxidase activity was 0.34 U/ml in raw goat milk and the thiocyanate content was 4.98 ppm (Table 2.1).

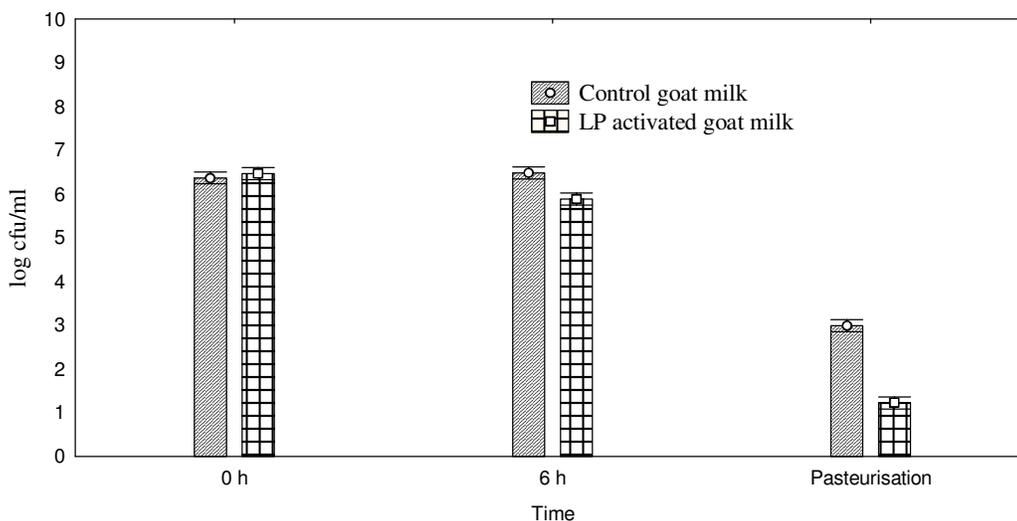
**Table 2.2 Analysis of variance for *Listeria monocytogenes* ATTC 7644 counts in control and lactoperoxidase activated raw and pasteurised goat milk**

Treatment	Degrees of freedom	P-value
Lactoperoxidase	1	0.000
Heat (Pasteurisation vs. raw milk)	2	0.000
Lactoperoxidase * heat	2	0.000



**Figure 2.2 Overall effect of the Lactoperoxidase system on the growth of *Listeria monocytogenes* ATTC 7644 in raw and pasteurised goat milk**

The LP main effect significantly ( $p \leq 0.05$ ) influenced the LM ATTC 7644 count (Fig. 2.2, Table 2.2). The overall mean LM ATTC 7644 count at ambient temperature (30 °C) was log 5.2 cfu/ml for the control and log 4.5 cfu/ml for the LP activated goat milk. Therefore, the mean LM ATTC 7644 count for the LP activated samples was log 0.7 cfu/ml less than the LM ATTC 7644 count for the control. The overall LM ATTC 7644 count in the control milk was reduced by 80 % due to the activation of the LPS.



**Figure 2.3 Growth of *Listeria monocytogenes* ATTC 7644 in control and in lactoperoxidase activated, raw and pasteurised goat milk at 0h, 6h and after pasteurisation**

Initially, at 0h, the mean LM ATTC 7644 count was log 6.4 cfu/ml for the LP activated goat milk and log 6 cfu/ml for the control (Fig. 2.3). Six hours after LP activation the mean LM ATTC 7644 count was log 5.9 cfu/ml for the LP activated milk and log 6.5 cfu/ml for the control. Therefore, after 6h of LP activation there was a significant ( $p \leq 0.05$ ) (Table 2.2) reduction of log 0.5 cfu/ml LM ATTC 7644, in the LP activated goat milk. In the control, the LM ATTC 7644 increased by log 0.5 cfu/ml. The level of LM ATTC 7644 count in the LP activated goat milk was reduced by 75 % in relation to the control milk.

After pasteurisation the mean LM ATTC 7644 count was 1.2 log cfu/ml for the LP activated pasteurised goat milk and log 2.9 cfu/ml (Fig. 2.3) for the control pasteurised. After pasteurisation the LM ATTC 7644 count was significantly less ( $p \leq 0.05$ ), (Table 2.2) by log 1.7 cfu/ml than the control. The LM ATTC 7644 count in LP activated pasteurised goat milk decreased by log 4.7 cfu/ml compared to the control pasteurised milk which decreased by log 3.6 cfu/ml after 6h at ambient temperatures. The LM ATTC 7644 count in the LP activated was 98 % less than the LM ATTC 7644 count in the control milk.

#### 2.4.2 Effect of the Lactoperoxidase system on the growth of *Listeria monocytogenes* ATTC 7644 in raw and pasteurised goat milk goat milk cottage cheese

