



**Application of the lactoperoxidase system to improve the
quality and safety of goat milk and goat milk cheese**

By

Eyassu Seifu Defabachew

Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy (PhD)

in the

**Department of Food Science
Faculty of Natural and Agricultural Sciences**

University of Pretoria

Pretoria

Republic of South Africa

October 2003



DECLARATION

I declare that the thesis which I hereby submit for the degree of PhD at the University of Pretoria is my own work and has not previously been submitted by me for a degree at any other University or institution of higher education.

A handwritten signature in black ink, appearing to be 'A. J. ...'.



DEDICATION

This thesis is dedicated to my brother Derebe Nigatu who like any one of us had plans and aspirations for life but died at the age of thirty.

ACKNOWLEDGEMENTS

My sincere gratitude and acknowledgements goes to all individuals and institutions that have supported me in many ways as follows:

My promoter Dr. E. M. Buys for her constant guidance and supervision during the course of the study.

My co-promoter Prof. E. F. Donkin for his interest in my work and for his support and cooperation during the execution of the study.

Prof. B. H. Bester for his encouragement and valuable comments during the planning and inception of the study.

The Agricultural Research and Training Project (Ethiopian Government) for fellowship financial support and Alemaya University for study leave permit.

Dr. Riëtte de Kock and Mrs. Marise Kinnear for their assistance during the sensory evaluation of the cheese samples.

Dr. F. Kanfer and Dr. Mike van der Linde for their assistance during the statistical analysis of the data.

Dr. I-M Petzer of the Veterinary Faculty of the University of Pretoria for allowing me to use her laboratory and for her cooperation while conducting the experiment on some of the pathogens and Mr. Theo van der Schans for his technical assistance during this period.

Dr. Marijke M. Henton and Dr Anita Michel of the Onderstepoort Veterinary Institute for allowing me to use their laboratories and for their cooperation while conducting my experiment on *Brucella melitensis* and *Mycobacterium paratuberculosis*, respectively.



Mr. P. Schoeman and the Onderstepoort Teaching Animal Unit staff for their assistance during the collection of the goat milk samples.

All the academic and technical staffs of the Department of Food Science who rendered me their assistance one way or another during my stay in the Department.

Prof. J. G. Van der Walt of the Department of Anatomy and Physiology of the Faculty of Veterinary Science for allowing me to use their spectrophotometer while conducting my experiment at Onderstepoort.

ABSTRACT

Application of the lactoperoxidase system to improve the quality and safety of goat milk and goat milk cheese

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Eyassu Seifu Defabachew

Promoters: Dr. E. M. Buys
Professor E. F. Donkin
Department: Food Science
Degree: Ph.D.

The lactoperoxidase system is an endogenous antibacterial system present in milk and is used for preservation of raw milk especially under situations where it is not possible to cool the milk. In this thesis the inhibitory effect of the lactoperoxidase (LP) system against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Brucella melitensis* was investigated in Saanen and South African Indigenous goat milks. The effect of the LP system on the activity of mixed strain and single strain mesophilic cheese starter cultures was investigated in Saanen goat milk, and a starter culture that is resistant to the LP system was identified. The suitability of Saanen goat milk preserved by the LP system for the manufacture of Gouda cheese was determined.

The LP system exhibited a bactericidal effect against *L. monocytogenes* and *Br. melitensis* both in Saanen and Indigenous goat milks during a storage period of 6 h at 30 °C. The LP system showed a bactericidal effect against *S. aureus* in Saanen goat milk; however, it was bacteriostatic against *S. aureus* in Indigenous goat milk. On the other hand, the LP system only reduced the rate of multiplication of *E. coli* both in Saanen and Indigenous goat milks. The same pathogen showed different degrees of sensitivity to the LP system in the milk of the two goat breeds probably because of the difference in the LP system components in the milk of the two goat breeds. Saanen goat milk had a mean LP activity of 0.79 ± 0.18 U/ml and a mean thiocyanate concentration of 2.78 ± 1.21 ppm.

However, Indigenous goat milk had a mean LP activity of 0.26 ± 0.10 U/ml and a mean thiocyanate concentration of 4.58 ± 1.92 ppm.

Most of the mesophilic cheese starter cultures examined were found to be sensitive to the LP system, but varied in their susceptibility to inhibition. The activity of the mixed starter cultures CHN11, CHN22, CHN19, DCC240 and Flora Danica Normal was strongly inhibited by the LP system. However, the mixed starter culture LL 50C showed resistance to the LP system and could be used for cheesemaking from goat milk preserved by the LP system. The single strain culture *Lactococcus lactis* subsp. *lactis* NCDO 605 was inhibited by the LP system. However, the cultures *Lactococcus lactis* subsp. *diacetylactis* NCDO 176 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313 were insensitive to the LP system.

Gouda cheese made from LP-activated Saanen goat milk had significantly ($p < 0.05$) lower levels of coliform and coagulase positive staphylococci compared to cheese made from the untreated control milk. The LP-treatment did not affect the gross chemical composition of Gouda cheese made from Saanen goat milk. The level of proteolysis in cheeses made from LP-treated and control Saanen goat milks was comparable. However, the level of lipolysis in cheese made from LP-activated Saanen goat milk was significantly ($p < 0.05$) lower than that made from the control goat milk at the end of the ripening period. The lower lipolytic activity of cheese made from LP-activated goat milk might be of significant importance in reducing the strong flavour associated with goat milk cheeses. Significant differences ($p < 0.05$) in sensory attributes were observed between cheeses made from the LP-treated and the untreated control Saanen goat milk. Gouda cheeses made from LP-treated goat milk had a mild flavour as compared to the control. The results of this study indicate the potential of using the LP system to improve the quality and safety of goat milk and goat milk cheeses.

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CHAPTER 1

General Introduction and Literature Review

1.1. General Introduction

One of the great challenges facing the world over the next decades is the production of sufficient food to satisfy the demands of a growing human population. The world population reached 6.1 billion in mid-2000 and is currently growing at an annual rate of 1.2 percent, or 77million people per year according to the report of the United Nations Population Division (UNPD, 2001). The world population is expected to be between 7.9 and 10.9 billion by the year 2050 and most of this growth will be in developing countries (UNPD, 2001). The increase in world population projected over the next 50 years will affect food production and delivery systems. Currently about 800 million people are undernourished, most of them living in underdeveloped countries in Africa and Asia (McQueen, 2000).

Due to the rapidly increasing human population, the demand for animal protein and energy sources, particularly milk and milk products, is on the rise in the tropical developing world. The increased demand can be met by increasing ruminant livestock population as suggested by Devendra and McLeroy (1982). The contribution of small ruminants in general and goats in particular in meeting this demand will be very high. Goats are important milk producers in several parts of the tropics and contribute significantly to human nutrition in many developing countries (Devendra, 1999). Although goat milk is very nutritious and an acceptable food in several parts of the tropics, its production and handling remains a major problem limiting its consumption. The dispersed nature of production across the diversity of small farms, small volumes and seasonality of milk production, high ambient temperatures, poor handling systems, lack of cooling facilities, poorly developed road systems, lack of well organized transportation and communication systems all create a considerable challenge to goat milk production in several developing countries (Chamberlain, 1989; World Bank, 1991; Devendra, 1999; Mikkelsen, 2001). One of the main constraints is the distance from the production point to the main population centres where there is the biggest and constant demand. As a result, there is often unavoidable time lapse between milking and delivery of the milk to a processing plant or to consumers and it often exceeds five hours, very negatively

affecting the quality of milk, which is often rejected by dairy processing plants and is also not acceptable to consumers (Barabás, 1995).

Most of the population in developing countries starve not only because of lack of food but also because of lack of well organized food distribution systems from areas of surplus production to areas of low production. Equally important is lack of infrastructures and facilities to preserve the already available food as a result of which considerable amounts are lost because of spoilage (FAO, 1997). According to the World Bank, 21 million tonnes of milk or the equivalent of 5000 million US Dollar representing 60 % of the value of milk equivalent imports by developing countries is spoiled or under-valued per annum due to lack of appropriate milk collection systems in developing countries (Lambert, 2001). In West Africa alone, the World Bank estimates that 5 million litres of milk (20 % of production) per year is lost because of lack of means to preserve it while it is being transported to cooling centres or dairies (FAO/IDF, 2002).

In countries with advanced dairy industries, cooling is used to safeguard the bacterial quality of the raw milk during on-farm handling, storage and transportation. However, in most developing countries, this method would be too sophisticated and expensive, or perhaps not even possible due to the absence of a reliable electricity supply or economic constraints. Under these circumstances, it would be more advantageous to have access to an alternative, safe and suitable method of preservation to protect raw milk from bacterial deterioration during the collection and transportation of milk to the dairy plant. To this end, a method for increasing the storage stability of milk at high ambient temperatures has been developed. The method makes use of a naturally occurring antibacterial system in milk known as the lactoperoxidase (LP) system. The LP system has been recommended for preservation of raw milk in areas where it is not possible to use mechanical refrigeration for technical and/or economic reasons (IDF, 1988; FAO, 1999). The LP system consists of three components: the enzyme lactoperoxidase, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). Lactoperoxidase is normally found in a sufficient amount in milk; however, SCN^- and H_2O_2 are the limiting factors and need to be added from exogenous source to activate the LP system. The enzyme lactoperoxidase catalyses

the oxidation of SCN^- by H_2O_2 , and generates the hypothiocyanite (OSCN^-) ion, which has proven antibacterial activity (Reiter, 1985a).

Milk in general and goat milk in particular is a highly nutritious food ideally suited for the growth of both spoilage and pathogenic organisms. Outbreaks of milk-borne illness date back from the inception of the dairy industry. Various bacterial infections have been linked to consumption of raw goat milk (Vasavada, 1986). Consumption of goat milk or cheese containing *Brucella melitensis* is an important source of human brucellosis worldwide and has caused several outbreaks (Garin-Bastuji & Verger, 1994). A recent report (Morgan, Bonnin, Mallereau & Perrin, 2001) indicated that *Listeria monocytogenes* was able to survive in soft lactic cheeses made from raw goat milk. Furthermore, goat milk cheese is generally manufactured in small 'artisanal' units from raw goat milk using traditional technologies (Lodi, Brasca, Carcano & Sangalli, 1996; Klinger & Rosenthal, 1997). Cheeses manufactured under these conditions may not have the minimum hygiene and sanitary guarantees necessary to obtain constant product quality (Emaldi, 1996).

Activity of the LP system has been shown to inhibit the growth of many bacterial species in cow milk (Pruitt & Reiter, 1985; Reiter, 1985a; Naidu, 2000). However, the antibacterial activity of the LP system on bacteria depends on the species and/or strain of bacterium used, temperature of incubation, type of medium (milk) used for activation of the system, and the concentrations of the LP system components (Sarkar & Misra, 1992; Fuglsang, Johansen, Christgau & Adler-Nissen, 1995). The LP activity and SCN^- contents of goat milk are different from cow milk (Fonteh, Grandison & Lewis, 2002). Variations in these components were reported even between breeds of goats (Zapico, Gaya, De Paz, Nuñez & Medina, 1991). Thus, it can be expected that goat milk LP system may exhibit different degrees of inhibition on pathogenic bacteria.

One of the key elements and concerns regarding the use of the LP system for preservation of raw milk is the possible effect which it may have on starter cultures and thus on milk which is to be processed into products such as cheese. The activation of the LP system

was found to inhibit the activity of lactic starter cultures (de Valdez, Bibi & Bachmann, 1988) in cow milk. However, the activity of individual starter cultures varies to a considerable extent with the type of milk used (Dutta, Kuila, Ranganathan & Laxminarayana, 1971; Pettersson, 1988). Thus, it can be expected that the effect of goat milk LP system on cheese starter cultures may be different from that of cow milk.

Milk production from goats in South Africa has increased over the last two decades and projects aimed at promoting goat milk production by householders and small-scale farmers are underway (Donkin, 1998). In South Africa, goat milk is produced by several small-scale milk producers and processed into various types of cheeses (USAID & ARC, 1998a). Gouda is one of the various types of cheeses made from goat milk by these producers. The high pH of Gouda cheese and the absence of enough lactic acid to suppress spoilage bacteria emphasize the importance of using milk of good bacteriological quality for making this cheese (Chapman & Sharpe, 1990). The possibility of making acceptable varieties of cheese from cow (Lara, Mendoza, de la Cruz & Garcia, 1987), sheep (Uceda, Guillen, Gaya, Medina & Nuñez, 1994;) and buffalo (Abdou, Dawood, Abd El-Hady & El-Nagar, 1996) milks preserved by the LP system has been reported. However, to date no work has been reported on the cheesemaking properties of LP-treated goat milk. Owing to the difference in composition between goat and cow milk, the cheesemaking characteristic of goat milk is different from cow milk (Chandan, Attaie & Sahani, 1992; Park, 2001). As a result, activation of the LP system in goat milk may have different effect on the quality of goat milk cheese.

In this thesis, the inhibitory effect of the LP system on the growth and survival of selected food-borne pathogens in the milk of two goat breeds was investigated; the effect of the LP system on the activity of various mesophilic cheese starter cultures was determined in goat milk and an LP-resistant culture which could be used for cheesemaking from goat milk preserved by the LP system was identified; the suitability of goat milk preserved by the LP system for the manufacture of Gouda cheese and the effect of this treatment on the biochemical, microbiological and organoleptic properties of Gouda cheese was investigated.

1.2. Literature Review

1.2.1. Goats as important milk producers in the tropics

Since the dawn of civilization, man has used the milk of different domestic animals as food. However, cattle, buffaloes, goats, sheep and camels are the major livestock species which provide milk in the tropics. Among these species, cows are the main milk producers in the tropics (Chamberlain, 1989); however, the goat by virtue of its origin, distribution and unique anatomy and physiology, and behavioural characteristics represents an under-utilised resource that at the same time has great potential for increasing milk production in the tropics (Knights & Garcia, 1997).

The suitability of the goat for milk production in the tropics is related to many factors: their small size relative to the cow which makes them better suited to the needs and capabilities of smallholders; their fast reproductive rate and short generation interval; their disease tolerance and adaptation to both hot and dry tropical areas; their adaptability to a wide range of feeds; their efficiency of converting high fibre roughages to human food (Devendra, 1978; Devendra, 1981; Matthewman, 1985; DeVries, 1988; Knights & Garcia, 1997). Moreover, goats have also been claimed to have greater milk production efficiency in terms of live weight, in view of their lower maintenance needs in comparison to cows and buffaloes when reared under the same conditions (Devendra, 1975; Devendra, 1978; Knights & Garcia, 1997).

Goat milk is produced in many parts of the world in particular in Southeast Asia mainly in India and Bangladesh; in the Near East countries such as Iraq, Cyprus, Turkey, Syria, Iran; in African countries such as Libya, Morocco, Sudan, Niger and Somalia; in European countries such as Greece, Spain and France (Devendra, 1975, 1980, 1981). The contribution of goat milk to overall milk production in some individual countries could be considered significant, reaching levels of 44 % in Mali, 29 % in Somalia, 24 % in Iran and 16 % in Sudan (Knights & Garcia, 1997). In the Caribbean, for example, Haiti and Bahamas, the contribution of goats to milk production is approximately 50 % of the total milk produced (Matthewman, 1985). However, it should be noted that these are only estimates and since because of difficulty of measuring yield and lack of formal marketing

for goat milk, it is difficult to get the exact figures. In each of these countries, goat milk is used for diversified purposes. For instance, in the USA, which has plenty of cow milk, goat milk finds a market because of its alleged superiority in nutritional quality or reported value as a source of milk for individuals suffering from allergies to the proteins of cow milk (Jenness, 1980). In brief, goat milk is used by necessity in some countries, by choice in others and by a combination of the two in still others.

1.2.2. The dairy goat industry in South Africa

The dairy industry of South Africa is regarded as a mixture of ‘several worlds’ which is ‘developed’ on the one hand, with large milk producers using modern production methods, processing facilities and supermarket distribution, and ‘developing’ on the other hand, with many small milk producers using relatively primitive production and distribution methods (Hermann, 1996). Du Toit (1997) on his report on small-scale milk production and processing in South Africa indicated that thousands of people are entering the field of agriculture as small farmers. He further pointed out that these farmers would contribute considerably towards achieving household food security in the country. Most of these reports regarding the dairy industry of South Africa focused mainly on cow milk production. There is limited information on goats and goat milk production in South Africa. However, currently there is a growing interest in the dairy goat sector. More and more farmers and small-stock owners are considering the milking of goats as a possible farming alternative. The national programme on commercialisation of Indigenous goats launched by the Animal Nutrition and Products Institute of the Agricultural Research Council emphasizes the importance of this sector (USAID & ARC, 1998b).

1.2.2.1. Dairy goat breeds in South Africa

According to Campbell (1995), the various goat breeds in South Africa are classified as follows:

Milk goats: These are improved dairy types of goat breeds such as Saanen, British Alpine and Toggenburg which were imported from Europe for milk production. The Saanen goat breed, originating in Switzerland, is the major goat milk producer in South

Africa. The British Alpine (originating in the United Kingdom) and Toggenburg (originating in the province of Ober-Toggenburg in Switzerland) are also milked in some places in South Africa.

Angora goats: These goats were imported from Turkey for mohair production.

Boer goats: These are an indigenous breed, developed in South Africa for meat production.

Indigenous goats: These constitute the largest number and are mainly owned by the black communities in rural areas. The Indigenous goats possess important economic traits such as viability and high survival rate, good mothering ability, low maintenance requirements, disease resistance such as against heartwater, and resistance against ticks and other external parasites (Campbell, 1995).