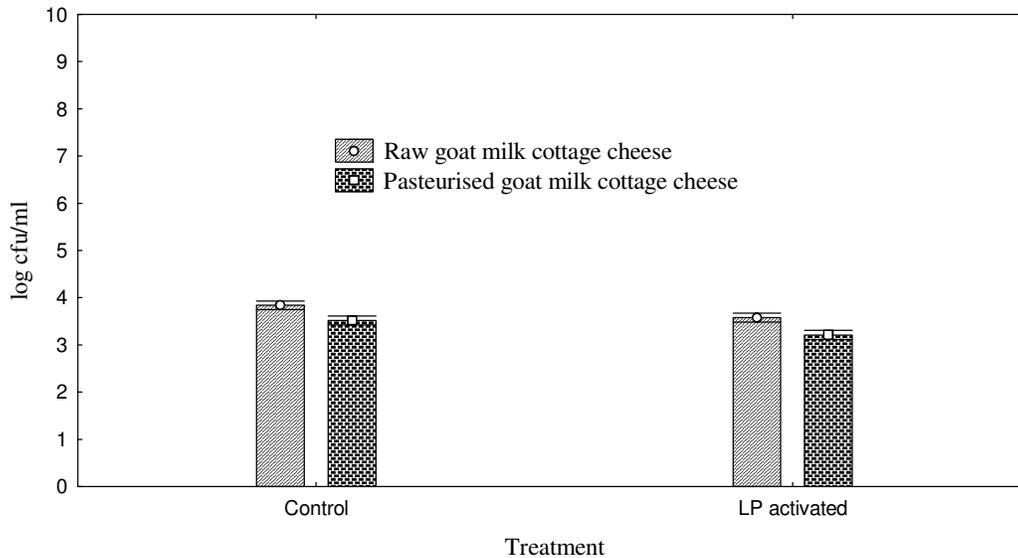
**Table 2.3 pH changes for control and LP activated cottage cheese made from pasteurised goat milk during storage of 10 days at 4 °C (n=4)**

Days (P= 0.00)	Treatment (P=0.99)	
	Control	LP activated
1	4.90	4.80
2	4.70	4.70
5	4.33	4.25
10	4.33	4.29

The initial pH for the control pasteurised goat milk cottage cheese significantly ( $p \leq 0.05$ ) decreased, 4.9 to 4.33, over a period of 10 days at 4 °C. The pH for the LP activated pasteurised goat milk cottage cheese followed the same trend during storage decreasing significantly ( $p \leq 0.05$ ) to 4.29 after 10 days of storage at 4 °C (Table 2.3). However there was no significant difference between the treatments, LP activated and control samples.

**Table 2.4 Analysis of variance for the growth of *Listeria monocytogenes* ATTC 7644 in the control, lactoperoxidase activated raw and pasteurised goat milk cottage cheese stored for 10 days at 4 °C**

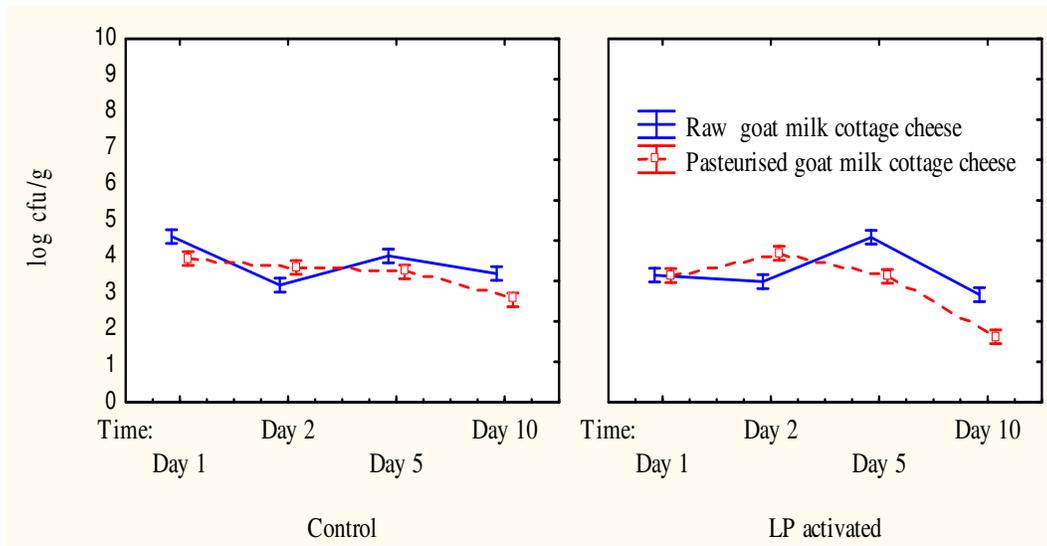
<b>Treatment</b>	<b>Degree of freedom</b>	<b>P-values</b>
Lactoperoxidase	1	0.000
Pasteurisation (pasteurised vs. raw)	1	0.000
Time (1, 2, 5 & 10 days)	3	0.000
Lactoperoxidase * pasteurisation	1	0.607
Lactoperoxidase * time	3	0.000
Pasteurisation * time	3	0.000
Lactoperoxidase* pasteurisation * time	3	0.000



**Figure 2.4 Overall effect of the lactoperoxidase activation on the growth of *Listeria monocytogenes* ATCC 7644 in raw and pasteurised goat milk cottage cheese stored for 10 days at 4 °C**

The overall mean LM ATCC 7644 count for the control raw goat milk cottage cheese was log 3.8 cfu/g, where as for the control pasteurised goat milk cottage cheese was log 3.6 cfu/g (Table 2.4). Therefore, the overall LM ATCC 7644 count for the control pasteurised goat milk cottage cheese was only log 0.2 cfu/g, lower than the LM ATCC 7644 count for the control raw goat milk cottage cheese. In the LP activated goat milk cottage cheese the LM ATCC 7644 count for the LP activated raw goat milk cottage cheese was log 3.5 cfu/g, where as for the LP activated pasteurised goat milk cottage cheese was log 3.2 cfu/g (Table 2.4).

There was no difference between the LM ATCC 7644 counts for the raw goat milk cottage cheese samples, control or LP activated. This was also the trend for the pasteurised samples control or LP activated. Furthermore, the main effect of heat and LP activation decreased the count for the LP activated pasteurised goat milk cottage cheese by log 0.4 cfu/g less than the control pasteurised goat milk cottage cheese.



**Figure 2.5 Effect of the lactoperoxidase activation on the growth of *Listeria monocytogenes* ATCC 7644 in control raw, control pasteurised and lactoperoxidase activated raw and lactoperoxidase activated pasteurised goat milk cottage cheese during storage at 4 °C for 10 days**

For the control raw goat milk cottage cheese, the LM ATCC 7644 count was log 4.5 cfu/g on day 1. The count decreased to log 3.2 cfu/g on day 2 and on day 5 the count increased by log 0.5 cfu/g to log 4.0 cfu/g. On the final day of storage the LM ATCC 7644 count declined to log 3.5 cfu/g (Fig. 2.5). During the storage period of 10 days at 4 °C the LM ATCC 7644 count decreased significantly ( $p \leq 0.05$ ) (Table 2.4) by log 1 cfu/g. The level of LM ATCC 7644 count was 90 % less on the last day of storage compared to the first day of storage.

For the control pasteurised goat milk cottage cheese the LM ATCC 7644 count was 3.9 log cfu/g on day 1 and on day 2 the count remained similar, at log 3.7 cfu/g. On day 5 the count decreased to log 3.5 cfu/g and reached lower levels of log 2.8 cfu/g on day 10 (Fig. 2.5). During the 10 days of storage there was a significant ( $p \leq 0.05$ ) (Table 2.4) decrease of log 1.1 cfu/g LM ATCC 7644 in control pasteurised goat milk cottage cheese. The LM ATCC 7644 count was 92 % lower on day 10 compared to the first day of storage.

The LM ATCC 7644 count for the LP activated raw goat milk cottage cheese was, initially log 3.48 cfu/g, on day 2 the count remained stable. On day 5 the count reached a level of

log 4.5 cfu/g. Over the next 5 days the counts decreased to levels of log 2.9 cfu/g. The count decreased significantly ( $p \leq 0.05$ ) by log 0.59 cfu/g over a period of 10 days (Fig. 2.5) (Table 2.4). The LP activated raw goat milk cottage cheese LM ATTC 7644 count decreased by 74 % during 10 days of storage.

For the LP activated pasteurised goat milk cottage cheese the LM ATTC 7644 count was log 3.48 cfu/g initially. The count increased to log 4.1 cfu/g after 2 days of storage. After day 2 the count decreased to reach levels of log 3.46 cfu/g on day 5. On the last day of storage LM ATTC 7644 count further decreased reached log 1.79 cfu/g (Fig. 2.5). During the entire storage period the LM ATTC 7644 count decreased significantly ( $p \leq 0.05$ ) by log 1.69 cfu/g. The LP activated pasteurised goat milk cottage cheese LM ATTC 7644 count was 98 % less on the last day compared to the first day of storage.

## CHAPTER 3 DISCUSSION

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### 3.1 Lactoperoxidase and thiocyanate content in raw goat milk

The thiocyanate content required to activate the LPS is 15 ppm (Bjock *et al.*, 1979). This is said to be sufficient to obtain a significant improvement in the microbial quality of stored raw milk (Bjock *et al.*, 1979). In the present study an average thiocyanate content of 4.98 ppm was found in raw goat milk. This amount is lower than the one recommended by (Bjock *et al.*, 1979). Other researchers have reported different results. Seifu *et al.*, (2004a) reported 4.58 ppm in indigenous South African goat milk and 2.78 ppm in Saanen goat milk. Zapico, Medina, Gaya and Nunez, (1991) reported 4.5 ppm and Saad de Schoos *et al.*, (1999) reported 10.29 ppm thiocyanate content in Creole goat milk. The differences in thiocyanate content in milk is said to be due to variations in the diet (Reiter, 1985) and genetic differences of the animal.

Various factors have been found to influence the lactoperoxidase concentration in goat milk, namely breed, the animal and the days of lactation. Zapico *et al.*, (1991) reported 0.95 U/ml in Vereta and 2.15 U/ml in Murciano goats. In this study the mean LP activity of goat milk was 0.34 U/ml, which differs from other reports by Zapico *et al.*, (1991), Fonteh *et al.*, (2002) and Seifu *et al.*, (2004a). Fonteh *et al.*, (2002) reported the highest LP activity of 0.25 U/ml and the lowest LP activity of 0.01 U/ml. The lactoperoxidase activity in goat milk has been found to be lower compared to cow milk (Fonteh *et al.*, 2002). Variations in LP activity have been observed in individual goats and between weeks of sampling with the highest LP activity observed during mid lactation (Fonteh *et al.*, 2002). Seifu *et al.*, (2004a) reported mean LP activity of 0.26 U/ml in Indigenous goat milk and 0.79 U/ml in Saanen goat milk.

The quantity of peroxidases found in different milk has been observed to be a contributing factor to the differences in bactericidal properties of the LPS (Pruitt and Kamau, 1991). Seifu *et al.*, (2004a) found that the LPS in Saanen goat milk had a higher inhibitory affect on *Staphylococcus aureus* where as in Indigenous goat milk the LP activation was only bacteriostatic. Seifu *et al.*, (2004a) attributed the higher inhibitory effect of LP in Saanen goat milk to the higher LP activity in the Saanen goat milk than Indigenous goat milk. In another study Zapico *et al.*, (1991) found that goat milk activation of the lactoperoxidase

enzyme had less inhibitory affect than in cow milk. This was also said to be due to the lower LP activity in goat milk compared to cow milk.