1.2.2.2. Goat milk and milk products in South Africa

Official statistics are not available for the total goat milk production in South Africa; however, the total goat milk production for the estimated 15 000 milk does in South Africa is projected at 1 350 000 litres per annum (USAID & ARC, 1998a). Of this total amount, Fairview produces approximately 408 500 litres, Stillerust approximately 156 000 litres, Andanté approximately 99 000 litres, with the smaller operators making up the rest (USAID & ARC, 1998a).

Frozen and fresh goat milk is sold through health food stores, some supermarkets and home industry stores. Fairview is the main goat cheese producer in South Africa, with total cheese production estimated at 40 000 kg per annum. About 70 percent of the cheese produced consists of Gotino and Rabiola, while the remaining 30 percent is Chevin and Camembert. Lamontanara in Bonnievale produces mainly Capino Romano goat cheese. Andanté Farm produces several cheese types including Capino Romano, Chevin, Gouda, St. Maure and Feta, as well as drinking yoghurt (USAID & ARC, 1998a).

In many rural communities of South Africa, small-scale and subsistence farmers milk goats for household consumption (Casey & van Niekerk, 1988). Very little is known about the milk production potential of South African Indigenous goat breeds. However, the available reports to date indicate the potential of South African Indigenous goats for milk production. Mmbengwa (1999) reported that the Boer goat does produced more milk than the Indigenous goats. Boer goats kept under intensive feeding regime produced 3.7 ± 1.4 l/day at peak milk production period whereas Indigenous goats kept under the same feeding regime produced 1.9 ± 0.7 l/day at peak milk production. However, when kept under extensive feeding programme, Boer does produced a maximum of 1.1 ± 0.7 l/day whereas Indigenous does produced a maximum milk yield of 1.1 ± 7.1 l/day. On the other hand, Donkin (1997) investigated the feasibility of crossbreeding (50 % Saanen x 50 % South African Indigenous goats) of goats for milk production. He reported that mean lactation yields of Saanen goats at 1st, 2nd and 3rd parities were 579, 838 and 758 kg, respectively. Mean lactation yields for Crossbred goats were 317, 446 and 438 kg at 1st, 2nd and 3rd lactations, respectively. However, the mean milk yield of Indigenous goats at a mean lactation length of 94 days was 23 kg. Milk composition analyses for Saanen goats were 3.43 % fat, 2.88 % protein and 4.49 % lactose. Crossbred goats had a mean fat, protein and lactose contents of 5.47, 3.88 and 4.81 %, respectively. Indigenous goat milk had a mean fat, protein and lactose contents of 9.33, 5.04 and 5.12 %, respectively. Habteyohannes (2001) reported that Indigenous goat milk had higher quality than Saanen goat milk as measured by chemical and microbiological parameters. He also pointed out the possibility of manufacturing acceptable qualities of Gouda-type cheese from the milk of Indigenous goat breeds.

1.2.3. Nutritional advantages of goat milk

Goat milk has great potential role in human nutrition and has a number of relative advantages when compared with milk of other species. Goat, cow, and human milks are approximately isocaloric; each furnishes about 750 Kcal energy per litre. However, the protein content of goat milk is much higher than that of human milk in relation to total calories (Jenness, 1980; Devendra & Burns, 1983). In goat and cow milk, fat, protein and lactose account for about 50, 25, and 25 % of the energy, but in human milk they furnish

55, 7, and 38 %, respectively (Jenness, 1980). The higher protein, non-protein-nitrogen, and phosphate in caprine milk gives it greater buffering capacity compared to cow milk (Park, 1992). High buffering capacity of goat milk appears to be useful for treatment of gastric ulcers (Haenlein & Caccese, 1984; Park, 1994).

Goat milk proteins are digested more readily and their amino acids absorbed more efficiently than those of cow milk (Jenness, 1980; Haenlein, 1992). Goat milk appears to form a softer, more friable curd when acidified. This may be due to the fact that little if any α_1 -casein is found in goat milk. It seems logical that smaller, more friable curds would be attacked more rapidly by stomach proteases. The soft curd of goat milk may be an advantage for adult humans suffering from gastrointestinal disturbances and ulcers (Haenlein, 1996). Goat milk has been recommended as a substitute for patients allergic to cow milk (Haenlein, 1978; Hagglund, 1992; Park, 1994). Between 40-100 % of patients allergic to cow milk proteins tolerate goat milk (Park, 1992, 1994; Klinger & Rosenthal, 1997).

Both goat and cow milk fats contain adequate concentration of essential fatty acids (linoleic, linolenic and arachidonic acids) for human infants, although concentrations are slightly higher in some specimens of goat milk fat (Jenness, 1980; Devendra & Burns, 1983). The average fat globule size in goat milk ($3.5\mu\text{M}$) is significantly smaller than that of cow milk ($4.5\mu\text{M}$) (Knights & Garcia, 1997). Goat milk has higher proportions of small fat globules as compared to cow milk (Juárez & Ramos, 1986); this facilitates easy digestion of goat milk fat as compared to that of cow milk fat (Jenness, 1980; Devendra & Burns, 1983; Chandan *et al.*, 1992). It is supposed that lipases can attack fat in goat milk more rapidly because of greater surface exposed. Nearly 20 % of the fatty acids of goat milk fat are in the category of short and medium chain length (4 to 12 carbons) such as caproic acid (C_6), caprylic acid (C_8), and capric acid (C_{10}) compared to cow milk which contains only 10 to 20 % of fatty acids of this category (Devendra, 1975; 1980). This difference may contribute to more rapid digestion of goat milk fat since lipases attack ester linkages of such fatty acids more readily than they do those of longer chains (Jenness, 1980). In addition, these fatty acids are commonly used in the treatment of

patients with various malabsorption disorders because of their unique metabolic ability to provide energy, lower serum cholesterol, inhibit and limit cholesterol deposition in tissues and dissolve cholesterol gallstones (Haenlein, 1992; Park, 1994).

Goat milk is adequate for the human infant in vitamins A and niacin and supplies generous excesses of thiamin, riboflavin, and panthothenate (Jenness, 1980; Haenlein, 1988). It is, however, deficient in vitamins C, D, B₁₂, pyridoxine, and folic acid (Chandan *et al.*, 1992; Park, 1994). Thus, when used for infant feeding, goat milk must be corrected by appropriate fortification. One of the most important contributions of goat milk to human nutrition is the calcium and phosphate that it supplies. Goat milk contains about 1.2 g calcium and 1 g phosphate per litre; these concentrations are similar to those in cow milk (Jenness, 1980). Human milk contains much less of these minerals with only one-fourth as much calcium and one-sixth as much phosphate. Thus goat milk provides a great excess of Ca and P in relation to energy to human infant. Both calcium and phosphorus of goat milk are absorbed by the human infant (Jenness, 1980).

1.2.4. Microbiological and public health aspects of goat milk

Fresh milk obtained from a healthy udder under sanitary (good hygienic) conditions contains relatively few microorganisms (Burgess, Heggum, Walker & Schothorst, 1994); but subsequently becomes contaminated by man, his practices and the environment. Contrary to the naturally enclosed and intact delivery system nature designed for milk, man subjects the milk to air, agitation, and bacterial contamination. It is this exposure to man's environment which affects the nutritional integrity of milk.

The number and types of microorganisms present in milk and dairy products at any particular period depend on the microbial quality of the raw material, the conditions under which the products were produced and also on the temperatures and duration of storage (Burgess *et al.*, 1994). The temperature of milk has a significant effect on the rate of bacterial development and, consequently, on the spoilage of milk. It is generally concluded that if milk is not cooled and does not reach the processor within five hours after milking, it will not be suitable for processing (Barabás, 1995). In the tropical

countries of Africa with high ambient temperatures, the general lack of refrigeration facilities at the farm and household levels imply that raw milk will acidify very fast unless otherwise protected. Goat milk is mostly produced on small farms from small herds which frequently are a source of milk to the immediate neighbourhood (Chamberlain, 1989). Much of goat milk in developing countries is sold raw and may be collected, transported, and held two to five days or longer before further processing (Chamberlain, 1989). Because of limited supply, the common practice is to pool milk of different age in order to obtain sufficient amounts to make further processing feasible. Therefore the bacteriological quality of raw goat milk should be of major concern to the dairy producer, the dairy industry and the general public. Highly contaminated milk is likely to receive a low grade at the dairy plant and therefore, a reduced revenue to the producer. The dairy industry cannot produce high quality products from poor quality raw milk, the consequence of which may be poor consumer satisfaction or even public-health risks of milk-borne diseases (Vasavada & Cousin, 1993).

Goat milk has a highly variable microbial count. Some authors claim that goat milk contains significantly lower bacterial counts than cow or buffalo milk and that a variety of microorganisms can exist in goat milk without being pathogenic (Haenlein, 1992). However, a survey by Jensen and Hughes (1980) in New South Wales showed that goat milk in Australia was of a poorer microbiological quality. They reported that a larger proportion of samples (24.4 %) had standard plate counts of $> 10^6$ cfu/ml and 23.7 % contained coliforms > 10 cfu/ml. Similarly, Cox and MacRae (1989) reported that the keeping quality of both raw and pasteurised goat milk produced in Queensland, Australia was generally poor. They reported that raw factory goat milk had a mean total aerobic count of $6.15 \log_{10}$ cfu/ml and pasteurised goat milk had a mean total aerobic count of $3.71 \log_{10}$ cfu/ml. Whereas the mean coliform count in raw factory goat milk was $2.34 \log_{10}$ cfu/ml; however, coliforms were not detected in pasteurised goat milk. A report from Portugal (Barbosa & Miranda, 1986) indicated that raw goat milk had aerobic plate count of 2×10^6 cfu/ml at 30 °C, lactic acid bacteria count of 5×10^5 cfu/ml, and yeast and mould counts of 2×10^3 cfu/ml. On the other hand, Tirard-Collet, Zee, Carmichael and Simard (1991) reported that 68 - 80 % of goat milk samples from Quebec, Canada,

had mesophilic counts of $< 10^5$ cfu/ml, coliform counts of $< 10^3$, and yeast and mould counts varying from 10 to 100 cfu/ml.

Like cow milk, goat milk is a good medium for the growth of pathogens and toxin production. Public health hazards related to consumption of unpasteurised goat milk have been reported (Darnton-Hill, Coveney & Davey, 1987; Little & Louvois, 1999). Park and Humphrey (1986) found mean count of 3.3×10^3 cfu/ml *Staphylococcus* spp. in raw goat milk. De Buyser, Dilasser, Hummel and Bergdoll (1987) isolated staphylococci from 81 of 238 samples from 120 goats representing six different French goat breeds. Based on their data they concluded the possibility of enterotoxin production in goat milk. Parente and Mazzatura (1991) noted that *Staphylococcus aureus* could survive sub pasteurisation treatments and proliferate in goat milk and cheese. Unpasteurised or raw goat milk poses a public health hazard due to staphylococci poisoning (Chubb, Orchard & McInness, 1985). Chubb *et al.* (1985) detected coagulase positive staphylococci in 40 % of samples of milk taken aseptically from individual goats in New South Wales, Australia which showed no signs of clinical mastitis. High prevalence (71 %) of *Staphylococcus* spp. in goat milk was reported by Contreras, Corrales and Sierra (1993). Kalogridou-Vassiliadou (1991) reported that 59.1 % of raw goat milk samples in Greece were positive to staphylococci. Harvey and Gilmour (1988) also reported that staphylococci were the dominant species isolated from goat milk in Northern Ireland. Thus, the prevalence of staphylococci in goat milk suggests that consumption of raw goat milk may be a serious public health risk due to enterotoxin production by staphylococci.

Escherichia coli is the predominant bacterium of the facultatively anaerobic flora of intestines of many animal species. While most *E. coli* strains are non-pathogenic, some of them may cause disease. Pathogenic strains of *E. coli* produce several toxins categorized either as enterotoxins or cytotoxins (Henton & Hunter, 1994). Enteropathogenic or enterotoxigenic strains of *E. coli* have been responsible for outbreaks involving milk and cheese (Burgess *et al.*, 1994). Bielaszewska, Janda, Bláhová, Minaříková, Jíková, Karamali, Laubová, Šikulová, Preston, Khakhria, Karch, Klazarová and Nyč (1997) reported human infection by verocytotoxin producing *E. coli* O157: H7 associated with

the consumption of raw goat milk. Jensen and Hughes (1980) found high levels of *Escherichia coli* in many goat milk samples from New South Wales, Australia.

Listeria monocytogenes is a pathogen of major concern to the dairy industry as food-borne listeriosis has been related to consumption of contaminated milk and milk products (Griffiths, 1989). Dairy related incidents of listeriosis have implicated raw milk, poorly pasteurised milk and cheese (Burgess *et al.*, 1994; Boggs, Whitwam, Hale, Briscoe, Khan, MacCormack, Maillard, Grayson, Sigmon, Readon & Saah, 2001). A large proportion of healthy goats are subclinical carriers of *L. monocytogenes*, excreting organisms in their faeces and milk when stressed (Schneider, 1994). *Listeria monocytogenes* has the ability to survive in semi-soft cheese made of unpasteurised goat milk during a ripening period of 2-3 months (Tham, 1988). Similarly, a recent report indicated the ability of *L. monocytogenes* to survive in soft lactic cheeses made from raw goat milk (Morgan *et al.*, 2001).

Human brucellosis still constitutes an important health problem in most developing countries in which high rates of *Brucella melitensis* infections are found among goats (Bryan, 1983). *Brucella melitensis* is highly pathogenic to humans (Garin-Bastuji & Verger, 1994; Ryser, 1998) causing one of the most serious zoonoses in the world. Goats are the primary reservoir of *Br. melitensis* (Ryser, 1998). Wallach, Samartino, Efron and Baldi (1998) reported the prevalence of high rates of *Br. melitensis* infection among goats. Consumption of raw goat milk and goat milk cheese has been reported to be the probable means of brucellosis infection (Ryser, 1998). Thapar and Young (1986) reported an outbreak of human brucellosis in the United States caused by *Br. melitensis* due to consumption of unpasteurised goat milk cheese. Similarly, Wallach, Miguel, Baldi, Guarnera, Goldbaum and Fossati (1994) reported an outbreak of a *Br. melitensis* infection in an Argentine family due to consumption of unpasteurised goat milk cheese.

Mycobacterium paratuberculosis causes Johne's disease in cattle, sheep, goats and other ruminants (Chiodini, van Kruiningen & Merkal, 1984). Due to the similarity between Johne's disease and Crohn's disease of humans, implication of this organism with the

latter is also under discussion (Hammer, 1999). Milk has been suggested as a possible vehicle for transmission of this organism to humans. Detectable quantities of *M. paratuberculosis* have previously been reported in the milk of both clinically infected (Taylor, Wilks & McQueen, 1981) and subclinically infected (Streeter, Hoffsis, Bech-Nielsen, Schulaw & Rings, 1995) cattle with Johne's disease. Milk can be contaminated with *M. paratuberculosis* by direct shedding or faecal contamination (Hammer, 1999). *Mycobacterium paratuberculosis* was reported to be resistant to pasteurisation of milk (Hammer, 1999). In addition, a high resistance of *M. paratuberculosis* to environmental factors, particularly to acids, could be of influence on products made from raw and pasteurised milk, for example in cheese (Hammer, 1999). A survey conducted in England, Wales and Northern Ireland to determine the incidence of *M. paratuberculosis* in goat milk indicated that < 1 % of raw goat milk sample tested positive for the presence of *M. paratuberculosis* (Grant, O'Riordan, Ball & Rowe, 2001). They concluded that raw goat milk from these regions of the UK may not represent significant vehicles of transmission of *M. paratuberculosis* to humans. Since the prevalence of *M. paratuberculosis* may vary in different geographical regions, the presence of *M. paratuberculosis* in goat milk reported by Grant *et al.* (2001) suggests the importance of further studies on *M. paratuberculosis* in goat milk.

One of the concerns regarding goat milk by the general public is the perception that goat milk or goat milk products have a "goaty" flavour because of a long history of widespread negative popular misconception (Haenlein, 1992) against goats. Well-produced and well-handled goat milk is indistinguishable in taste and odour from good quality cow milk (Haenlein, 1992). Pinkerton (1991) reported that the major factors affecting acceptance of goat milk and its products by the general public are: unfamiliarity with goat milk and its products; poor public image of goat milk and goats; and lack of public knowledge of, and appreciation for, the unique qualities of goat milk. Milk in general, and goat milk in particular has its unique characteristic flavour but not unacceptable smell or odour. Proper handling of milking goats and bucks by separation, good management and hygiene can eliminate the poor attitude by consumers towards goat milk (Haenlein, 1992).

1.2.5. Goat cheese production

The production of cheese from goat milk has a very long history. In Homer's *Odyssey*, there is a vivid description of the manufacture of cheese from goat milk. Ipcrates (460-356 BC) also mentioned the production of cheese from goat milk. For countries with difficult natural conditions, seasonal and irregular rainfall and eroded soils, goats are the dominant animals for the production of milk, meat, fibre, hides and manure. The cheeses and yoghurts produced are the most important sources of protein for the local and rural population of such counties. The cheeses are, in general, made in small-scale units, by traditional technology, the result of a pastoral art (Kalantzopoulos, 1993). The products obtained have a special taste and flavour, very different from that of cheese made from cow milk. The market for these specialized cheeses is expanding, and the image of "healthy food" attached to goat milk and milk products has increased their economic importance beyond traditional and geographic borders (Chandan *et al.*, 1992).

Most goat milk is transformed into cheese and only a limited amount is used for direct consumption and for yoghurt production, especially for consumption on the producing farms (Kalantzopoulos, 1993). The processing of goat milk into cheese and cheesemaking at an industrial level is growing especially in the Mediterranean region (Kalantzopoulos, 1993).