### **3.2 Effect of the lactoperoxidase activation on the growth of *Listeria monocytogenes* ATTC 7644 in raw goat milk after storage at ambient temperatures for 6h**

The LP main effect significantly ( $p \leq 0.05$ ) influenced the LM ATTC 7644 count. The overall mean LM ATTC 7644 count at ambient temperature (30 °C) was log 5.2 cfu/ml for the control and log 4.5 cfu/ml for the LP activated goat milk. The activation of lactoperoxidase in this study caused a significant ( $p \leq 0.05$ ) log 0.5 cfu/ml decrease after 6h at ambient temperature. This suggests that the LP activation had a positive impact on LM ATTC 7644 counts in goat milk when stored at ambient temperatures for 6h. These results are in agreement with a previous study by Boussouel *et al.*, (2000) who reported a bacteriostatic phase of about 50h on *L. monocytogenes*, when lactoperoxidase was activated in skim milk.

In a study by Rodriguez, Tomillo, Nunez and Medina, (1997) at 8 °C activation of the LPS was responsible for a reduction of 0.37 log units of *L. monocytogenes* compared to the control. Haddadin, Ibrahim and Robinson, (1996) noted that, the shelf life of the LP activated milk was increased to 18h at 22 °C. In similar study Seifu *et al.*, (2004a) reported a decrease of 0.12 log units *L. monocytogenes* in LP activated indigenous milk compared to the control which had an increase of 0.92 log units after 6h at ambient temperatures. The reduction of *L. monocytogenes* is said to be due to the production of the hypothiocyanate anion which is the major antimicrobial of the LPS (Kamau *et al.*, 1990a). The results from this study indicated that the LPS can therefore be used to prevent *L. monocytogenes* from increasing in raw milk for 6h, when inadequate cooling is not available. The log 0.5 cfu/ml reduction of LM ATTC 7644 counts in LP activated goat milk shows that goat milk lactoperoxidase is capable of reducing *L. monocytogenes* in goat milk when stored at ambient temperatures compared to the control.

The increase of LM ATTC 7644 (log 0.5 cfu/ml) after 6h at ambient temperature indicates that LM ATTC 7644 in untreated milk can multiply and increase when kept at ambient temperatures. The LM ATTC 7644 count in untreated goat milk was lower than the count in LP activated sample at 0h by (log 0.4 cfu/ml). The LM ATTC 7644 in untreated goat

milk was able to grow faster than the LM ATTC 7644 in LP activated sample and was (log 0.5 cfu/ml) more than the LP activated sample after 6h. This increase may be attributed to the favorable conditions in milk as well as the ambient temperatures the milk was kept at for 6h. The nutrients in milk as well as the high water activity make it ideal for microbial growth (Varnam and Sutherland, 1994). The favorable conditions in untreated milk may have reduced the lag phase of the organism therefore enabling it to grow faster than the LP activated samples. This is in agreement with results by Haddadin *et al.*, (1996) who also reported that untreated samples of bovine milk became unacceptable after 3h and the rate of deterioration of *L. monocytogenes* accelerated after 3h.

### **3.3 Effect of the lactoperoxidase activation in combination pasteurisation on the growth of *Listeria monocytogenes* ATTC 7644 in goat milk**

The LM ATTC 7644 count in LP activated pasteurised goat milk decreased by log 4.7 cfu/ml compared to the control pasteurised goat milk which decreased by log 3.6 cfu/ml after being kept at ambient temperatures for 6h. The higher reduction observed in LP activated milk may be due to a combined effect of the LP activation and pasteurisation on the organism. This indicates that combining the LPS and pasteurisation may have a synergistic effect on *L. monocytogenes* in goat milk. These results are in agreement with studies done by Kamau *et al.*, (1990a) who reported that the increase in heat sensitivity was more pronounced in the presence of the LPS. This may be due to the fact that when two or more stress factors such as acids, salts and other antimicrobial agents are present, they can enhance thermal destruction of bacteria (Kamau *et al.*, 1990a). These researchers found that the LPS eliminated the lag phase and increased the death rate of *L. monocytogenes*. They also noted that combination of heating and LP activation increased the maximum specific death rates of *L. monocytogenes* in milk. Therefore the reduction of LM ATTC 7644 count may imply that pasteurisation together with lactoperoxidase activation may be more effective than pasteurisation alone in controlling the growth of *L. monocytogenes* in goat milk after a storage period of 6h at ambient temperatures.

### **3.4 Effect of the lactoperoxidase activation on the growth of *Listeria monocytogenes* ATTC 7644 in control raw, control pasteurised, LP activated raw and LP activated pasteurised goat milk cottage cheese.**

Overall the LPS significantly ( $p \leq 0.05$ ) affected LM ATTC 7644 count in goat milk cottage cheese over a period of 10 days at 4 °C. This is due to LP activation in the goat milk which led to differences of the LM ATTC 7644 count in raw goat milk used during cheese making.

A decrease in the LM ATTC 7644 count in control raw goat milk cottage cheese count, log 1 cfu/g, (90 % reduction) and in LP activated raw goat milk cottage cheese, log 0.59 cfu/g, (74 % reduction) was observed over a storage period of 10 days at 4 °C. There was also a decrease observed in LM ATTC 7644 counts of control raw and LP activated goat milk cottage cheese on day 2 of storage. This could be attributed to the physical entrapment of LM ATTC 7644 in the curd as well as the lowering of the pH therefore reducing the bacterial count (Ramsaran, Chen, Brunke, Hill and Griffiths, 1998). Reactions in the cytoplasm do so in an aqueous environment, therefore the reduction of water during draining might have changed the water activity, slowed the active growth of the organism resulting in reduced growth (Adams and Moss, 2004).

Ramsaran *et al.*, (1998) indicated that the counts in raw milk Feta cheese decreased below those of initial inoculum, 24h into curd formation, the LP activated remained at this level for some days before decreasing further during storage. The results of this study are in agreement with reports by Rogga *et al.*, (2005) who found that the populations of *L. monocytogenes* declined from day 0 to 3 followed by smaller declines from days 3 to 28 at 4 °C in untreated samples. In similar study Millet, Saubusse, Didiene, Tessier and Montel, (2006) attributed the decrease of *L. monocytogenes* in the core of raw milk Saint-Nectaire-type cheeses from manufacture until day eight of ripening to pH and the lactic acid content.

In this study on day 5, the LM ATTC 7644 level increased in both the control raw and LP activated raw goat milk cottage cheese, this increase may indicate that the *L. monocytogenes* trapped in the curd might have survived and multiplied therefore, increasing in the control raw and LP activated raw goat milk cottage cheese (Ramsaran *et al.*, 1998). The LM ATTC 7644 count of the control raw goat milk cottage cheese decreased more during the 10 days of storage than the LM ATTC 7644 count of the LP activated raw goat milk cottage cheese which may have been as a result of the differences

in the increase of the LM ATTC 7644 count observed on day 5 in LP activated raw goat milk cottage cheese (log 4.5 cfu/g) compared to control raw goat milk cottage cheese (log 4.0 cfu/g). After day 5 both LM ATTC 7644 count in control raw and LP activated raw goat milk cottage cheese decreased and reached  $10^3$  cfu/g and  $10^2$  cfu/g respectively.

However, after 10 day storage at 4 °C the LP activated raw goat milk cottage cheese LM ATTC 7644 count was 1 log lower than the control raw goat milk cottage cheese on the last day of storage, day 10. This can be attributed to the combined effect of the low pH and LP activation on the organism as more than one stress factors are said to be more deleterious to bacteria (Kamau *et al.*, 1990a).

In the control pasteurised goat milk cottage cheese the LM ATTC 7644 count decreased by log 1.1 cfu/g (92 % reduction) over the storage period from 1 to 10 days at 4 °C, where as during the same period the LM ATTC 7644 count in pasteurised LP activated goat milk cottage cheese the count decreased by log 1.69 cfu/g (98 % reduction). The decrease in LM ATTC 7644 count was therefore more extensive in LP activated pasteurised goat milk cottage cheese compared to the other treatments. In contrast to the trend for the LM ATTC 7644 count in the control raw and the LP activated raw goat milk cottage cheese, the LM ATTC 7644 count in the control pasteurised goat milk cottage cheese and LP activated pasteurised goat milk cottage cheese, decreased steadily over the storage period for 10 days of storage at 4 °C.

After 10 days of storage at 4 °C the LM ATTC 7644 level in LP activated pasteurised goat milk cottage cheese had decreased more than any of the other treatments over 10 day storage at 4 °C from log 3.48 to log 1.79. cfu/g. The increased reduction of LM ATTC 7644 count in LP activated pasteurised goat milk cottage cheese compared to other treatments may be due the combined effects of pasteurisation, decreasing pH and lactoperoxidase activation on *L. monocytogenes*. Presence of one or more stressful factors in food is also more deleterious because these factors act as hurdles to the microorganism (Beales, 2004). This could also be attributed to heat injury of *L. monocytogenes*, and the organism may have not been able to recover, and therefore multiply. However, these stress factors did not completely eliminate the organism which was inoculated at a level of  $10^7$ /ml. The results of this study differ from results by Denis and Ramet, (1989), who

found that the activation of the LP decreased *L. monocytogenes* counts on the surface of soft cheese ranging from 10 to 10<sup>6</sup> cfu/g to below detection level.

In a study by Galdiero *et al.*, (1997) no growth of *L. monocytogenes* was observed at pH 4.5 at 4 °C but noted growth at 22 °C and 30 °C. These results differ with the results of the current study as at pH 4.33 and pH 4.25, on day 5 of storage, the LM ATTC 7644 count reported was log 4.0 and 3.5 cfu/g in the raw control and raw LP activated goat milk cottage cheese. Where as in pasteurised control and pasteurised LP activated goat milk cottage cheese the counts were log 3.5 and 3.46 cfu/g respectively. These results show that the LM ATTC 7644 in this study survived a pH of 4.3 at 4 °C. This may be due to the high initial number (10<sup>7</sup>/ml) of inoculated LM ATTC 7644 count in raw goat milk. The higher the number of organisms the higher the degree of resistance, this is said to be due to the production of protective enzymes produced by the cells or the presence of microorganisms with differing degrees of natural resistance, making the organism more resistant (Jay, 2000). Therefore the ability of the LPS and pasteurisation to reduce the LM ATTC 7644 count by 98 % reduction in goat milk cottage cheese during 10 day storage at 4 °C may be an indication that this combination may be used to improve the safety of cottage cheese.

### **3.5 Conclusion**

These results indicate that the activation of the LPS significantly decreased the LM ATTC 7644 count in goat milk, during a period of 6h, at ambient temperatures. Furthermore, combined pasteurisation and LP activation had a synergistic effect on the LM ATTC 7644 count in goat milk. The LM ATTC 7644 count declined in cottage cheese made from both control and LP activated goat milk. However, a greater decrease was observed in LP

activated pasteurised goat milk cottage cheese over the storage period of 10 days at 4 °C. Consequently, the increased reduction of the LM ATTC 7644 count in goat milk and goat milk cottage cheese by combining LPS and pasteurisation indicates that, this combination may be used to reduce the multiplication of LM ATTC 7644 for production of safer products like goat milk and goat milk cottage cheese.