The milk, cheeses and meat of goats constitute a fundamental base for the nutrition of a number of peoples in the world especially in areas where the husbandry of cows is very difficult. Three categories of cheese are produced from goat milk (Kalantzopoulos, 1993):

- i) the first includes traditional cheeses of many varieties made on farms, which are more or less well known.
- ii) the second includes traditional cheese made partly on a farm scale under improved conditions and partly on a modern industrial scale. This is the case in France, the only country in the world with a production of more than 90 varieties of goat milk cheese.

- iii) the third group of cheeses are produced from mixed sheep and goat milk. Owing to the fact that in most countries goats and sheep are farmed in mixed flocks and because of the seasonality and small quantity of milk from these two species, producers mix the two types of milk in order to increase the quantity for cheesemaking.

While there are wide variations in the flavour, body, texture and specific nutritional qualities of goat cheeses, they have some characteristics in common. On the basis of their manufacturing protocol, three groups of goat cheese can be distinguished (Kalantzopoulos, 1993). The first group contains cheese made from lactic acid curd coagulated by the indigenous lactic acid microflora. When fresh, these cheeses have moisture content of 80 % but they are susceptible to evaporation. The curd of this group of cheeses is generally fragile and does not permit the production of large cheeses. The cheese varieties Alicante, Cadiz and Soria of Spain belong to this group. Other members are those ripened after immersion in olive oil, e.g. Sourke of Syria, Malaga of Spain and Ladotyri of Greece.

The second group contains cheeses made from rennet-coagulated curds which drain freshly (Kalantzopoulos, 1993). These cheeses contain 50 % moisture, which is important for the development of the lactic acid bacteria, which are required to complete the ripening process. The cheeses of this group are divided into three types: cheese in brine, soft or semi-soft cheeses, and surface ripened cheese. Examples are Feta (Greece), Saint Maure and Crottin (France), Altenburg (Germany), Blanco (Libya) and Akari (Syria).

The third group contains cheese prepared from rennet-coagulated curds which are cooked to accelerate draining, e.g. Aseredo (Mexico), Salamora (Turkey) and Rumalia (Greece and Bulgaria) (Kalantzopoulos, 1993). Similar cheeses include Gjetot (Norway) with a semi-strong flavour, Quesco Echilago (Mexico), Lighwan (Iran) and Banon (France), Caprino a pasta cruda and Caprino semicotta (Italy).

The technology and composition of most goat cheeses, especially the traditional ones, are not well documented (Godina, 1986). The production of traditional goat cheeses is usually carried out on individual farms by shepherds themselves and their families. Most of these cheeses are produced traditionally and recipes and manufacturing methods are passed from generation to generation without a standard technology, standard regulations or pasteurisation (Klinger & Rosenthal, 1997). Cheeses made under these conditions do not have the minimum hygiene and sanitary guarantees necessary to obtain constant product quality. The real value of goat milk cheese is through industrialization under technical and scientific conditions capable of providing products with the indispensable guarantees of quality and constancy (Godina, 1986).

1.2.6. Constraints to goat milk production

In many tropical countries, goats are usually kept on marginal areas where the land is not suitable for cultivation and where rugged and mountainous terrain predominate (Fatouros, 1986). As a result collection of goat milk from these regions is difficult. Poor road systems and lack of well-organized transportation make collection and distribution of goat milk difficult in many parts of the tropics (Fatouros, 1986; Chamberlain, 1989). Consequently, access to scattered goat milk producers in tropical areas is difficult and often impossible in wet weather. Animals are milked by hand; milk is not cooled on farms and remains at high ambient temperatures above 30 °C resulting in a substantial loss of this important nutrient. Small quantities of milk produced per animal per farmer in isolated areas and irregular milk supplies further complicate goat milk collection in the tropics (Fatouros, 1986; Chamberlain, 1989; Klinger & Rosenthal, 1997). Milk quality on farms may be poor due to unhygienic milking conditions, dirty containers and poor-quality cleaning water, lack of electricity and refrigeration. As a result milk and milk products are too expensive for those that require them most (Chamberlain, 1989).

In tropical countries there appear to be four main choices for keeping spoilage to a minimum: moving milk quickly to the customer within two to three hours of milking; making products which will keep without refrigeration; adding preservatives to the fresh

milk; or cooling the milk as soon as possible on the farm or at a collection centre (Chamberlain, 1989; Empson & Bachmann, 1990).

1.2.7. Milk collection and preservation

Milk and milk products have made very significant contributions to human nutrition ever since the earliest civilizations, although there are wide variations in the traditional role of milk in the diet of people in different sub-continent. Because of its constituents, milk could greatly improve people's health in developing countries where a large number of the population, especially pregnant and nursing women and children, suffer from severe malnutrition and where people survive on high starch, low protein foods (Chamberlain, 1989).

Dairying provides one of the most cost-effective methods of converting crude animal feed resources into high-quality protein-rich food for human consumption. However, since milk is a very perishable foodstuff special measures and considerations are necessary to ensure that it reaches the market in an acceptable condition. The collection of milk from the farmers and transportation to the dairy is the most critical link in the total handling chain of milk. This problem is recognized worldwide (Barron del Castillo, 1990; Claesson, 1992).

The most commonly used method to stop or retard the deterioration of milk on its way from the farmer to the dairy is cooling (Claesson, 1992; Lambert, 2001). However, in many parts of the world, this is not possible for various reasons, such as lack of available capital, lack of electricity, less developed road systems, high operational costs, frequent break downs of equipment, lack of spare parts and difficulties in repair of equipment in rural areas. Prevailing high ambient temperatures often further compound the problem of milk collection in these areas. This causes a considerable loss of fresh milk, and in many regions only a minor part of the production reaches the dairy industry in an acceptable condition for use as human food. In many regions most of the evening milk is spoiled after storage over-night (Claesson, 1992; Jandal, 1997).

To solve this problem, a new method to increase the storage stability of milk at high ambient temperatures has been developed (IDF, 1988; Claesson, 1992; FAO, 1999). The method makes use of a naturally occurring antibacterial system in milk known as the lactoperoxidase system, which is activated by increasing the concentrations of two components or activators (thiocyanate and hydrogen peroxide) reacting with each other. This reaction is catalysed by the enzyme lactoperoxidase which is naturally present in milk and leads to the formation of antibacterial compounds.

1.2.8. The lactoperoxidase system

The lactoperoxidase (LP) system consists of three components: LP, thiocyanate, and hydrogen peroxide and it is active only in the presence of all these three components. Lactoperoxidase catalyses the oxidation of thiocyanate by H_2O_2 and generates an intermediate product with antibacterial properties. These products have a broad spectrum of antimicrobial effects against bacteria, fungi and viruses (de Wit & van Hooydonk, 1996; Kussendrager & van Hooijdonk, 2000).

1.2.8.1. Components of the lactoperoxidase system

1.2.8.1.1. Lactoperoxidase

Lactoperoxidase (EC 1.11.1.7.) is a member of the peroxidase family, a group of natural enzymes, widely distributed in nature and found in plants and animals, including man (Kussendrager & van Hooijdonk, 2000). Lactoperoxidase is an oxidoreductase secreted into milk, and plays an important role in protecting the lactating mammary gland and the intestinal tract of the newborn infants against pathogenic microorganisms (Naidu, 2000). The enzyme LP is a normal component of bovine and human milk and is present in all mammalian milks, which have been tested so far (Pruitt & Kamau, 1991).

Lactoperoxidase is found in the mammary, salivary, and lachrymal glands of mammals and in their respective secretions, i.e., milk, saliva, and tears (Wolfson & Sumner, 1993). The involvement of LP in the inhibition of microbial growth was first suggested by Hanssen (1924). The peroxidases in mammary, salivary and lachrymal glands are

chemically and immunologically similar (Linden & Lorient, 1999). The peroxidase isolated from milk is given the name lactoperoxidase (Reiter & Härnolv, 1984).

The biological significance of LP is its involvement in the natural host defence system against invading microorganisms (Reiter, 1985a). In addition to its antimicrobial action, degradation of various carcinogens and protection of animal cells against peroxidative effects have been reported (Kussendrager & van Hooijdonk, 2000). Perraudin and Reiter (1998) reported that the LP system acts as an antioxidant thereby protecting mammalian cells against the highly reactive and damaging oxygen-derived species. The reactions of LP in the so-called LP system are harmless to mammalian cells (Björck, 1990). Reiter and Härnolv (1984) indicated that mammalian cells are not affected by oxidation products of SCN⁻ and the LP system is not only atoxic to human cells but may protect these cells against toxic effects of H₂O₂.

1.2.8.1.1.1. Physico-chemical properties of lactoperoxidase

Bovine LP consists of a single polypeptide chain containing 612 amino acid residues (Cals, Maillart, Brignon, Anglade & Dumas, 1991). Its molecular weight is approximately 78 Kda (Björck, 1990; Björck, 1992; Ekstrand, 1994). Lactoperoxidase is a basic protein with a high isoelectric point of 9.6 (Ekstrand, 1989). Lactoperoxidase is a glycoprotein (Björck, 1990; Björck, 1992) and at least 10 fractions of LP are known (Kussendrager & van Hooijdonk, 2000). There is no significant difference in enzymatic activity between the various LP fractions (Paul & Ohlsson, 1985). Lactoperoxidase contains one haem group (protohaem 9) and about 10 % carbohydrate (Paul & Ohlsson, 1985; Board, 1995). The haem group in the catalytic centre of the LP molecule is a protoporphyrin IX, covalently bound to the polypeptide chain through an ester bond (Rae & Goff, 1989; Colas, Kuo & Ortiz de Montellano, 2002). The iron content of LP is 0.07 %, corresponding to one iron per LP molecule, being part of the haem group (Paul & Ohlsson, 1985; Fox & McSweeney, 1998). Lactoperoxidase has an absorbance maximum at 412 nm (Fox & McSweeney, 1998) and its purity ratio is measured at A₄₁₂/A₂₈₀ and it is approximately 0.95 (Kussendrager & van Hooijdonk, 2000).

Lactoperoxidase is one of the most heat stable enzymes in milk (Fox & McSweeney, 1998). Its destruction has been used as an index of pasteurisation efficiency of milk. Lactoperoxidase is only partially inactivated by short time pasteurisation at 74 °C, leaving sufficient activity to catalyse the reactions between thiocyanate and hydrogen peroxide (Wolfson & Sumner, 1993). Korhonen (1980) reported that LP retains its activity in normal pasteurisation of cow milk (63 °C for 30 minutes or 72 °C for 15 seconds) but is destroyed at 80 °C in 2.5 seconds. De Wit and van Hooijdonk (1996) reported that complete inactivation of LP in cow milk requires 15 seconds at 78 °C. A recent report by Marks, Grandison and Lewis (2001) confirms the fact that normal pasteurisation of milk does not inactivate LP in milk. They reported that after pasteurisation of cow milk at 72 °C for 15 seconds, an active LP system was found to greatly increase the keeping quality of the milk inoculated with *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus thermophilus*. However, activation of the LP system in cow milk heated at 80 °C for 15 seconds had little or no effect on these bacteria. Lactoperoxidase is less heat stable under acidic conditions (pH 5.3), possibly due to release of Ca from the molecule (Kussendrager & van Hooijdonk, 2000). The calcium ion concentration appeared to have a large influence on the heat stability of LP.

The optimum pH of the LP catalysed reaction studied using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate lies between pH 5 and 6, depending on the concentration of ABTS and H₂O₂ (Kussendrager & van Hooijdonk, 2000). Lactoperoxidase is relatively stable against a number of proteolytic enzymes; however, it appears to be very sensitive to light in the presence of riboflavin (de Wit & van Hooijdonk, 1996; Kussendrager & van Hooijdonk, 2000). The LP molecule has a high tendency to adhere to surfaces, which can cause a marked decrease in activity of dilute LP solutions in glass vessels (Paul & Ohlsson, 1985; Björck & Mullan, 1993). Adherence to surfaces may lead to aggregation and turbidity, which seems to depend on the hydrophobicity of the surface.

1.2.8.1.1.2. Concentration of lactoperoxidase in milk

Because of the various chromogens used for its assay and the variability in the assay conditions, data for LP activity in the literature vary widely. A recommended method for measuring the LP activity in milk is by using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as a chromophore and measuring the absorbance at 412 nm (Shindler & Bardsley, 1975; Pruitt & Kamau, 1994). This is a very sensitive and nowadays the most commonly used chromogen for measuring LP activity.

In bovine milk, LP is the second most abundant enzyme after xanthine oxidase (Pruitt & Kamau, 1991; de Wit & van Hooydonk, 1996). Its concentration in bovine milk is around 30 mg/l constituting about 1 % of the whey protein (Reiter, 1985a). The levels of LP and thiocyanate in different types of milk are given in Table 1.1. The LP concentration is low in bovine colostrum, unlike other antibacterial proteins; however, it increases rapidly to reach a maximum after 3-5 days postpartum (Korhonen, 1980; Reiter, 1985a). Variations in enzyme level were reported to depend on the sexual cycle of the cow, season, feeding regime and breed (Reiter, 1985a; Kussendrager & van Hooijdonk, 2000). Cow milk contains from 1.2 to 19.4 units/ml LP and is about 20 times richer in peroxidase activity than human milk (Gothefors & Marklund, 1975). A recent report indicated that the mean LP activity in cow milk ranged from 1.5-2.7 U/ml with an overall mean of 2.3 U/ml (Fonteh, Grandison & Lewis, 2002). However, Reiter (1985a) reported lower (1.42 U/ml) LP activity in bovine milk. Human milk LP activity is low and ranges from 0.06 to 0.97 units/ml (Wolfson & Sumner, 1993; Reiter, 1985a). The highest level of LP activity (22 units/ml) has been reported for guinea pig milk (Stephens, Harkness & Cockle, 1979). The LP activity of goat milk reported in the literature varies greatly. Lactoperoxidase activity in goat milk ranging from 0.05-3.55 U/ml (Zapico, Gaya, Nuñez & Medina, 1990), 0.48-9.28 U/ml (Saad de Schoos, Oliver & Fernandez, 1999), and 0.04-0.16 U/ml (Fonteh *et al.*, 2002) have been reported. Mean LP activity of 0.77 U/ml (Medina, Gaya & Nuñez, 1989) and 3.46 U/ml (Althaus, Molina & Rodríguez, 2001) have been reported for ewe milk. On the other hand, LP level of 0.9 U/ml (Härnolv & Kandasamy, 1982) was reported for buffalo milk.

Table 1.1. Concentrations of lactoperoxidase and thiocyanate in different types of milk

Type of milk	Lactoperoxidase		Thiocyanate	
	Units/ml	References	ppm	References
Cow	1.4	Stephens <i>et al.</i> , 1979	3.2-4.6	Björck, Claesson and Schuthes, 1979
Ewe	0.14-2.38	Medina <i>et al.</i> , 1989	10.3-20.6	Medina <i>et al.</i> , 1989
Goat	1.55	Zapico <i>et al.</i> , 1990	4.03	Zapico <i>et al.</i> , 1990
	4.45	Saad de Schoos <i>et al.</i> , 1999	10.29	Saad de Schoos <i>et al.</i> , 1999
Buffalo	0.9	Härnulv and Kandasamy, 1982	5.6	Härnulv and Kandasamy, 1982
Guinea pig	22	Stephens <i>et al.</i> , 1979	NA	
Human	0.06-0.97	Reiter, 1985a	2.6	Metwally and Nasr, 1992
Limit for bactericidal activity	0.02	Reiter and Härnulv, 1984	15	Björck <i>et al.</i> , 1979

Where: NA is information not available.

1.2.8.1.2. The thiocyanate ion

Thiocyanate (SCN^-) is widely distributed in animal tissues and secretions. It is present in the mammary, salivary and thyroid glands and their secretions, in organs such as the stomach and kidney and in fluids such as synovial, cerebral, cervical, and spinal fluids, lymph and plasma (Reiter & Härnolv, 1984). Its concentration partly depends on the feeding regime of the animal (Reiter, 1985a). In bovine milk, the SCN^- concentration reflects blood serum levels and varies with breed, species, udder health and type of feed (Kussendrager & van Hooijdonk, 2000). Levels between 1 and 15 ppm have been reported (Reiter and Härnolv, 1984). Davidson (1997) reported that fresh cow milk contains 1 to 10 mg of thiocyanate per litre, which is not always sufficient to activate the LP system. The thiocyanate concentrations in human saliva and in human gastric juices have been reported to vary between 50 to 300 ppm and 40 to 50 ppm, respectively (Korhonen, 1980; Björck *et al.*, 1979). These values are much higher than the concentration (15 ppm) of SCN^- required for the activation of the LP system.

There are two major dietary sources of SCN^- , glucosinolates, and cyanogenic glucosides (Wolfson & Sumner, 1993). Vegetables such as cabbage, kale, brussel sprouts, cauliflower, turnips and rutabaga, are particularly rich in glucosinolates, which upon hydrolysis yield thiocyanate in addition to other reaction products (Reiter & Härnolv, 1984). Cyanogenic glucosides are also found in cassava, potatoes, maize, millet, sugar cane, peas, and beans. When hydrolysed, glucosides release cyanide, which in reaction with thiosulphate (metabolic product of sulphur-containing amino acids) is detoxified by conversion into thiocyanate (Reiter & Härnolv, 1984). The latter reaction is catalysed by the enzyme rhodanase (Reiter & Härnolv, 1984).

1.2.8.1.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is the third component of the LP system. Hydrogen peroxide is not normally detected in raw milk (Pruitt & Kamau, 1991; FAO, 1999). It may be generated endogenously, for example, by polymorphonuclear leucocytes in the process of phagocytosis (de Wit & van Hooydonk, 1996). Many lactobacilli, lactococci, and streptococci produce sufficient H_2O_2 under aerobic conditions to activate the LP system

(Wolfson & Sumner, 1993; Kussendrager and van Hooijdonk, 2000). Hydrogen peroxide may be added or may be generated by the addition of H₂O₂ generating systems such as sodium percarbonate, glucose oxidase, etc. (Kussendrager & van Hooijdonk, 2000).