## CHAPTER 4 GENERAL DISCUSSION

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The main objective of this study was to determine of the effect of the LPS alone and in combination with pasteurisation on the growth of *L. monocytogenes* in goat milk and in goat milk cottage cheese during a shelf life of 10 days at 4 °C.

Milk is a highly nutritious and perishable raw material, making it an ideal product for bacteria to multiply rapidly and unsuitable for processing or for consumption (Varnam and Sutherland, 1994). In developing countries about 20 % of milk produced is lost or undervalued because of poor microbial quality (Lambert, 2001). Goat milk production in some countries is done by small scale farmers, who sometimes have no access to refrigerated transport (Barabas, 1995). This result in milk reaching processing plants in an unacceptable state, hence it is undervalued. In countries like Ethiopia raw milk is left to ferment at ambient temperatures during the manufacturing of Ergo, which may end up being contaminated by different bacteria including pathogens (Ashenafi, 1994). Indigenous antibacterial systems in milk can therefore be applied practically to preserve milk. The LPS is one of methods which can be used.

### 4.1 Methodology

On arrival at the pilot plant (University of Pretoria, Food Science Department), the goat milk was pasteurised at 63 °C for 30 minutes and cooled to 25 °C. The pasteurisation was meant to inactivate bacteria that might have been present in the milk due contamination during handling. Milk was inoculated with  $10^7$  *L. monocytogenes* ATTC 7644, this level was chosen to clearly see differences between treatments as at low levels the organism could have been eliminated therefore not showing differences.

Thiocyanate content was determined according to the method by (IDF, 1988). The samples were deproteinised with trichloroacetic acid, after deproteinisation the samples were filtered with a suitable filter paper and the filtrate mixed with ferric nitrate reagent and absorbance measured at 460nm. Sources of thiocyanate are the glucosinolates and cyanogenic glucosides found in vegetables (Wolfson and Sumner, 1993). Glucosinolates present in vacuoles are hydrolysed and the hydrolysis products include thiocyanates, isothiocyanates, nitriles and others reaction products (Vaughn and Berhow, 2005). The

thiocyanate content of goat milk in this study was  $4.98 \pm 0.25$  ppm, these results differ from reports by Seifu *et al.*, (2004a) who reported different thiocyanate content in both the Indigenous and the Saanen goat milk.

To determine the lactoperoxidase activity of goat milk a method by Kumar and Bhatia, (1999) was used. The LPS is an indigenous antimicrobial in milk. It has been found in relatively high concentrations in bovine and buffalo milk. In goat milk the lactoperoxidase activity has been found to be lower than that of bovine and buffalo milk. The lactoperoxidase enzyme catalyses the reaction of thiocyanate and hydrogen peroxide converting thiocyanate to hypothiocyanous acid. Only small concentrations of hydrogen peroxide are required as high concentrations destroy the lactoperoxidase enzyme. The system is mainly inhibitory in mixed flora but it is bacteriostatic against gram negative bacteria like *Escherichia coli* (Farrag, El-Gazzar and Marth, 1992). According to Seifu *et al.*, (2004a) the LPS was bactericidal to *L. monocytogenes* at 30°C in both Saanen and Indigenous goat milk.

The efficacy of LPS is generally evaluated by assessing the enzyme activity, the method is colourmetric and involves the oxidation of 2,2'-azino-bis-3ethylbenz-thiazoline-6-sulphonic acid (ABTS) by hydrogen peroxide. Oxidation of ABTS produces a green colour whose absorbance is measured by the use of spectrophotometer with the rate of oxidation being directly proportional to the enzyme activity (Fonteh, Gradison and Lewis, 2005).

Five mg of ABTS (Sigma) was dissolved in phosphate buffer ( $0.1 \text{ mol}^{-1}$  pH 6.0) and made up to the volume of 100 ml to determine the lactoperoxidase activity. Hydrogen peroxide stock solution was prepared, the goat milk lactoperoxidase was prepared by dissolving it in phosphate buffer saline. The ABTS solution, peroxidase sample were added in a cuvette and the reaction was initiated by addition of 0.1 mol of hydrogen peroxide and absorbance measured at 412 nm as a function of time for 2 min at 10 s intervals. The LPS was activated by addition of sodium thiocyanate as a source of  $\text{SCN}^-$  to increase the  $\text{SCN}^-$  and sodium per carbonate was added as a source of  $\text{H}_2\text{O}_2$  (IDF, 1988). Raw goat milk in this study had a lactoperoxidase activity of  $0.34 \pm 0.08$  U/ml, Seifu *et al.*, (2004a) reported different lactoperoxidase activity in both the indigenous and the Saanen goat milks. Zapico

*et al.*, (1991) also reported deferring lactoperoxidase activity in Vereta and Murciano-Granadina goats. These differences are attributed to variations in the diet and genetic differences of the animal (Reiter, 1985).

The microbiological safety of goat milk and goat milk cottage cheese was determined by enumeration of *L. monocytogenes* on Listeria selective agar (Oxford Formulation) (Oxoid). The Listeria selective medium utilises the selective inhibitory components lithium chloride, acriflavine, colistin sulphate, cefotetan, cycloheximide or amphotericin B and fosfomycin. It also uses the indicator system aesculin and ferrous iron. The organism hydrolyses aesculin, producing black zones around the colonies due to formation of black iron phenolic compounds derived from the aglucon. Listeria selective agar supplement (Oxoid) was added to enhance more recovery of the organism. The incubation temperature used was 37 °C, this temperature is the one usually used, with the majority of laboratories using 24h incubation and about 20% using of 48h incubation (Augustin and Carlier, 2006).

*L. monocytogenes* was prepared by transferring a loopful from the original cultures on to slants of tryptone soy agar supplemented with 0.6% yeast extract and incubated at 37 °C for 24h. The slants were maintained at 4 °C until used. Working cultures were obtained by transferring a loopful from the slants onto Blood tryptose agar and incubated at 37 °C. Physiological saline solution was used to mix the suspensions.

The surface plate method was used for enumeration of *L. monocytogenes* in goat milk and goat milk cottage cheese. The most common selective media are those of Curtis Mitchell, King and Griffin, (1989) which are the Aloa, Oxford formulation and the PALCAM agar. The routine method recommends the use of PALCAM agar and the OXFORD agar while the international reference method recommends the use of PALCAM agar (Augustin and Carlier, 2006). Other alternative media are the rapid *L. monocytogenes* and Compass *L. monocytogenes*. The culture media used by most laboratories is the PALCAM agar which is used by more than 50% where as the ALOA, rapid *L. monocytogenes* and Compass *L. monocytogenes* media are used by 35% of the laboratories. Jatisatiern and Busse, (1989) reported that Oxford agar had an advantage over other agars, in that *Listeria* spp. could be visually differentiated from other bacteria without the need for any further tests. With

Oxford formulation agar all species are isolated by these methods and are morphologically distinguishable from each other.

For differentiation of *L. monocytogenes* substrates have been added to detect phospholipase (Notermans Dufrenne, Jleimeister-Wachter, Domann and Chakraborty, 1991) or  $\beta$ -glucosidase and enhanced haemolysis (Beumer, Giffel and Cox, 1997). The ALOA media isolates stresses cultures of *L. monocytogenes* serovars being tested, and they grow on the medium as bluish colonies surrounded by a distinctive opaque halo and gave a productivity ratio of at least 0.95. Non-pathogenic *Listeria* spp. produced bluish colonies without a halo. ALOA detected 4.3% more positives from naturally contaminated dairy and meat samples, only 13.9% false negatives were found compared with 38.9% using PALCAM/Oxford. The effectiveness of Oxford selective medium together with the use of selective enrichment media on *L. monocytogenes* have been confirmed by Van Kessel, Karns, Gorski, Mc-Cluskey and Perdue, (2004).

#### **4.2 Survival of *Listeria monocytogenes* ATTC 7644 in goat milk at 0h, 6h after pasteurisation.**

The milk was inoculated with LM ATTC 7644 and analysed at 0h and 6h as well as after pasteurisation. This was done to determine the effect of the LPS after 6h, which is the time the milk should take from the farm to the processing plant for it to be suitable for processing (Barabas 1995). This was important in this study as in situations where refrigerated transportation is not feasible the LPS can be an option to retard or reduce the bacterial growth during collection and transportation to the processing plant.

The highest count reported, of *L. monocytogenes* shed by a naturally infected cow is about  $1-2 \times 10^4$  cfu/ml Farber, Sanders, Speirs, D'Aousy, Emmons, and Mckellar, (1988). Farber and Peterkin, (1991) suggested that the infectious dose of *L. monocytogenes* ranged from  $2.7 \times 10^6$  to  $3.4 \times 10^9$  cells of *L. monocytogenes* per gram or millilitre of in food. Exposure to the organism does not always cause a disease in healthy humans. The likelihood of systematic infection may depend on the host susceptibility, the virulence of the organism and the infectious dose. Serotypes mainly involved in human listeriosis are 4b, 1/2a and 1/2b. Strains of *L. monocytogenes* may affect the survival of the organism in food. Gahan *et al.*, (1996) reported that acid tolerant and acid adapted strains had a greater

survival than the non-adapted strains of *L. monocytogenes*. Because of their lower hurdle effect when compared to hard cheeses, soft cheeses have shown a higher incidence and potential for survival of *L. monocytogenes* (Farber and Peterkin, 1991; Ramsaran *et al.*, 1998).

In this study the levels of LM ATTC 7644 count were reduced by log 0.5 cfu/ml in LP activated goat milk after 6h storage at ambient temperatures where as the control increased by log 0.5 cfu/ml. Combination of pasteurisation and LP activation further reduced the count by log 4 cfu/ml where as the control was reduced by log 3 cfu/ml. Therefore with naturally infected *L. monocytogenes* reported at  $1-2 \times 10^4$  cfu/ml (Farber *et al.*, 1988) and this present study showing that combination of pasteurisation and LP activation may reduce the LM ATTC 7644 count in goat milk cottage cheese by log 4 cfu/g after 10 day storage at 4 °C. Combination of pasteurisation and LP activation may lead to elimination if the milk was naturally contaminated at the levels reported, increasing the safety of the product.

The results in this study indicate that the LPS significantly reduced the growth of *L. monocytogenes* in goat milk after 6h at ambient temperatures as well as after pasteurisation. At 0h LM ATTC 7644 count in the control was lower than the count in the LP activated goat milk. After 6h at ambient temperatures the LM ATTC 7644 count in the control was higher than the LM ATTC 7644 count in the LP activated goat milk. Seifu *et al.*, (2004a) reported a decrease of 0.12 log units *L. monocytogenes* in LP activated indigenous milk compared to the control which had an increase of 0.92 log units after 6h at ambient temperatures. This was probably due to the fast multiplication of *L. monocytogenes* in the control as a result of the favourable conditions in untreated milk as well as no inhibitory stresses.