Hydrogen peroxide is the only approved additive for the preservation of milk in the absence of refrigeration. It may be added at a concentration of 100-800 ppm (Lück, 1962). Hydrogen peroxide is highly toxic for mammalian cells. However, at low (100 µM or less) concentrations and in the presence of LP and SCN⁻ mammalian cells are protected from this toxicity (Pruitt & Kamau, 1991).

1.2.8.2. Reaction mechanisms of the lactoperoxidase system

Lactoperoxidase together with myeloperoxidase (MP), eosinophil peroxidase (EP) and thyroid peroxidase (TP) constitutes the mammalian peroxidase super family II which is distinguished from the peroxidase super family I (enzymes from plant, fungi and bacteria) in that the prosthetic haem group is covalently attached to the protein matrix (Furtmüller, Jantschko, Regelsberger, Jakopitsch, Arnhold & Obinger, 2002). Most peroxidases, including LP contain ferri-protoporphyrin IX as a prosthetic group (Rae & Goff, 1996; Naidu, 2000). A characteristic feature of haemoprotein peroxidase is their ability to exist in various oxidation states. There are five known enzyme intermediates. The major intermediates for LP are ferric peroxidase (the native enzyme), Compound I, Compound II, Compound III, and ferrous peroxidase (Pruitt & Kamau, 1991).

The peroxidative reactions are complex and follow different pathways depending upon the concentration of H₂O₂ and whether or not exogenous electron donors are present (de Wit & van Hooydonk, 1996). The first step in the enzymatic mechanism is the initiation reaction of the resting LP (Fe³⁺) to its ground state, using H₂O₂:



followed by the propagation reactions as illustrated in Figure 1.1. The superoxide radical (HO₂) plays an important role in termination of the catalytic reactions to the resting LP (de Wit & van Hooydonk, 1996).

The propagation reaction includes the conversion of LP from the ground state into the so-called Compound I state by reaction with H₂O₂. At low SCN⁻ (< 3 μM) and halide concentrations, Compound I reacts with H₂O₂ and with any one-electron donor that may be present (such as proteins, peptides, etc.) to form Compound II, which is continuously reduced to the ground state at a low rate. At an excess of H₂O₂ (> 0.5 mM), Compound II may react with H₂O₂ to form Compound III, leading to a ferrylperoxidase adduct. Compound III is involved in metabolic reactions, leading to irreversible inactivation of LP. The oxidant in peroxidase-catalysed halogenation is not H₂O₂ itself but rather the reaction product of peroxidase with H₂O₂, known as Compound I (de Wit & van Hooydonk, 1996), that is, the thiocyanate ion (SCN⁻) is oxidized by Compound I by a direct two-electron transfer of oxidizing equivalents (Pruitt & Kamau, 1991). The net reaction is:



Where X represents the halide or the thiocyanate ion and XO is the oxidized product. The products of peroxidation of two-electron donors kill or inhibit the growth and metabolism of many species of microorganisms (Pruitt & Reiter, 1985). Myeloperoxidase catalyses the oxidation of chloride (Cl⁻) (at neutral pH), iodide (I⁻), bromide (Br⁻) and SCN⁻ (Furtmüller *et al.*, 2002). Similarly, EP also catalyses all the above-mentioned substrates except that, it catalyses the oxidation of Cl⁻ only at acidic pH (Furtmüller *et al.*, 2002). Lactoperoxidase on the other hand, catalyses I⁻ and SCN⁻ and to a limited extent Br⁻, SCN⁻ being the physiological substrate. However, LP cannot catalyse the oxidation of Cl⁻; i.e., Cl⁻ cannot function as substrate for LP. The relative rates of Compound I reduction of LP is SCN⁻ > I⁻ > Br⁻ (Furtmüller *et al.*, 2002).

In general, peroxidation of H₂O₂ by LP can occur through three different cycles, resulting in divergent anti-microbial activities (de Wit & van Hooydonk, 1996) as follows:

- 1) In the presence of sufficient oxidizing halide or SCN⁻ as 2-electron donor for Compound I, giving optimal activation of LP.
- 2) In the presence of insufficient halide or SCN⁻ of appropriate redox potential, resulting in dominating 1-electron donors and accumulation of Compound II and reversible inactivation of LP.

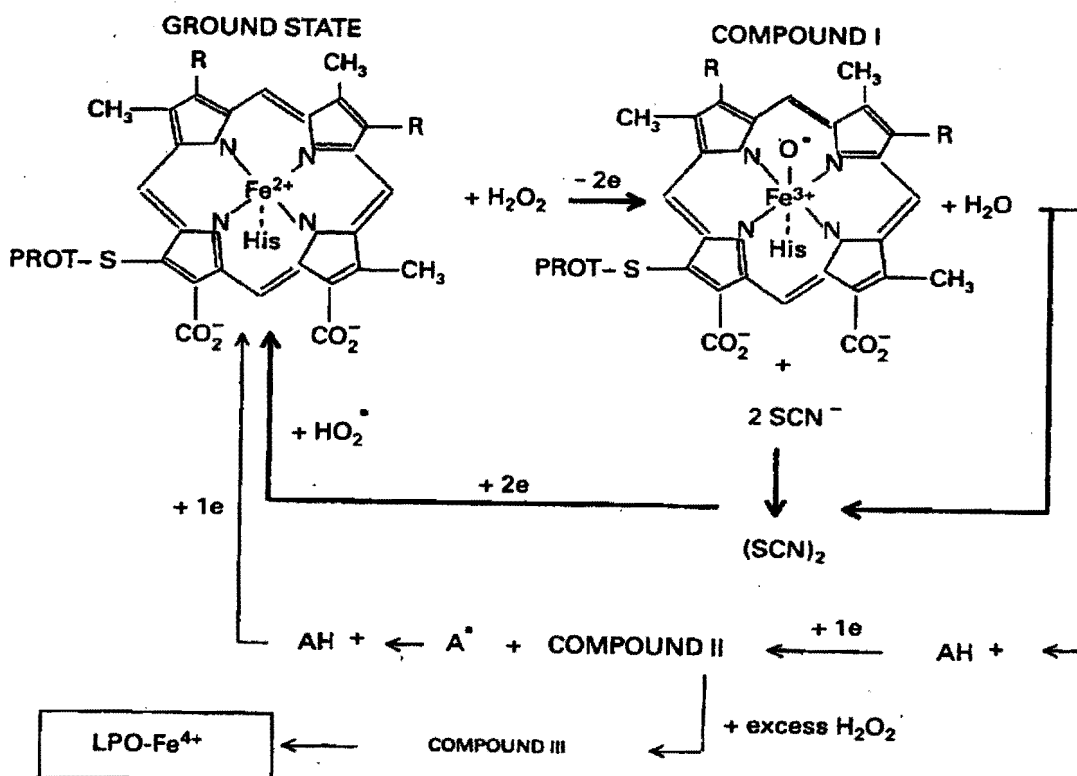


Figure 1.1. Pathways in the lactoperoxidase-catalysed reaction mechanism. The normal peroxidatic cycle includes compound I. Insufficient 2-electron donors lead to compound II, and excess of H_2O_2 results in the formation of compound III (de Wit & van Hooydonk, 1996).

- 3) In the presence of an excess of H_2O_2 resulting in the formation of Compound III, associated with irreversible inactivation of LP.

1.2.8.2.1. The peroxidation of thiocyanate

Lactoperoxidase-catalysed reactions yield short-lived intermediate oxidation products of SCN^- (Pruitt, Tenovuo, Andrews & McKane, 1982), which show the antibacterial activity. A proposed scheme for the peroxidase-catalysed oxidation of SCN^- is shown in Figure 1.2 (Thomas & Aune, 1978). The major intermediate oxidation product of the LP-catalysed oxidation of SCN^- is the hypothiocyanite ion ($OSCN^-$) (Aune & Thomas, 1977; Hoogendoorn, Piessens, Scholtes & Stoddard, 1977; Pruitt *et al.*, 1982). However, the

reaction system is complex and OSCN^- may not be the first product released from the enzyme's active site. Other short-lived intermediates which may be formed in varying amounts depending upon reaction conditions include thiocyanogen ($(\text{SCN})_2$), cyanogen thiocyanate (NC-SCN), cyanosulphurous acid (HO_2SCN) and cyanosulphuric acid (HO_3SCN) (Pruitt & Kamau, 1991).

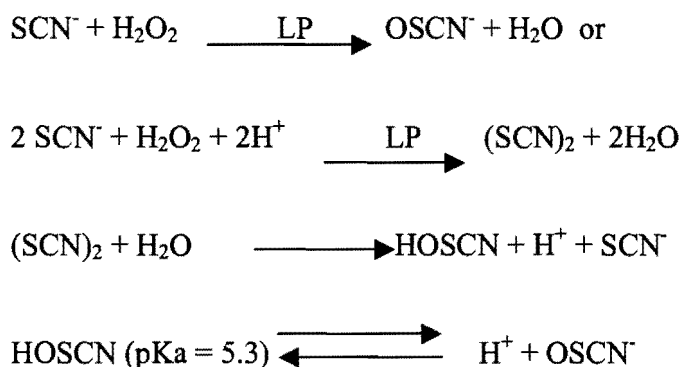
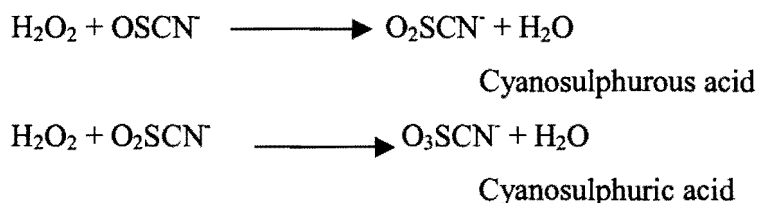
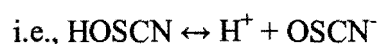


Figure 1.2. Oxidation of thiocyanate by lactoperoxidase catalysed reactions.

Whether the reaction proceeds by direct oxidation of SCN^- to OSCN^- or by oxidation of SCN^- via thiocyanogen ($(\text{SCN})_2$), the major product at neutral pH is OSCN^- . At neutral pH an excess of H_2O_2 (e.g. in saliva) results in formation of highly reactive, short-lived antimicrobial products in addition to OSCN^- (Björck & Claesson, 1980; Pruitt *et al.*, 1982) such as cyanosulphurous acid and cyanosulphuric acid.



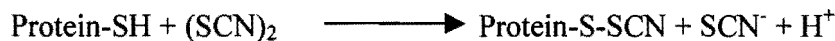
OSCN^- is in equilibrium with hypothiocyanous acid (HOSCN) at $\text{pKa} = 5.3$ (Pruitt & Kamau, 1991).



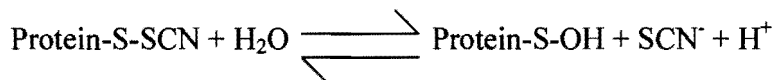
Both forms exert antibacterial activity but there is evidence that the uncharged HOSCN is more bactericidal. The stability of hypothiocyanite, OSCN⁻, is affected by many factors, such as pH, light, metals (Fe, Ni, Cu, Mn), glycerol and ammonium sulphate as well as by the presence and removal of LP; however, it is very heat stable (Thomas, 1985).

The products HOSCN and OSCN⁻ react rapidly with protein sulphydryl groups to yield sulphenyl thiocyanates (R-S-SCN). At low concentrations of sulphydryls (R'-SH), the R-S-SCN may react to form mixed disulphides (R-S-S-R'). At higher concentrations of R'-SH, the R-S-SCN may be reduced back to R-SH. The R-S-SCN may also be hydrolysed to sulphenic acids (R-S-OH) (Pruitt & Kamau, 1991).

Lactoperoxidase catalyses the incorporation of SCN⁻ into protein substrates. The reaction of (SCN)₂ or OSCN⁻ with proteins oxidizes the protein sulphydryls to sulphenyl thiocyanate derivatives (Thomas & Aune, 1978).



Sulphenyl thiocyanate derivatives can undergo further modifications, including reversible hydrolysis to yield sulphenic acids.



The oxidation of sulphydryl (SH) groups in microbial enzymes and other proteins is considered to be the key to the antimicrobial action of the LP system. This activity can be inhibited by reducing agents containing sulphydryl (SH) groups such as cysteine, glutathione, mercapto-ethanol, dithiothreitol and sodium hydrosulphite, either by direct binding to the haem group of the enzyme or by scavenging thiocyanate ions. Neither HOSCN nor OSCN⁻ appears to oxidise SH groups of milk proteins such as β -lactoglobulin (de Wit & van Hooydonk, 1996).

The structural damage of microbial cytoplasmic membranes by the oxidation of SH-groups results in leakage of potassium ions, amino acids and polypeptides into the medium. Subsequently uptake of glucose, amino acids, purines, pyrimidines in the cell and the synthesis of proteins, DNA and RNA is also inhibited (Reiter & Härnolv, 1984).

1.2.9. Antimicrobial spectrum of the lactoperoxidase system

The LP system could elicit bacteriostatic and/or bactericidal activity on a variety of susceptible microorganisms including bacteria, fungi and viruses (Table 1.2). The molecular mechanism (s) of such inhibitory effects depend on the type of electron donor, test media, temperature, and pH and could range from oxidative killing to blockage of glycolytic pathways or interference in cytopathic effects (Naidu, 2000).

1.2.9.1. Antibacterial effect of the lactoperoxidase system

Different groups of bacteria show a varying degree of sensitivity to the LP system. Gram-negative, catalase-positive organisms, such as pseudomonas, coliforms, salmonellae and shigellae, are not only inhibited by the LP system but also, depending on the medium conditions (pH, temperature, incubation time, cell density) may be killed provided that H₂O₂ is supplied exogenously (Björck, Rosén, Marshall & Reiter, 1975; Reiter, Marshall, Björck & Rosén, 1976). Gram-positive, catalase negative bacteria, such as streptococci and lactobacilli are generally inhibited but not killed by the LP system (Oram & Reiter, 1966). This difference in sensitivity to the LP system can probably be explained by the differences in cell wall structure and their different barrier properties (Reiter & Härnolv, 1984; de Wit & van Hooydonk, 1996). The inner membrane of Gram-negative bacteria appears to be more extensively damaged by LP-treatment than is that of Gram-positive species (Marshall & Reiter, 1980).

Lactoperoxidase present in various secretions oxidizes SCN⁻ in the presence of H₂O₂ to an unstable oxidation product hypothiocyanite (OSCN⁻), which is bactericidal for enteric pathogens including multiple antibiotic resistant strains of *E. coli* (Naidu, 2000). The system damages the inner membrane causing leakage and cessation of uptake of nutrient,

leading eventually to death of the organisms and lysis. The antimicrobial activity of the LP system against *E. coli* seems to be related to the oxidation of bacterial sulphhydryls (Thomas & Aune, 1978). The oxidation of sulphhydryls to sulphenyl derivatives inhibits bacterial respiration. However, a recent report by Shin, Hayasawa and Lönnnerdal (2001) indicated that the inhibitory effect of the LP system against *E. coli* is related to the inhibition of dehydrogenases in the respiratory chain of *E. coli*. The LP system could inhibit the growth of enterotoxigenic *E. coli* strains that cause scouring in neonatal and post-weaning piglets (Grieve, Dionysius & Vos, 1992). Antimicrobial activity of the LP system against verotoxigenic *E. coli* O157:H7 has also been reported (Heuvelink, Bleumink, Van Den Biggelaar, Te Giffel, Beumer & De Boer, 1998). Activation of the LP system in goat milk resulted in lower *E. coli* counts in LP-activated milk as compared to the control during storage of goat milk at 8 °C for 5 days (Zapico, Gaya, Nuñez & Medina, 1995). In the same study these authors also reported that activation of the LP system in goat milk was bactericidal against *Pseudomonas fluorescens* and resulted in mean decreases in the levels of *P. fluorescens* by 1.69 log units at 4 °C and 1.85 log units at 8 °C during the first 24 h.

The LP system exerts both bacteriostatic and bactericidal activities against strains of *Salmonella typhimurium* (Purdy, Tenovuo, Pruitt & White, 1983). The bactericidal activity was dependent on the permeability of the bacterial cell wall. The rough mutant TA1535, with the most permeable cell envelope, was killed both at neutral and acid pH, whereas very little or no killing was observed with the intact cells of the wild-type parent strain. Wolfson and Sumner (1994) also reported that the LP system showed both bacteriostatic and bactericidal activities against *S. typhimurium* in trypticase soy broth. The bactericidal effect was dependent on the initial inoculum level. Inoculum levels of 10^2 cfu/ml were killed at 37 °C as opposed to the bacteriostatic activity with inoculum levels of 10^6 - 10^7 cfu/ml.

Campylobacter jejuni is a major cause of acute enteritis in humans and milk has been associated with several outbreaks of *C. jejuni* enteritis (Pruitt & Kamau, 1991). The bactericidal effect of the LP system against *C. jejuni* in milk has been demonstrated

(Beumer, Noomen, Marijs & Kampelmacher, 1985). On the other hand, Borch, Wallentin, Rosén and Björck (1989) showed that the LP system has a strong bactericidal activity against strains of *C. jejuni* isolated from poultry. The bactericidal effect of the LP system against this pathogen is more pronounced at higher temperatures (Beumer *et al.*, 1985).