After 6h at ambient temperatures the milk was pasteurised at 72 °C for 15s. Pasteurisation has been found to be able to destroy spoilage as well as pathogenic bacteria in milk (Jay, 2000). In this study pasteurisation alone reduced the LM ATTC 7644 count by log 3.6 cfu/g while pasteurisation and the LPS reduced the LM ATTC 7644 count by log 4.7 cfu/g which is log 1.1 cfu/g higher than the count in pasteurised milk only. This agrees with the studies by Kamau *et al.*, (1991) who reported a higher destruction of LM ATTC 7644

ATTC 7644 count in milk when pasteurisation was combined with the LPS. The availability of two or more stress factors has been found to synergistically enhance the destruction of bacteria in food (Kamau *et al.*, 1991).

#### **4.3 Survival of *Listeria monocytogenes* ATTC 7644 in goat milk cottage cheese during storage at 4 °C for 10 days**

In raw goat milk cottage cheese the LM ATTC 7644 count decreased in control raw goat milk cottage cheese as well as in LP activated raw goat milk cottage cheese. The LP activated raw goat milk cottage cheese had the lowest LM ATTC 7644 count at log 2.9 cfu/g compared to log 3.5 cfu/g of the control raw goat milk cottage cheese after 10 day storage at 4 °C. The LM ATTC 7644 count reduced from the  $10^6$  cfu/ml initially obtained in raw goat milk to the levels of  $10^3$  cfu/g and  $10^2$  cfu/g in control raw goat milk and LP activated raw goat milk respectively. This could be due to the combination of inhibitory factors in LP activated raw goat milk cottage cheese which are the decreasing pH and the LP activation.

The overall decrease in control raw goat milk cottage cheese could be due to the susceptibility of *L. monocytogenes* to pH in raw goat milk cottage cheese. Low pH is said to affect microorganisms by leaking hydrogen ion across the membrane and acidifying the internal pH, becoming toxic due to low acid therefore affecting the biochemical reactions and the macromolecular structure of microorganisms (Foster, 2000; Hill *et al.*, 2002). In this study the assumption is that weak acid, which is lactic acid from the lactic acid bacteria might have entered the cell undissociated, disassociating inside the cell therefore decreasing the internal pH (Phan-Thanh *et al.*, 2000).

In pasteurised goat milk cottage cheese the LM ATTC 7644 count decreased steadily during storage at 4 °C for a period of 10 days. The LP activated pasteurised goat milk cottage cheese decreased more and was log 1.79 cfu/g compared to the control pasteurised goat milk cottage cheese with log 2.9 cfu/g on the last day. The decrease in LM ATTC 7644 count in pasteurised goat milk cottage cheese was from  $10^6$  cfu/ml in goat milk to  $10^2$  and 10 in LP activated pasteurised and control pasteurised goat milk cottage cheese respectively. This indicates that the combination pasteurisation and LP activated caused

more decrease of the LM ATTC 7644 count in goat milk cottage cheese compared to other treatments but the organism was not completely eliminated. Griffiths, (1989) found that *L. monocytogenes* if present in numbers in excess of  $10^2$  cfu/ml prior to cheese making may survive cheese making and may remain viable in the final product even after the storage period.

Rogga *et al.*, (2005) reported that the survival of *L. monocytogenes* was independent from the initial level of contamination of *L. monocytogenes* in Galotyri cheese. The lowest pH limit *L. monocytogenes* can resist depending on the medium composition, the strain and its physiological state. This therefore suggest that even at lower levels of contamination other factors such as the adaptability of strain and how tolerant the stain is to acid may influence the survival in cheeses contaminated by low amount of the *L. monocytogenes* (Rogga *et al.*, 2005). Phan-Thanh *et al.*, (2000) reported acidic pH limit of 4 and 3.5 for strains LO28 and EGD respectively. In this study the lowest LM ATTC 7644 count reported was log 1.79 cfu/g in goat milk cottage cheese after 10 day storage at 4 °C at the pH of 4.29 where the inoculation level was  $10^7$  cfu/ml in goat milk used for cheese making.

## CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

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The Lactoperoxidase system was inhibitory against LM ATTC 7644 in goat milk stored at ambient temperatures for 6h. Furthermore, after pasteurisation the level of LM ATTC 7644 count decreased in LP activated pasteurised goat milk resulting in the lowest count compared to other treatments. Thus the combined pasteurisation and LP activation had a synergistic effect on the LM ATTC 7644 count in goat milk. The mean LP activity of goat milk in this study was 0.34 U/ml and the thiocyanate content was 4.98 ppm.

The LM ATTC 7644 count declined in cottage cheese made from both control and LP activated goat milk. In pasteurized cottage cheese the level of LM ATTC 7644 decreased more than the level in raw cottage cheese. However, a greater decrease was observed in LP activated pasteurised goat milk cottage cheese over the storage period of 10 days at 4 °C. Consequently the increased reduction of the LM ATTC 7644 count in goat milk and goat milk cottage cheese by combining LPS and pasteurisation may indicate that, this combination may be used to reduce the multiplication of LM ATTC 7644 for production of safer products like goat milk and goat milk cottage cheese.

This study demonstrated that a combination of lactoperoxidase and pasteurisation reduced LM ATTC 7644 in goat milk and goat milk cottage cheese after inoculation of  $10^7$  cfu/ml at the pH of 4.29. Future research may be necessary to look at the survival of more acid tolerant or acid adapted strains of *L. monocytogenes* inoculated in low levels of  $10^2$  cfu/ml in goat milk and goat milk cottage cheese, also a research on the implementation of the system should be done to demonstrate the effectiveness of this system in countries with high ambient temperatures (around 30 °C).

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