The inhibition of *Bacillus cereus* by the LP system was reported by Tenovuo, Mäkinen and Sievers (1985). The growth inhibition was directly proportional to the quantity of OSCN⁻ ions present. This inhibition was associated with reduced extracellular release of collagenase activity from the cells. Similarly, Zajac, Björck and Classon (1981) tested the antibacterial effect of the LP system against both vegetative cells and spores of four different strains of *Bacillus cereus* isolated from cow milk. The antibacterial effect was substantial against the vegetative cells of all strains tested. However, little effect was observed against the corresponding spore preparations.

Staphylococcus aureus is a major causative agent of bovine mastitis and poses a human health problem since this pathogen can be shed into milk from mastitic udders (Hunter, 1984). Although *S. aureus* is readily destroyed by proper pasteurisation, enterotoxins produced by strains of this pathogen can withstand pasteurisation and cause food poisoning in the absence of viable cells (Smith, Buchanan & Palumbo, 1983). The LP system is both bactericidal and bacteriostatic against *S. aureus* in milk (Kamau, Doores & Pruitt, 1990a).

Listeria monocytogenes is an intracellular pathogen capable of survival and growth within phagocytes, a characteristic associated with virulence (Wilder & Edberg, 1973). Listeric infections can cause septicaemia, meningitis and abortion and the main groups at risk include neonates, pregnant women and other immuno-compromised individuals (Marth, 1988). The risk of listeriosis is further amplified by the ability of *L. monocytogenes* to grow at refrigeration temperatures (Wilkins, Bourgeois & Murray, 1972) and by its relatively higher heat resistance compared to other non-spore-forming bacteria (Doyle, Glass, Beery, Garcia, Pollard & Schultz, 1987). The LP system is both

bactericidal and bacteriostatic against *L. monocytogenes* (Dennis & Ramet, 1989; Earnshaw & Banks, 1989; Kamau *et al.*, 1990a; Siragusa & Johnson, 1989). The bactericidal effect of the LP system against *L. monocytogenes* depends on initial inoculum concentration, culture medium and storage temperature (Dennis & Ramet, 1989). The inhibitory effect of the LP system against *L. monocytogenes* is inversely related to incubation temperature (Dennis & Ramet, 1989).

Kamau, Doores and Pruitt (1990b) reported that the LP system enhanced thermal destruction of *L. monocytogenes* and *S. aureus*. The most rapid killing of *L. monocytogenes* occurred when samples were heated soon after activation of the LP system. According to Kamau *et al.* (1990b), activation of the LP system followed by heating can increase the margin of safety with respect to milk-borne pathogens.

Effects of the LP system on *L. monocytogenes* (strains V7, Scott A and California) were determined by El-Shenawy, Garcia and Marth (1990) using a semi synthetic medium, raw cow milk and a buffer solution. Each medium was inoculated to contain three levels of the pathogen (low, 30 to 50 cfu/ml; medium, 10^4 cfu/ml; and high, 10^7 cfu/ml) and incubated at 4 or 35 °C. Low numbers of bacteria were completely inactivated within 2 to 4 h (35 °C) or 12 to 24 h (4 °C). When substrates contained medium or high populations, a limited bactericidal effect occurred and the LP system failed to cause permanent injury of *L. monocytogenes* at 4 or 35 °C. El-Shenawy *et al.* (1990) believed that the efficacy of the LP system as an antimicrobial agent was related to strain and number of pathogen, suspending medium, and incubation temperature. The activity of raw cow milk LP system on four *L. monocytogenes* strains (Scott A, 5069, ATCC 19119 and NCTC 11994) at refrigeration temperatures was studied by Gaya, Medina and Nuñez (1991). The LP system exhibited a bactericidal activity against *L. monocytogenes* at 4 and 8 °C; the activity was dependent on temperature, length of incubation, and strain of *L. monocytogenes* tested (Gaya *et al.* 1991). A significant decrease in the counts of three *Listeria monocytogenes* strains (Scott A, 5069 and NCTC 11994) was observed in activated lactoperoxidase system in goat milk at refrigeration temperatures (Zapico, Gaya, Nuñez & Medina, 1993). Inhibitory activity of the LP system on *L. monocytogenes*

Table 1.2. Antimicrobial activity of the lactoperoxidase system

Microorganism	Donor	Inhibitory effect	References
Gram positive bacteria			
<i>Bacillus cereus</i>	SCN ⁻	collagenase production	Tenovuo <i>et al.</i> (1985)
<i>Lactobacillus casei</i>	SCN ⁻	growth inhibition	Iwamoto, Kakamura, Watanabe and Tsunemitsu (1972)
<i>Staphylococcus aureus</i>	SCN ⁻	amino acid uptake	Kamau <i>et al.</i> (1990a)
<i>Streptococcus cremoris</i>	SCN ⁻	oxygen uptake	Modi, Deodhar, Behere and Mitra (1991)
<i>Streptococcus lactis</i>	SCN ⁻	growth inhibition	Marshall and Reiter (1980)
<i>Streptococcus agalactiae</i>	SCN ⁻	sugar transport	Mickelson (1977)
<i>Streptococcus mutans</i>	SCN ⁻	glucose uptake	Loimaranta, Tenovuo and Korhonen (1998)
	SCN ⁻	glucosyltransferases	Korpela, Yu, Loimaranta, Lenander, Vacca, Wunder, Bowen and Tenovuo (2002)
<i>Streptococcus salivarius</i>	SCN ⁻	acid production	Carlsson, Iwami and Yamada (1983)
Gram negative bacteria			
<i>Actinobacillus actinomycetemcomitans</i>	SCN ⁻ /I	bactericidal	Ihalin, Loimaranta, Lenander & Tenovuo (1998)
<i>Escherichia coli</i>	SCN ⁻	dehydrogenases inhibition	Shin <i>et al.</i> (2001)
<i>Fusobacterium nucleatum</i>	SCN ⁻ /I	bactericidal	Ihalin, Loimaranta, Lenander & Tenovuo (2001)
<i>Helicobacter pylori</i>	SCN ⁻	bactericidal	Shin, Yamauchi, Teraguchi, Hayasawa & Imoto

Table 1.2. Continued

			(2002)
<i>Salmonella typhimurium</i>	SCN ⁻	bactericidal/bacteriostatic	Purdy <i>et al.</i> (1983)
<i>Porphyromonas gingivalis</i>	SCN ⁻ /I ⁻	bactericidal	Ihalin <i>et al.</i> (2001)
	SCN ⁻	bactericidal	Fadel and Courtois (1999)
<i>Pseudomonas aeruginosa</i>	SCN ⁻	bactericidal	Reiter <i>et al.</i> (1976)
<i>Pseudomonas fluorescens</i>	SCN ⁻	bactericidal	Björck <i>et al.</i> (1975)
<i>Yersinia enterocolitica</i>	SCN ⁻	bactericidal/bacteriostatic	Farrag, El-Gazzar and Marth (1992a)
Fungi			
<i>Candida albicans</i>	SCN ⁻	loss of viability	Lenander (1992)
<i>Rhodotorula rubra</i>	SCN ⁻	bacteriostatic	Popper and Knorr (1997)
<i>Saccharomyces cerevisiae</i>	SCN ⁻	bacteriostatic	Popper and Knorr (1997)
<i>Byssoschlamys fulva</i>	SCN ⁻	bacteriostatic	Popper and Knorr (1997)
<i>Aspergillus niger</i>	SCN ⁻	bactericidal	Jacob, Antony, Sreekumar and Haridas (2000)
<i>Penicillium chrysogenum</i>	SCN ⁻	bactericidal	Jacob, Antony, Sreekumar and Haridas (2000)
Viruses			
Polio and Vaccinia viruses	I ⁻ /Br ⁻	virucidal	Belding, Klebanoff and Ray (1970)
HIV-1	I ⁻	loss of viral replication	Yamaguchi, Semmel, Stanislawski, Strosberg and Stanislawski (1993)
	SCN ⁻	reversetranscriptase inhibition	Wang, Ye and Ng (2000)

depended on temperature, length of incubation, and strain of *L. monocytogenes* tested. The LP system only retarded growth of *L. monocytogenes* at 20 °C in raw goat milk.

Exposure of susceptible cells to the LP system could cause rapid inhibition of bacterial metabolism resulting in leakage of amino acids and potassium ions. Various microbial cellular events including carbohydrate transport and utilization; oxygen uptake; amino acid and purine transport (protein and nucleic acid synthesis); production and excretion of extracellular products (i.e., lactic acid, H₂O₂, collagenase) and growth may be inhibited (Naidu, 2000)

The inhibitory effect of the LP system on *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis*, and *Streptococcus salivarius*, in particular, the rate of acid production and oxygen uptake by intact cells, the activity of glycolytic enzymes in cell-free extracts, and the levels of intracellular glycolytic intermediates have been reported (Carlsson *et al.*, 1983). Acid production, oxygen uptake, and consequently H₂O₂ excretion were inhibited in all the strains by the LP system. *Streptococcus sanguis* and *S. mitis* showed more resistance to the LP system than *S. mutans* and *S. salivarius*. They attributed this to higher activity of NADH-OSCN oxidoreductase in the former strains. The primary target of OSCN in the glycolytic pathway was glyceraldehyde 3-phosphate dehydrogenase (Carlsson *et al.*, 1983).

1.2.9.2. Antifungal effect of the lactoperoxidase system

The ability of the LP system to degrade aflatoxin in the presence of sodium chloride (225 µM) and H₂O₂ (50 µM) has been reported (Doyle & Marth, 1978). Increasing the amount of LP from 50 to 500 units/ml of reaction mixture resulted in augmenting the rate of degradation of aflatoxin B1 from 3.6 to 5.1 % /24 h. When comparable amounts of LP were present, aflatoxin G1 was degraded approximately 1.5 times faster than was aflatoxin B1.

Popper and Knorr (1997) reported antifungal activity of the LP system with glucose oxidase as H₂O₂ source in salt solution and in apple juice. *Rhodotorula rubra* and

Saccharomyces cerevisiae cultivated aerobically in apple juice and agar-grown *Mucor rouxii*, *Aspergillus niger* and *Byssoschlamys fulva* were tested. The antifungal activity of LP was tested with initial counts of about 10^5 cfu/ml in salt solution supplemented with SCN^- (25 mg/L) and glucose (20 g/L) or in apple juice supplemented with the same amount of SCN^- . Antifungal activity was observed against all test organisms in both media. The yeast strains were found to be least stable while *B. fulva* was most resistant. However, a combination of LP (5 U/ml) with glucose oxidase (0.5 to 1 U/ml) caused total inactivation of this mould in salt solution within 2 h. The LP system also showed antifungal activity in apple juice at acid pH (3.2), although its effectiveness was reduced. In this medium, *B. fulva* was inactivated by LP (20 U/ml) and glucose oxidase (1 U/ml) within 4 h. Strains of *R. rubra* and *S. cerevisiae* were also inhibited in apple juice by LP (5 U/ml) and glucose oxidase (1 U/ml).

Jacob *et al.* (2000) reported that purified goat milk lactoperoxidase (gLP) showed high antifungal and antibacterial activity in a thiocyanate-hydrogen peroxide medium. Goat milk LP-thiocyanate- H_2O_2 system was found to inhibit the growth and proliferation of many fungal species. *Aspergillus flavus*, *Trichoderma* spp., *Corynespora cassicola*, *Phytophthora meadii* and *Corticium salmonicolor*; however, *Candida albicans* and *Pythium* spp. were not affected by the LP system. Goat milk LP-thiocyanate- H_2O_2 system showed antifungal activity against *Aspergillus niger*, *Alternaria* spp., *Penicillium chrysogenum* and *Claviceps* spp.

1.2.9.3. Antiviral effect of the lactoperoxidase system

Lactoperoxidase has been shown to kill both poliovirus and vaccina virus with halides (I, Br⁻) as electron donors (Belding *et al.*, 1970). These particular viruses are more resistant than are most others to the effects of drying, heat, and disinfectants. Yamaguchi *et al.* (1993) reported that LP and glucose oxidase are virucidal to HIV-1 in the presence of sodium iodide, as assessed by the loss of viral replication in a syncytium-forming assay or by the inhibition of cytopathic effects on infected cells. These *in vitro* findings demonstrate that the LP/ H_2O_2 /halide system provides potent virucidal activity against HIV-1.

1.2.10. Effect of the lactoperoxidase system in the preservation of raw milk

The most widely recommended industrial application of the LP system in food production is in the dairy industry for the preservation of raw milk during storage and/or transportation to processing plants. The International Dairy Federation has published a guideline for the use of the LP system for the preservation of raw milk especially in the absence of refrigeration (IDF, 1988).

Antimicrobial agents of the LP system in milk cause inhibition of various spoilage and pathogenic organisms, thus enhancing the microbiological quality of milk. The antibacterial activity of the LP system in milk against psychrotrophic spoilage organisms has been widely investigated (Björck *et al.*, 1975; Björck, 1978; Reiter *et al.*, 1976). Using a glucose/glucose oxidase system to generate H₂O₂, and supplementing milk with 0.17-0.26 mM SCN⁻, Björck *et al.* (1975) demonstrated that the LP system was bactericidal against *Pseudomonas* and *Escherichia coli*. When various Gram-negative rods isolated from milk were exposed to the LP system, at least 91 % of the inoculum was killed within 4 h at 30 °C (Björck *et al.*, 1975). Activation of the antibacterial LP system in milk by increasing SCN⁻ concentration to 0.25mM and adding an equimolar amount of H₂O₂ resulted in a substantial reduction of the bacterial flora and prevented the growth of psychrotrophic bacteria for up to 5 days (Björck *et al.*, 1975). The treatment neither altered physico-chemical properties of milk nor developed LP-resistant bacteria. Reiter *et al.* (1976) observed that the bactericidal effect of the LP system against *E. coli* was increased when SCN⁻ concentration was raised from 0.015 to 0.15 mM, and was reduced when the initial inoculum was increased. Björck (1978) observed a similar increase in the bactericidal effect of the LP system against *P. fluorescens* when both the SCN⁻ and H₂O₂ concentrations were increased up to an equimolar concentration of 0.3 mM.

Apart from controlling psychrotrophic spoilage bacteria, the LP system has been widely investigated for its potential to control mesophilic spoilage bacteria, especially in bovine

milk (Björck *et al.*, 1979; Reiter & Härnulv, 1982; Zajac, Gladys, Skarzynska, Harnülv & Björck, 1983; Kamau & Kroger, 1984). Preservation of buffalo milk by the LP system has also been reported both at ambient and refrigeration temperatures (Thakar & Dave, 1986; Chakraborty, Chaudry, Alex, Jacob & Soni, 1986). For bacteria that survive the initial bactericidal activity of the LP system, there is an extended lag phase or recovery period. The length of this lag period is highly temperature dependent, being much longer at cold storage than at high temperatures (Björck *et al.*, 1979; Zajac *et al.*, 1983; Kamau & Kroger, 1984). After recovery, most bacteria resume normal growth. The length of the antibacterial effect achieved by activation of the LP system is inversely related to the storage temperature of the milk (IDF, 1988). When milk is stored at 30, 25, 20 and 15 °C, the antibacterial effect of the LP system lasts for 7-8, 11-12, 16-17 and 24-26 h, respectively (IDF, 1988).

Field experiments in Kenya (Björck *et al.*, 1979) and Sri Lanka (Härnülv & Kandasamy, 1982) have demonstrated that a substantial improvement of the hygienic quality of raw milk can be achieved during collection and transportation after an activation of the LP system at the collection point or collecting centre. Similarly, experiments conducted in India showed that activation of the lactoperoxidase system extended the keeping quality of raw buffalo milk at 30 °C under both farm (Kumar & Mathur, 1989a) and field (Kumar & Mathur, 1989b) conditions.

Kamau, Doores and Pruitt (1991) found that the activated LP system in conjunction with pasteurisation extended the shelf-life of cow milk held at 10 °C by more than 20 days, compared to untreated milk. They found that observable growth of surviving natural milk microflora started after 12 days in LP system treated milk, compared to 4 days in untreated and H₂O₂-treated milk. After 22 days, viable counts in untreated and H₂O₂-treated milk reached 10⁶-10⁷ cells per ml compared to about 10³ cells per ml in LP system-treated milk.

Activation of the LP system by using 20:20 ppm SCN⁻: H₂O₂ or 60:60 ppm SCN⁻: H₂O₂ was found to be an effective means of preservation against microbial growth in raw ewe

and goat milk samples stored at 20 °C or 30 °C for at least 6 h (Gürsel, Atamer, Gürsoy, Şenel & Gençer, 1999). Similarly, Haddadin, Ibrahim and Robinson (1996) reported the possibility of extending the shelf-life of bovine, ovine and caprine milks by activation of the LP system.

Apart from its importance in the preservation of raw milk, the LP system can also be used to extend shelf-life of pasteurised milk (Martinez, Mendoza, Alacron & Garcia, 1988; Barrett, Grandison & Lewis, 1999). Sarkar and Misra (1994a) showed that when raw cow milk was LP-activated prior to pasteurisation, an improvement in the quality of pasteurised milk was observed probably due to reduced heat resistance of microorganisms as a result of the LP-treatment. The reduction of heat resistance of organisms due to the LP system has been reported by Kamau *et al.* (1990b).

1.2.11. Effect of the lactoperoxidase system on cheese starter cultures

Antimicrobial compounds formed by the LP system may interfere with the starter activity and affect the quality of the final product (Table 1.3). Reactivation of the LP system during the manufacture of fermented milk products poses manufacturing problems (Sarkar & Misra 1994b). An intermediate oxidation product (OSCN⁻) produced in LP-activated milk causes growth inhibition and reduced lactic acid production (Reiter, 1978; Roginski, Broome, Hungerford & Hickey, 1984) by the starter cultures.

The effect of the LP system on the behaviour of thermophilic lactic starter cultures commonly used in the dairy industry for cheesemaking was investigated by de Valdez *et al.* (1988). It was determined that the thermophilic starter cultures were very sensitive to the LP system and the activity of the cultures was strongly inhibited. Guirguis and Hickey (1987) indicated that thermophilic lactic starter strains were found to be sensitive to the LP system inhibition, but varied in their susceptibility to inhibition by the system. Basaga and Dik (1994) also reported that activation of the LP system delayed the coagulation time and reduced the activity of starter cultures used for yoghurt production.

In a study on the effect of the LP system on the growth and acid production by selected lactococci and their phage resistant mutants, it was observed that some phage-resistant mutants were more susceptible to the LP system than their parent organisms (Roginski & Hickey, 1991). A considerable reduction in biomass and acid production was also observed when the phage-resistant mutant of *Lactococcus lactis* spp. *lactis* ML8 was allowed to grow aerobically in sterile milk.

Inhibition of metabolic activity of thermophilic and mesophilic starter cultures in LP-activated milk is strain dependent (Reiter, 1978; Roginski *et al.*, 1984). Reduction in the rate of acid production by yoghurt cultures was observed in LP-activated cow milk (20:20 ppm SCN: H₂O₂) for the first 3 h (Sarkar & Misra, 1994b). The rate of acid production by *Lactobacillus acidophilus* and LF-40 cultures was also retarded. A decline in the rate of acid production by starter cultures in LP-activated milk may increase the production time significantly or may lead to an abnormal product. On the basis of susceptibility to the LP system, thermophilic starters can be categorized into three groups (Guirguis & Hickey, 1987):

- (i) the most sensitive groups of organisms generating H₂O₂, for example, *Lactobacillus acidophilus*, three strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (1373, 1489, LBr),
- (ii) organisms that are sensitive to inhibition by the LP system but do not have the ability to generate H₂O₂ and require an extraneous source of H₂O₂, for example, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Lactobacillus lactis* (3201) and
- (iii) organisms resistant to inhibition by the LP system, e.g. *Enterococcus faecium*, *Lactobacillus lactis* and one strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* (1243).

Table 1.3. Effect of the lactoperoxidase system on the activity of starter cultures

Starter cultures	Effect	References
Lactic acid bacteria	Reduced rate of acid production.	Kamau and Kroger (1984)
Thermophilic starters	Delay in coagulation time by 4.5 h when inoculated 2 h after LP activation.	de Valdez <i>et al.</i> (1988)
<i>Streptococcus thermophilus</i> + <i>Lactobacillus bulgaricus</i>	Reduced rate of acid production and delay in curdling time by 1.2 h.	Zall, Chen and Dzurec (1983) Kumar and Mathur (1989c)
LF-40	Reduced rate of acid production.	Sarkar and Misra (1994b)
<i>Lactobacillus acidophilus</i>	Reduced rate of acid production.	Sarkar and Misra (1994b)

1.2.12. Effect of the lactoperoxidase system on the manufacture of fermented milk products

Antimicrobial agents or compounds formed by the LP system exhibit antibacterial activity and a reduced starter activity during the preparation of fermented milk products. Factors affecting successful utilization of LP-treated milk for the manufacture of fermented milk products are (Sarkar & Misra, 1992):

- type of milk used
- type and strain of starter cultures used
- level of thiocyanate and H₂O₂ used to activate the LP system in milk
- natural levels of thiocyanate and H₂O₂ in milk
- temperature of heating LP-activated milk and antibacterial compounds formed
- time and temperature of incubation and
- rate of inoculation of starters.

The effect of the LP system on the manufacture of various fermented milk products is shown in Table 1.4. The possibility of making acceptable varieties of soft and hard cheeses from LP-treated cow milk has been reported (Zall *et al.*, 1983). Lara *et al.* (1987) reported that the yields of fresh type cheese (measured on wet or dry basis) of LP-treated cow milk were significantly higher than cheese made from control milks. They indicated that a complete transition of fat from LP-activated milk into the cheese was achieved, while only 85 % was retained in the cheese made from untreated milk. More than 2 kg of extra cheese can be recovered by the use of the LP system for every 100 kg of milk. They also pointed out that microbiological counts, pH and appearance were also favourable for cheeses made from LP system activated milks.

Activation of ewe milk LP system was found to be useful in preventing excessive proteolysis and softening of Manchego cheese texture caused by proteinases of Gram-negative psychrotrophs (Uceda *et al.*, 1994). Santos, López-Díaz, García-Fernández, García-López and Otero (1995) also reported that the activation of the LP system in pasteurised ewe milk used for manufacturing of fresh cheese seems to be a useful method for controlling the undesirable effects associated with cold tolerant microorganisms. Abdou *et al.* (1996) showed that treatment of cow and buffalo milks with LP and H₂O₂ increased cheese yield. They also reported that the LP-treatment produced cheese with satisfactory quality that scored highly in organoleptic testing.

There was no significant difference in the recovery of solids from milk in Mozzarella cheese made from LP-treated buffalo milk; however, a lower moisture retention and slow rate of acid development was observed (Kumar & Mathur, 1989c). Reduced starter activity in LP-activated milk was observed but organoleptically and chemically equivalent yoghurt, Cottage and Cheddar cheeses were manufactured using LP-treated cow milk (Sarkar & Misra, 1994c). Longer ripening period and slight reduction in yield of Cheddar cheese were observed while using LP-activated cow milk (Zall *et al.*, 1983). Fresh type cheese made from LP-treated cow milk had slower rate of acid development and lower moisture retention but the body and texture of the product were satisfactory

(Lara *et al.*, 1987). A higher yield of cheese was obtained when LP-treated cow milk held at ambient temperature for 4 h was used (Lara *et al.*, 1987).

The quality of Dahi, yoghurt and acidophilus milk made from LP-treated cow milk or buffalo milk was not drastically affected, but a slow rate of acid production was encountered while using LP-activated milk (Sarkar & Misra, 1994b). Buffalo milk subjected to LP-treatment had no difference in the body and texture of yoghurt but gave a lower flavour rating compared to the untreated control when incubated at 30 °C for 16 h (Kumar & Mathur, 1989c).

1.2.13. Potential applications of the lactoperoxidase system

The most widely recommended industrial application of the LP system in food production is in the dairy industry for the preservation of raw milk during storage and/or transportation to processing plants. However, other novel applications of the LP system are being explored. If the LP system is activated immediately prior to application of approved thermal processes, the shelf-life of dairy products may be extended significantly and high-temperature processes may be replaced with more economical lower temperature treatments. In addition to energy savings, LP-low temperature thermal processes may provide better nutrient and/or quality retention for highly heat-sensitive foods such as salad dressings, spreads, beverages, dips and desserts (Pruitt & Kamau, 1991).

Guthrie (1992) reported the application of the LP system for preservation of cosmetics and concluded that the LP system can provide a broad-spectrum antimicrobial activity against bacteria, yeasts and moulds when it is composed of LP, H₂O₂ and SCN⁻ at carefully selected weight ratios. Optimum results were obtained when H₂O₂ was generated enzymatically by the glucose oxidase-glucose system. A report by van Hooijdonk, Kussendrager and Steijns (2000) indicated the potential of the lactoperoxidase system for use in fish farming, oral hygiene and functional foods.

Table 1.4. Effect of the lactoperoxidase system on the manufacture of fermented milk products

Product	Type of milk	Effect of the lactoperoxidase system	References
Dahi	Buffalo	Lower diacetyl + acetoin content and proteolytic activity.	Sarkar and Misra (1994b)
Fresh cheese	Cow	Slow acid development, low moisture retention, satisfactory body and texture.	Zall <i>et al.</i> (1983)
Cheddar cheese	Cow	Delayed acid production, and longer manufacturing schedule (about 2 h), weak curd at cutting, dry and rubbery curd at cheddaring, slightly lower yield, slower ripening of cheese.	Zall <i>et al.</i> (1983)
Manchego cheese	Ewe	Prevents excessive proteolysis and softening.	Uceda <i>et al.</i> (1994b)
Cottage cheese	Cow	Taste and flavour of experimental cheese slightly different from the control, increased cheese yield.	Zall <i>et al.</i> (1983)
Acidophilus milk	Cow	Lower diacetyl + acetoin content and lower proteolytic activity.	Sarkar and Misra (1994b)

Table 1.4. Continued

Cultured milk	Cow	No significant difference in the quality, longer rennet coagulation time.	Kamau and Kroger (1984)
Mozzarella cheese	Buffalo	No difference in recovery of solids from milk, lower moisture retention, slow acid development, longer time (2 h) to reach stretching stage.	Kumar and Mathur (1989c)
Yoghurt	Cow	No difference in chemical composition and organoleptic qualities.	Zall <i>et al.</i> (1983)
	Buffalo	A delay in curdling time by 1 h 20 min, no effect on body and texture, lower flavour rating.	Kumar and Mathur (1989c)
Pickled cheese	Cow and buffalo	Manufacture of pickled soft cheese from unsalted milk was possible, shorter processing time and economic utilization of whey, higher whey expulsion and lower yield.	Hefnawy, Ewais and Abd El-Salam (1986)
	Cow and buffalo	Increased cheese yield and coagulation time, decreased curd tension, increased moisture content and decreased acidity, satisfactory quality and ranked high scores.	Abdou <i>et al.</i> (1996)

Hoogendoorn (1985) reported that activation of salivary peroxidase antimicrobial system in toothpaste and mouth-rinse reduced acid production by oral microorganisms and clinical studies have shown that plaque accumulation, gingivitis and early carious lesions and aphthous lesions may all be reduced by appropriate applications of the applied enzyme preparations. Toothpastes (Biotene™, Zendium) that comprise the complete system are commercially available (Stadhouders & Beumer, 1994; Naidu, 2000). *In vivo* experiments indicated the potential of the LP system in bacterial clearance from airways and its contribution to airway defences (Gerson, Sabater, Scuri, Torbati, Coffey, Abraham, Lauredo, Forteza, Wanner, Salathe, Abraham & Conner, 2000). Tenovuo (2002) reported that lactoperoxidase together with other salivary defence proteins such as lysozyme and lactoferrin exerts a wide antimicrobial activity against a number of bacterial, viral and fungal pathogens *in vitro*. As a result, lactoperoxidase has been used as preservatives in foods and pharmaceuticals as well as in oral health care products to restore saliva's own antimicrobial capacity in patients with dry mouth.

It is suggested that peroxidase in lachrymal fluids will act as an anti-infectious agent. The LP system has a potential use as protectant in ophthalmic fluids (de Wit & van Hooydonk, 1996). Investigations have indicated that inclusion of an activated LP system in milk replacers for calves gives improved growth rates and reduced frequency of diarrhoea (Björck, 1990). Reiter, Marshall and Philips (1980) demonstrated that feeding of LP supplemented milk could prevent incidence of scouring in calves and also improve weight-gain in LP-treated animals. A similar study by Reiter, Fulford, Marshall, Yarrow, Ducker and Knutsson (1981) indicated that the lactoperoxidase system significantly increased the live-weight gain and reduced mortality of calves given whole milk. *In vivo* experiments in cows indicated that the LP system plays a role in protecting the lactating mammary gland from infection with *Streptococcus uberis* (Marshall, Cole & Bramley, 1986). Addition of LP and lactoferrin to a milk replacer diet resulted in a significant ($p < 0.05$) reduction in the severity of diarrhoea in young calves (van Leeuwen, Oosting, Mouwen & Verstegen, 2000).

The LP system is also involved in the degradation of various carcinogens which have been reported to occur in human saliva, and the protection of human cells from the toxicity of hydrogen peroxide (Gothefors & Marklund, 1975). Stanislawski, Rousseau, Goavec and Ito (1989) reported tumoricidal potency of enzyme immunotoxins constructed of antibodies conjugated to glucose oxidase and LP. Halide-dependent cytolysis of B-16 melanoma (black tumour) cells mediated by LP and myeloperoxidase systems has been reported (Odajima, Onishi, Hayama, Motoji, Momose & Shigematsu, 1996). A significant suppression of black tumours was detected in the groups of mice inoculated with melanoma cells when exposed to the systems containing LP or myeloperoxidase (Odajima *et al.*, 1996).

The lactoperoxidase system acts in synergy with other preservatives, increasing their efficiency. Boussoel, Mathieu, Revol-Junelles and Milliére (2002) reported the synergistic bactericidal effect of the LP system and nisin against *Listeria monocytogenes* ATCC 15313 in cow skim milk. They reported that when the LP system was added to cells already in contact with 100 or 200 IU/ml nisin for a period of 4 h, the inhibitory activity was enhanced with no *L. monocytogenes* detectable after 72 or 48 h, and until 15 days. Similarly, Zapico, Medina, Gaya and Nuñez (1998) reported that addition of nisin and the LP system showed a synergistic bactericidal effect against the *L. monocytogenes* strains Ohio and Scott A in UHT skim milk, and resulted in counts up to 5.6 log units lower than the control. A study by McLay, Kennedy, O'Rourke, Elliot and Simmonds (2002) indicated the inhibition of bacterial food-borne pathogens by the LP system in combination with monolaurin in broth, cow milk and ground beef. They reported that combinations of LP (in the range 5-200 mg/kg) and monolaurin (in the range 50-1000 ppm) inhibited growth of *Escherichia coli* O157: H7 and *Staphylococcus aureus*. On the other hand, García-Graells, van Opstal, Vanmuysen and Michiels (2002) reported the potential of the LP system to improve the bactericidal efficiency of high-pressure treatment for food preservation. They found that at cell concentrations of 10^6 cfu/ml, the LP system strongly increased high-pressure inactivation of *E. coli* MG1655, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria innocua* and *Lactobacillus plantarum* in cow skim milk.

1.2.14. Objectives

The overall objectives of this research were to:

1. Assess the effect of the lactoperoxidase (LP) system on the growth and survival of selected pathogenic bacteria in the milk of Saanen and South African Indigenous goats.
2. Assess the effect of the LP system on the activity of various cheese starter cultures in Saanen goat milk and identify a starter culture which is resistant to the LP system.
3. Determine the suitability of Saanen goat milk preserved by the LP system for the manufacture of Gouda cheese and assess the effect of this treatment on the biochemical, microbiological and sensory properties of Gouda cheese.

Specific objectives of the work were to:

1. Determine the effect of the LP system on the growth and survival of *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Brucella melitensis*, and *Mycobacterium paratuberculosis* in the milk of Saanen and South African Indigenous goats.
2. Determine the effect of the LP system on the activity of selected Gouda cheese lactic starter cultures.
3. Identify a starter culture which is resistant to the LP system.
4. Determine the effect of the LP system on the gross chemical composition of Gouda cheese.
5. Determine the effect of the LP system on the microbiological quality of Gouda cheese.
6. Determine the effect of the LP system on proteolysis and lipolysis during ripening of Gouda cheese.
7. Evaluate the difference in sensory properties between Gouda cheeses made from LP preserved and untreated control Saanen goat milk.

1.2.15. Hypothesis

1. Activation of the LP system may strongly inhibit the growth and survival of pathogenic bacteria in goat milk because such inhibition was observed in cow milk; and also variations in the inhibitory effect of the LP system against pathogens may occur in Saanen and South African Indigenous goats milk because such variations were observed in milk from other species depending on the breed of the animal and strain of bacterium.
2. Activation of the LP system in goat milk may inhibit the activity of lactic cheese starter cultures because such inhibition was observed in cow milk and a starter culture which is resistant to the inhibitory effect of goat milk LP system may be obtained since the effect of the LP system on lactic starter cultures varies depending on the type of milk and species and/or strain of a starter culture used.
3. Preservation of goat milk by the LP system may improve the microbiological quality of Gouda cheese manufactured from it and it may not exhibit adverse effect on the chemical composition, sensory properties and biochemical changes during the ripening of Gouda cheese because cheese of acceptable chemical composition, sensory property and improved microbiological quality has been manufactured from other species milk preserved by the LP system.



CHAPTER 2

Antibacterial Activity of the Lactoperoxidase System Against Food-borne Pathogens in Saanen and South African Indigenous Goat Milk

Manuscript in press [Food Control]

Key Words: Lactoperoxidase system; Goat milk; *Escherichia coli*; *Staphylococcus aureus*; *Listeria monocytogenes*; *Brucella melitensis*.

Abstract

The effect of the lactoperoxidase (LP) system on the growth and survival of *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Brucella melitensis* and *Mycobacterium paratuberculosis* in the milk of Saanen and South African Indigenous goats was determined. The inhibitory effect of the LP system against these pathogens was measured in the goat milk samples kept at 30 °C for 6 h. The LP system exhibited a bactericidal effect against *L. monocytogenes* and *Br. melitensis* both in Saanen and Indigenous goat milks. The LP system was bactericidal against *S. aureus* in Saanen goat milk; however, it was bacteriostatic against *S. aureus* in Indigenous goat milk. On the other hand, the LP system only reduced the rate of multiplication of *E. coli* both in Saanen and Indigenous goat milks. No growth of *M. paratuberculosis* was observed both in the untreated control and the LP-activated milks of Saanen and Indigenous goats. The same pathogen showed different degrees of sensitivity to the LP system in the milk of the two goat breeds probably due to the difference in the LP system components in the two milk types. The bactericidal effect of the LP system against *L. monocytogenes* and *Br. melitensis* at 30 °C suggests that activation of the LP system in goat milk could be of great practical importance in controlling the growth of these pathogens, especially under situations where milk is collected and transported for a long time at high ambient temperatures.

2.1. Introduction

Goats are important milk producers in several parts of the tropics and significantly contribute to human nutrition in many developing countries (Devendra, 1999). Goat milk is a very nutritious and acceptable food in many parts of the tropics; however, its production and handling remains a major problem limiting its consumption. The dispersed nature of production across the diversity of small farms, problems of collection, poor handling systems, inadequate transport and refrigeration facilities all create a considerable challenge to goat milk production in several developing countries (Chamberlain, 1989; Devendra, 1999).

Milk in general and goat milk in particular is a highly nutritious food ideally suited for the growth of both pathogenic and spoilage organisms. Outbreaks of milk-borne illness date back from the inception of the dairy industry. Various bacterial infections have been linked to consumption of raw goat milk (Vasavada, 1986). Brucellosis for instance is one such disease which can be transmitted to humans through contaminated and untreated milk and milk products. Consumption of goat milk or cheese containing *Brucella melitensis* is an important source of human brucellosis worldwide and has caused several outbreaks (Garin-Bastuji & Verger, 1994).

In developed countries, the bacteriological quality of raw milk is safeguarded during collection, storage and transportation through refrigeration. However, cooling facilities are not available in rural areas of most developing countries. As a result, the lactoperoxidase (LP) system has been recommended for preservation of raw milk as an alternative to cooling (Björck, 1987). The LP system consists of three components: the enzyme lactoperoxidase, thiocyanate and hydrogen peroxide. Lactoperoxidase is normally found in a sufficient amount in milk; however, thiocyanate and hydrogen peroxide are the limiting factors and need to be added from exogenous source to activate the LP system. The enzyme lactoperoxidase catalyses the oxidation of thiocyanate by hydrogen peroxide, and generates the hypothiocyanite (OSCN⁻) ion, which has proven antibacterial activity (Reiter, 1985b).

Activity of the LP system has been shown to inhibit the growth of many bacterial species in cow milk (Pruitt & Reiter, 1985; Reiter, 1985b; Naidu, 2000). However, there is limited information on the effect of the LP system on pathogenic bacteria in goat milk. This study was therefore designed to determine the effect of the LP system on the growth and survival of *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Brucella melitensis* and *Mycobacterium paratuberculosis* in the milk of Saanen and South African Indigenous goats.

2.2. Materials and methods

2.2.1. Milk source and treatment of milk samples

Milk samples were obtained from two goat breeds viz., Saanen and South African Indigenous goats. The goats were fed on a commercial ration composed of 23.8 % yellow maize meal, 19.84 % malt dust, 4.96 % sunflower oil cake, 7.93 % yeast, 29.76 % eragrostis, 11.9 % molasses, 0.5 % mono calcium phosphate, 0.5 % salt, 0.5 % limestone powder, 0.3 % premix, and 0.02 % Romensin. The goats were milked by a milking machine following standard procedures. Milk samples from individual goats of each breed were pooled together and kept in a refrigerator at 4 °C until used. From the pooled milk samples of each goat breed, samples were taken for microbiological and chemical analyses. All the milk samples intended for microbiological analysis were heated in a thermostatic water bath at pasteurisation temperature of 63 °C for 30 minutes and cooled to 4 °C.

2.2.2. Maintenance of test organisms

The test organisms used were *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Brucella melitensis* and *Mycobacterium paratuberculosis*. *Staphylococcus aureus* ATCC 25923 and *L. monocytogenes* ATCC 19116 were obtained from the South African Institute of Medical Research. *Escherichia coli* was obtained from the Veterinary Faculty of the University of Pretoria, South Africa. A freeze dried culture of *Brucella melitensis* vaccine strain (Rev 1) and a wild strain, 903 A, of *M. paratuberculosis* isolated from sheep were obtained from the Bacteriology Division of

the Onderstepoort Veterinary Institute, South Africa. Stock cultures of *S. aureus* and *E. coli* were prepared by transferring a loopful from the original cultures onto nutrient agar slopes. The cultures were then incubated at 37 °C for 24 h and stored at 4 °C. Stock culture of *L. monocytogenes* was prepared by transferring a loopful from the original culture onto slants of tryptone soy agar supplemented with 0.6 % yeast extract after incubation at 37 °C for 24 h. The slant was maintained at 4 °C until used. Working cultures of *E. coli*, *S. aureus* and *L. monocytogenes* were obtained by transferring a loopful from the respective slants onto Blood Tryptose Agar (BTA) after incubation at 37 °C for 24 h. The *Br. melitensis* culture was reconstituted in sterile distilled water, grown on BTA plates and transferred onto slopes of Farrell's medium (Alton, Jones, Angus & Verger, 1988) and stored at 4 °C. Working cultures of *Br. melitensis* were obtained by transferring a loopful from the slants onto Farrell's medium after incubation at 37 °C for 5 days. *Mycobacterium paratuberculosis* culture was maintained on slants of Herrold's egg yolk medium at 4 °C.

2.2.3. Preparation of bacterial suspensions

The diluent used for the preparation of suspensions of *E. coli*, *S. aureus*, *L. monocytogenes* and *Br. melitensis* was a physiological saline solution. However, in the case of *M. paratuberculosis*, 10 % (w/w) Tween 80 saline solution was used. Five ml of physiological saline solution or Tween saline solution were measured into screw-capped test tubes and sterilized in an autoclave for 15 minutes at 121 °C. The suspensions of each pathogen were prepared by transferring fresh colonies grown on the surface of BTA plates (*E. coli*, *S. aureus* and *L. monocytogenes*), Farrell's medium (*Br. melitensis*) and Herrold's egg yolk medium (*M. paratuberculosis*) into sterile physiological saline or Tween saline solutions using sterile plastic loops. The respective suspensions were thoroughly mixed using a whirl mixer and adjusted by either adding more saline or more bacteria to the turbidity of McFarland Standard 1.0 (1 % BaCl₂.2H₂O; 1 % H₂SO₄) (Balows, Hausler, Herrman, Isenberg & Shadomy, 1991).

2.2.4. Inoculation of milk samples with pathogens

Milk samples were first brought to room temperature (≈ 25 °C) before inoculation with the specific pathogens. Four hundred ml of milk sample, which had been previously pasteurised at 63 °C for 30 minutes, was inoculated with 1 ml of the appropriate suspensions of each pathogen. After inoculation of the milk samples with *E. coli*, *S. aureus*, *L. monocytogenes* and *Br. melitensis*, 1 ml sample was drawn from each inoculated milk sample and transferred into 9 ml of sterile quarter strength Ringer's solution. Serial dilutions were made to determine the initial number of *E. coli*, *S. aureus*, *L. monocytogenes* and *Br. melitensis* in the milk samples. However, in the case of *M. paratuberculosis*, microscopic cell count using Breed's method (Harrigan, 1998) was used to determine the initial number of bacteria per ml. Each milk sample was aseptically divided into two lots, A and B, of 200 ml each. After a period of 1 h, to enable the bacteria to adapt, sample A was subjected to activation of the LP system whereas sample B was used as an untreated control.

2.2.5. Activation of the lactoperoxidase system

Prior to activation of the LP system, the natural thiocyanate (SCN^-) concentration in the milk samples was determined according to the International Dairy Federation (IDF, 1988). Sample A was subjected to the activation of the LP system by addition of sodium thiocyanate (Saarchem Pty Ltd., Midrand, South Africa) as a source of SCN^- to increase the SCN^- level to 14 mg/l. After 1 minute of thorough mixing, 30 mg/l sodium percarbonate (Sigma-Aldrich Chemical Co., Midrand, South Africa) was added as a source of hydrogen peroxide (H_2O_2) as recommended by the International Dairy Federation (IDF, 1988). The milk samples were then incubated at 30 °C for 6 h in a thermostatically controlled water bath. After 6 hours, samples were drawn from each milk sample to determine viable bacteria both in the LP-treated and the control milk samples and compared with the initial number of pathogens in the milk samples. The experiment was repeated seven times except for *E. coli* in Indigenous goat milk which was repeated six times.

2.2.6. Enumeration of pathogens

Escherichia coli was enumerated using 3M Petrifilm™ *E. coli* count plates (3M Microbiology Products, St. Paul, USA) after incubation at 32 °C for 24 h. *Staphylococcus aureus* was enumerated on Baird Parker Medium (Oxoid, Hampshire, England) after incubation at 37 °C for 48 h. *Listeria monocytogenes* was enumerated on Tryptone Soy Agar (Oxoid) supplemented with 0.6 % yeast extract after incubation at 37 °C for 48 h. *Brucella melitensis* was enumerated on Farrell's medium after incubation at 37 °C for 5 days. *Mycobacterium paratuberculosis* was enumerated on slopes of Herrold's egg yolk medium (HEYM) containing mycobactin J (Grant, O'Riordan, Ball & Rowe, 2001). Four slopes of HEYM were each inoculated with 125 µl of the suspension containing approximately 10⁸ cfu/ml of *M. paratuberculosis*. After even distribution of the suspension on the surface of HEYM, the slopes were incubated at 37 °C for 20 weeks. The growth of *M. paratuberculosis* on the slopes was periodically monitored.

2.2.7. Quantification and confirmation of viable bacteria after treatment

After the respective incubation periods, confirmatory tests for each pathogen except *M. paratuberculosis* were done. Colonies grown on Petrifilm™ *E. coli* count plates were confirmed by API E20 diagnostic kit (BioMérieux, Marcy-L'Etoile, France). *Listeria monocytogenes* colonies were confirmed by the API listeria kit (BioMérieux). *Staphylococcus aureus* colonies were confirmed by the staphylase test kit (Oxoid). *Brucella melitensis* colonies were confirmed by performing appropriate biochemical and cultural diagnostic tests (brucella typing) (Ribeiro & Herr, 1990). Suspected colonies were identified presumptively by Stamp's staining, urease test and hydrogen sulphide production and confirmed as *Br. melitensis* by phage and dye sensitivity procedures. Biovars were assigned by means of agglutination with monospecific anti-A and anti-M sera and growth in the presence of erythritol, penicillin, and streptomycin as described by Ribeiro and Herr (1990).

2.2.8. Thiocyanate content of the milk samples

Thiocyanate concentration in raw milk was determined spectrophotometrically as described by IDF (1988). Four ml of milk was mixed with 2 ml of 20 % (w/v)

trichloroacetic acid (Sigma-Aldrich Chemical Co.) solution. The mixture was blended well and then allowed to stand for 30 minutes. It was then filtered through Whatman No. 40 filter paper. The clear filtrate (1.5 ml) was then mixed with 1.5 ml of ferric nitrate reagent and the absorbance measured at 460 nm by Beckman DU 650 Spectrophotometer (Beckman Instruments Inc., Fullerton, USA). The thiocyanate concentrations were calculated from a standard curve. The analysis was repeated 10 times.

2.2.9. Lactoperoxidase activity of the milk samples

Lactoperoxidase activity of the milk samples was measured with one-step ABTS (2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulphonic acid)) (Pierce Chemical Co., Rockford, USA) solution as substrate. The assay mixture consisted of 1 ml of 0.1M phosphate buffer (pH 6.0), 5 μ l of milk sample and 1 ml of ABTS solution. Absorbance was measured at 412 nm as a function of time for 2 min at 10 second intervals using a Beckman DU 650 Spectrophotometer adjusted at 25 °C. The activity expressed in units/ml was calculated according to Kumar and Bhatia (1999). One enzyme unit was equivalent to that amount of enzyme catalysing the oxidation of 1 micromole of substrate (ABTS) per minute at 25 °C. The analysis was repeated 10 times.

2.2.10. Statistical analysis of the data

The mean initial and final colony counts of each pathogen in the control and the LP-treated milk samples were analysed and compared using the Wilcoxon-Mann-Whitney test (SAS, 1999). The mean initial colony counts of each pathogen were compared with the mean values in the control and the LP-treated milk samples after 6 h of incubation. Similarly, the mean colony count of each pathogen in the treated milk samples was compared with the mean values in the control milk samples at the end of the incubation period. Significant differences were calculated at 5 % significance level.

2.3. Results

2.3.1. Thiocyanate content and lactoperoxidase activity

The thiocyanate concentration and lactoperoxidase activity of both Saanen and Indigenous goat milks are given in Table 2.1. Indigenous goat milk had a higher thiocyanate content than Saanen goat milk. However, the lactoperoxidase activity of Saanen goat milk was higher than that of Indigenous goat milk.

Table 2.1. Thiocyanate concentration and lactoperoxidase activity in raw milk samples of Saanen and Indigenous goats (n = 10 ± SD)

Milk source	SCN ⁻ (ppm)	LP activity (U/ml)
Indigenous	4.58 ± 1.92	0.26 ± 0.10
Saanen	2.78 ± 1.21	0.79 ± 0.18

Where LP is lactoperoxidase; SCN⁻ is thiocyanate and SD is standard deviation.

2.3.2. Effect of the lactoperoxidase system on the growth of *Escherichia coli*

In Indigenous goat milk, the mean *E. coli* count increased both in the LP-treated and the control milk samples by 1.71 and 1.97 log units, respectively (Table 2.2) at the end of the incubation period. Even though *E. coli* continued to grow in the LP-activated milk samples, the final level of *E. coli* was 46 % less in the LP-activated milk samples as compared to the control. In Saanen goat milk, the mean *E. coli* count increased both in the LP-activated and the control milk samples by 0.22 and 1.07 log units, respectively after 6 h of incubation (Table 2.2). At the end of the incubation period, the mean *E. coli* count was 86 % less in the LP-activated Saanen goat milk samples as compared to its level in the control milk samples.

2.3.3. Effect of the lactoperoxidase system on the growth of *Staphylococcus aureus*

The mean *S. aureus* count increased both in the LP-treated and the control milk samples of Indigenous goats by 0.5 and 0.6 log units, respectively (Table 2.2) after 6 h of incubation. Activation of the LP system in the Indigenous goat milk reduced the rate of

growth of *S. aureus* by 18 % in the LP-activated milk samples as compared to its growth in the control milk samples. In Saanen goat milk, the mean *S. aureus* count decreased by 0.1 log units in the LP-treated milk and increased by 0.14 log units in the control milk at the end of the incubation period (Table 2.2). The level of *S. aureus* in the LP-activated Saanen goat milk after 6 h of incubation was 41 % lower than its level in the control milk.

2.3.4. Effect of the lactoperoxidase system on the growth of *Listeria monocytogenes*

In the Indigenous goat milk, the mean *L. monocytogenes* count decreased by 0.12 log units in the LP-treated milk samples compared to the initial count after 6 h of incubation (Table 2.2). However, in the control milk sample of Indigenous goats, the mean *L. monocytogenes* count increased by 0.92 log units after 6 h of incubation. The level of *L. monocytogenes* in LP-activated Indigenous goat milk samples after 6 h of incubation was 91 % less than its level in the control milk samples. In the Saanen goat milk, the mean *L. monocytogenes* count decreased by 0.24 log units in the LP-treated milk samples; however, it increased by 0.21 log units in the control milk samples after 6 h of incubation (Table 2.2). The final level of *L. monocytogenes* was 65 % lower in the LP-activated Saanen goat milk samples as compared to its level in the control.

2.3.5. Effect of the lactoperoxidase system on the growth of *Brucella melitensis*

The mean *Br. melitensis* count in Indigenous goat milk increased by 1.25 log units in the control milk samples but decreased by 0.1 log units in the LP-treated milk samples at the end of the incubation period (Table 2.2). The final *Br. melitensis* count in the LP-activated Indigenous goat milk was 95 % lower than its level in the control milk samples. Similarly, the mean *Br. melitensis* count in Saanen goat milk increased by 1.32 log units in the control milk samples and decreased by 0.36 log units in the LP-treated milk samples at the end of the incubation period (Table 2.2). Activation of the LP system in Saanen goat milk decreased the level of *Br. melitensis* by 98 % in the LP-activated milk samples as compared to the control.

Table 2.2. Growth (cfu/ml) of food-borne pathogens in lactoperoxidase activated and control milk samples of Saanen and South African Indigenous goats after 6 h incubation at 30 °C

Milk source	Pathogen ^a	Initial	LP-activated (6 h)	Control (6 h)	Percent ^b reduction
Indigenous	<i>Escherichia coli</i>	1.7 x 10 ^{6c}	8.7 x 10 ^{7d}	1.6 x 10 ^{8d}	46
	<i>Staphylococcus aureus</i>	1.6 x 10 ^{6c}	5.0 x 10 ^{6d}	6.1 x 10 ^{6d}	18
	<i>Listeria monocytogenes</i>	1.7 x 10 ^{6c}	1.3 x 10 ^{6c}	1.4 x 10 ^{7d}	91
	<i>Brucella melitensis</i>	6.2 x 10 ^{5c}	5.3 x 10 ^{5c}	1.1 x 10 ^{7d}	95
	<i>Mycobacterium paratuberculosis</i>	ND	ND	ND	
Saanen	<i>Escherichia coli</i>	1.1 x 10 ^{7c}	1.8 x 10 ^{7c}	1.3 x 10 ^{8d}	86
	<i>Staphylococcus aureus</i>	1.6 x 10 ^{7cd}	1.3 x 10 ^{7c}	2.2 x 10 ^{7d}	41
	<i>Listeria monocytogenes</i>	9.9 x 10 ^{6cd}	5.6 x 10 ^{6c}	1.6 x 10 ^{7d}	65
	<i>Brucella melitensis</i>	2.1 x 10 ^{6c}	9.1 x 10 ^{5c}	5.1 x 10 ^{7d}	98
	<i>Mycobacterium paratuberculosis</i>	ND	ND	ND	

Counts with different superscripts in the same row were significantly different ($p < 0.05$) from each other.

^aMean of 7 experiments except for *E. coli* in Indigenous goat milk which was 6.

^bPercent reduction in viable count was calculated as follows: 100 x
 (viable count in the control milk at 6 h – viable count in the LP-activated milk at 6 h) / (viable count in the control milk at 6 h).

Where: ND is growth not detected; LP is lactoperoxidase

2.3.6. Effect of the lactoperoxidase system on the growth of *Mycobacterium paratuberculosis*

The growth of *M. paratuberculosis* was monitored periodically over a period of 20 weeks. However, no growth of *M. paratuberculosis* was observed both in the LP-treated and the control milk samples of Saanen and Indigenous goats.

2.4. Discussion

Previous reports have indicated different levels of thiocyanate concentration and LP activity in goat milk. Zapico, Gaya, Nuñez and Medina (1990) reported LP activity of 1.55 units/ml and SCN⁻ content of 4.03 ppm in raw goat milk from Spain. The LP activity and SCN⁻ concentration of Creole goat milk were found to be 4.45 units/ml and 10.29 ppm, respectively (Saad de Schoos, Oliver & Fernandez, 1999). The SCN⁻ content of the Indigenous goat milk (Table 2.1) observed in this study is higher than the value reported by Zapico *et al.* (1990); however, it is lower than the value reported by Saad de Schoos *et al.* (1999) for Creole goat milk. On the other hand, the SCN⁻ content of Saanen goat milk (Table 2.1) observed in this experiment is lower than the values reported by Zapico *et al.* (1990) and Saad de Schoos *et al.* (1999). The SCN⁻ concentration in milk varies with breed, species and feeding regime of the animal (Kussendrager & van Hooijdonk, 2000). However, the goats used in this study were fed on the same ration during the experiment. The LP activity of both Saanen and Indigenous goat milks (Table 2.1) observed in this study is lower than the values reported by Zapico *et al.* (1990) and Saad de Schoos *et al.* (1999). The variations in LP activity and SCN⁻ concentration in Saanen and Indigenous goat milks might have been attributed to the inherent genetic difference between the goats. Zapico *et al.* (1991) reported significant differences in LP activity and SCN⁻ concentration in the milk of Verata and Murciano-Granadina goats. The SCN⁻ concentrations and LP activities of Saanen and South African Indigenous goat milks reported here are the first information on these two breeds and will thus contribute to the literature data base regarding LP system components in goat milk.

Testing for *Escherichia coli* as an indicator of faecal contamination and/or poor hygienic practices has been done traditionally in the dairy industry. It is now recognized that some

strains of *E. coli* may be enteropathogenic or enterotoxigenic. Both these groups have been responsible for outbreaks involving milk and cheese (Burgess *et al.*, 1994; Kuntz, T. B. & Kuntz, S. T., 1999; Allerberger, Wagner, Schweiger, Rammer, Resch, Dierich, Friedrich & Karch, 2001; McIntyre, Fung, Paccagnella, Isaac-Renton, Rockwell, Emerson & Preston, 2002). Activation of the LP system both in Saanen and Indigenous goat milks did not inhibit *E. coli* totally; however, it reduced the rate of growth of *E. coli* in the LP-activated milk samples as compared to the control. The lower inhibitory effect of the LP system against *E. coli* in the present study compared to other research might be associated to the higher incubation temperature (30 °C) employed. *Escherichia coli* is a mesophilic bacterium with an optimum growth temperature between 30-37 °C. At this higher temperature, *E. coli* might have been at its highest metabolic activity and thus the oxidation product of the LP system might not have been able to counteract the multiplication of *E. coli*. Farrag, El-Gazzar and Marth (1992b) reported that the inactivation of *E. coli* O157: H7 by the LP system in cow milk was inversely related to incubation temperature. The higher the incubation temperature, the lower the inhibitory effect of the LP system against *E. coli* and vice versa. The inhibitory effect of the LP system against *E. coli* in cow milk has also shown to be related to the initial number of bacteria used (Reiter *et al.*, 1976; Farrag *et al.*, 1992b). These researchers reported that the lower the initial level of inoculum used, the higher is the inhibitory effect. Thus, the lower inhibitory effect of the LP system against *E. coli* observed in the present study may also be attributed to the higher initial inoculum ($10^6 - 10^7$ cfu/ml) used.

Staphylococcus aureus is a major causative agent of bovine and caprine mastitis and poses a human health problem since this pathogen can be shed into milk from mastitic udders (Hunter, 1984; Kalogridou-Vassiliadou, 1991). *Staphylococcus aureus* may cause human illness by the production of heat resistant enterotoxins and could cause food poisoning in the absence of viable cells (Smith, Buchanan & Palumbo, 1983). Valle, Gomez-Lucia, Piriz, Goyache, Orden and Vadillo (1990) reported that 48.8 % of *S. aureus* strains isolated from goat milk were toxigenic. Dairy related outbreaks of staphylococcal intoxication have been attributed to raw milk, dried milk and cheese (Burgess *et al.*, 1994; De Buyser, Dufour, Maire & Lafarge, 2001; Carmo, Dias, Linardi,

Sena, Santos, Faria, Pena, Jett, Heneine, 2002; Leclerc, Dufour, Lombard, Gauchard, Garin-Bastuji, Salvat, Brisabois, Poumeyrol, De Buyser, Gnanou-Besse & Lahellec, 2002). The bactericidal effect of the LP system against *S. aureus* in cow milk has been reported by Kamau, Doores and Pruitt (1990a) and Kangumba, Venter and Coetzer (1997). In the present study, the LP system inhibited the growth of *S. aureus* in Saanen goat milk; however, it only reduced the rate of growth of *S. aureus* in the Indigenous goat milk. The inhibitory effect of the LP system against *S. aureus* in Saanen goat milk might be associated with the higher LP activity in Saanen goat milk (Table 2.1). It has been reported that goat milk LP alone inhibits bacterial growth (Jacob, Manoj & Haridas, 1998) even in the absence of a medium containing thiocyanate and H₂O₂. Pruitt and Kamau (1991) also reported that the variability of the bactericidal properties of milk is caused by variations of the quantities of peroxidases contained in different milk samples. It seems that *S. aureus* needs a high concentration of LP to be effectively inhibited by the LP system. Jacob, Antony, Sreekumar and Haridas (2000) reported that the minimum inhibitory concentration of purified goat milk lactoperoxidase against *S. aureus* in thiocyanate-H₂O₂ system was 182 µg/ml.

The levels of *E. coli* and *S. aureus* found in goat milk under normal conditions are low. Foschino, Invernizzi, Barucco and Stradiotto (2002) reported a mean *E. coli* level of 2.9 cfu/ml and a mean *S. aureus* count of 1.2×10^3 cfu/ml in raw goat milk. Since the antibacterial activity of the LP system is greater when the initial number of bacteria is low, the bacteriostatic effect of the LP system against *E. coli* and *S. aureus* observed at the inoculum level used in the present study (10^6 - 10^7 cfu/ml) could be of significant importance in controlling the multiplication of these organisms in raw goat milk under practical conditions.

Listeria monocytogenes is a pathogen of major concern to the dairy industry as food-borne listeriosis has been related to consumption of contaminated milk and milk products (Griffiths, 1989; Kozak, Balmer, Byrne & Fisher, 1996). Listeric infections can cause abortion, septicaemia and meningitis (Marth, 1988). Dairy related incidents of listeriosis have implicated raw milk, poorly pasteurised milk and cheese (Burgess, *et al.*, 1994; De

Buyser *et al.*, 2001; Rudolf & Scherer, 2001; Leclerc *et al.*, 2002). The risk of listeriosis is amplified by the ability of *L. monocytogenes* to grow at refrigeration temperatures and its relative heat resistance compared to other non-spore-forming bacteria (Doyle, Glass, Beery, Garcia, Pollard & Schultz, 1987). The LP system is both bactericidal and bacteriostatic against *L. monocytogenes* in cow milk (Siragusa & Johnson, 1989; El-Shenawy, Garcia and Marth, 1990; Gaya, Medina and Nuñez, 1991). The bactericidal effect of the LP system against *L. monocytogenes* at 30 °C both in Saanen and Indigenous goat milks observed in this study is in line with the findings of Kamau *et al.* (1990a). They reported that the LP system in bovine milk had a bactericidal effect against *L. monocytogenes* during the initial period at 35 °C. The inhibitory effect of the LP system against *L. monocytogenes* observed in the present study suggests that activation of the LP system may help in controlling the growth of *L. monocytogenes* during collection and transportation of goat milk at ambient temperatures, as is the case in many developing countries.

Activation of the LP system exhibited a bactericidal effect against *Br. melitensis* in both Saanen and Indigenous goat milks. To date, no work has been reported on the effect of the LP system on *Br. melitensis* either in cow or goat milk. However, Kangumba *et al.* (1997) studied the effect of the LP system on *Brucella abortus* but did not find any inhibitory effect by the LP system. *Brucella melitensis* is highly pathogenic to humans (Garin-Bastuji & Verger, 1994; Ryser, 1998) causing one of the most serious zoonoses in the world. Goats are the primary reservoir of *Br. melitensis* and consumption of raw goat milk and goat milk cheese has been reported to be the probable means of brucellosis infection (Ryser, 1998). Thus, the bactericidal effect of the LP system against *Br. melitensis* observed in this study could be of practical importance in controlling the proliferation of *Br. melitensis* in goat milk.

Mycobacterium paratuberculosis causes paratuberculosis or Johne's disease in cattle, sheep, goat and other ruminants (Forshell, 2001). Due to similarities between Johne's disease and Crohn's disease of humans, *M. paratuberculosis* has been implicated as an etiological agent in Crohn's disease (Hammer, 1999). Milk has been suggested as a

possible vehicle of transmission of the organism from animals to humans (Hammer, 1999). Due to its possible resistance to pasteurisation temperatures (Grant, Ball & Rowe, 1999), *M. paratuberculosis* has been a subject of major concern to the dairy industry. *Mycobacterium paratuberculosis* is a slow growing organism and it requires a specific growth factor (mycobactin J) to grow on common media for mycobacteria (Hammer, 1999). Primary culture requires incubation times up to 4 months or longer (Hammer, 1999). The culture of *M. paratuberculosis* from milk presents a number of difficulties due to the lack of an appropriate selective culture media and the organism's particularly slow growth rate in relation to other milk microorganisms (Grant & Rowe, 2001). A range of solid and liquid culture media are used for the enumeration of *M. paratuberculosis* in milk (Grant & Rowe, 2001): HEYM (solid), BACTEC radiometric medium (liquid), Dubos broth (liquid), modified Middlebrook agar (solid) and Lowenstein-Jensen medium (solid). Most of these methods were used for the isolation of *M. paratuberculosis* from faeces to aid the diagnosis of paratuberculosis, rather than from milk. To date there is no standardised method developed for the isolation and enumeration of *M. paratuberculosis* from milk and milk products (Grant & Rowe, 2001). The resistance of *M. paratuberculosis* to adverse physical or chemical factors depends on the medium in which it is suspended, the strain used and the method of quantifying *M. paratuberculosis* (Collins, Spahr & Murphy, 2001). The inability of *M. paratuberculosis* to grow both in the LP-treated and the control milk samples of Saanen and Indigenous goats suggest that the goat milk samples used in the experiment might have inhibited the growth of or were not suitable medium for the growth of the *M. paratuberculosis* strain used in the experiment. Further investigations may help to elucidate this.

In general, the pathogens studied in this experiment except *M. paratuberculosis* showed different degrees of sensitivity to the LP system in goat milk. Among the pathogens tested in this study, *S. aureus* was less inhibited by the LP system and *Br. melitensis* was more sensitive to the LP system. This could be associated to the inherent genetic difference in sensitivity to the LP system by the different bacteria. Korhonen (1980) reported that different groups of bacteria show a varying degree of resistance to the LP

system and depending on the bacterial species and strain of the same bacterium the effect can be bacteriostatic or bactericidal.

2.5. Conclusions

Activation of the LP system exhibited bactericidal effect against *L. monocytogenes* and *Br. melitensis* both in Saanen and Indigenous goat milks. The LP system showed bactericidal effect against *S. aureus* in Saanen goat milk; however, it was bacteriostatic against *S. aureus* in Indigenous goat milk. On the other hand, the LP system only reduced the rate of multiplication of *E. coli* both in Saanen and Indigenous goat milks. The same pathogen showed different degrees of sensitivity to the LP system in the milk of the two goat breeds indicating breed difference in the activity of the LP system in goat milk. The bactericidal effect of the LP system against *L. monocytogenes* and *Br. melitensis* both in Saanen and Indigenous goat milks at such high concentrations (10^6 - 10^7 cfu/ml) and at high temperature (30 °C) suggests that activation of the LP system in goat milk could be a promising alternative in controlling the growth of these pathogens especially during collection of milk at high ambient temperatures and in areas where milk cooling facilities are not available.

Acknowledgements

This study was sponsored by the Agricultural Research and Training Project of Alemaya University (Ethiopia). Prof. B. H. Bester is highly appreciated for his encouragement and valuable comments during the inception of the experiment. I also extend my thanks to Mr. Theo van der Schans for his technical assistance. The assistance of Dr. Frans Kanfer and Dr. Mike van der Linde during the statistical analysis of the data is highly appreciated. My thanks are also due to the Onderstepoort Teaching Animal Unit staff for assistance in collecting the goat milk. I am also grateful to Dr. Marijke M. Henton for providing me the *Br. melitensis* culture.

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CHAPTER 3

Effect of the Lactoperoxidase System on the Activity of Mesophilic Cheese Starter Cultures in Goat Milk

Published: International Dairy Journal

Key words: Mesophilic cheese starter cultures; Goat milk, Lactoperoxidase system.



Abstract

The effect of goat milk lactoperoxidase (LP) system on the activity of commercially available mesophilic cheese starter cultures was investigated. The growth and acid production of the starter cultures were measured at 2 h intervals for 8 h in goat milk kept at 30 °C. Most of the starter cultures examined were found to be sensitive to the LP system, but varied in their susceptibility to inhibition. The activity of the mixed starter cultures CHN11, CHN22, CHN19, DCC240 and Flora Danica Normal was strongly inhibited by the LP system. However, the mixed starter culture LL 50C showed resistance to the LP system. The single strain culture *Lactococcus lactis* subsp. *lactis* NCDO 605 was inhibited by the LP system. However, the cultures *Lactococcus lactis* subsp. *diacetylactis* NCDO 176 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313 were insensitive to the LP system. The results of this study indicate the need for routine screening of starter cultures for resistance to the LP system before using them for cheesemaking from goat milk preserved by the LP system.

3.1. Introduction

The use of starters in cheese manufacture is as old as cheesemaking (Pettersson, 1988). Lactic acid starters are mainly prepared cultures of mesophilic and/or thermophilic lactic acid bacteria intended to be used in the manufacture of cheese, cultured butter, fermented milks or fermented milk products that are used to initiate desirable changes (IDF, 1991a). The primary function of starter cultures in cheesemaking is to ferment the lactose of the milk to lactic acid, which aids in separation of curd from whey during cheese manufacture, modifies texture of cheeses and enhances preservation (Cox, Stanley & Lewis, 1978; IDF, 1980a). Starters also have other important functions in cheese manufacture such as flavour and aroma development and controlled proteolysis of cheese curd during the ripening process. Some starter cultures also contribute to the formation of gas, which is desirable in some varieties of cheese (Cox *et al.*, 1978; IDF, 1980a).

Different factors can inhibit the activity of starter cultures such as bacteriophages, presence of agglutinins in raw milk, the lactoperoxidase (LP) system, lysozyme, lactoferrin, antibiotics, bacteriocins and chemical sanitizers (Cox *et al.*, 1978; Lewis, 1987; Frank & Hassan, 1998). The LP system is the most significant microbial inhibitor in raw milk (Frank & Hassan, 1998). Lactoperoxidase is an enzyme that is naturally present in raw milk and together with thiocyanate and hydrogen peroxide constitutes the LP system. Lactoperoxidase is usually present in sufficient amount in milk; however, the level of thiocyanate is more variable in milk and depends on the feeding of the animal (FAO, 1999). The third component, hydrogen peroxide, is not normally detected in raw milk (Pruitt & Kamau, 1994; FAO, 1999). The enzyme lactoperoxidase catalyses the oxidation of thiocyanate by hydrogen peroxide and generates the hypothiocyanite (OSCN⁻) ion, which has proven antibacterial activity (Reiter, 1985b).

The LP system has been recommended for preservation of raw milk in areas where it is not possible to use mechanical refrigeration for technical and/or economic reasons (IDF, 1988; FAO, 1999). Due to the growing interest in the use of the LP system for preservation of raw milk, there is a possibility that LP-activated milk could be used for cheese manufacture. However, one of the concerns of the LP system for the manufacture

of fermented dairy products, such as cheese, is its inhibitory effect on starter cultures. Earlier reports (Sarkar & Misra, 1992) indicated that activation of the LP system resulted in a reduced rate of acid production in fermented milk, which may lead to an abnormal product or extend the production time significantly. Therefore it is necessary to identify starter cultures, which are resistant to the inhibitory effect of the LP system.

Goats are important milk producers in several parts of the tropics and significantly contribute to human nutrition in many developing countries (Devendra, 1999). Milk production from goats in South Africa has increased over the last two decades (Donkin, 1998) and the majority of goat milk producers in South Africa are small-scale farmers and they process the milk into various types of cheese. The potential of the LP system for preservation of raw goat milk has been reported (Haddadin, Ibrahim & Robinson, 1996).

Activation of the LP system in cow milk was found to inhibit the activity of thermophilic lactic starter cultures (de Valdez, Bibi & Bachmann, 1988; Basaga & Dik, 1994). The activity of individual starter cultures varies to a considerable extent with the type of milk used (Dutta *et al.*, 1971; Pettersson, 1988). Rysstad and Abrahamsen (1983) found variations in the biochemical performance of two mixed strain starters, Christian Hansen 01 (CH01) and Flora Danica Normal, grown in goat and cow milk. They attributed this difference to the variation in the composition of goat and cow milk and suggested the use of different starters for fermented dairy products made from goat milk. Similarly, the acidifying and proteolytic activities of a given strain of lactic starter culture were found to be different in cow and goat milk (Lodi, Brasca, Carcano & Sargalli, 1996). Thus, it can be expected that the effect of goat milk LP system on cheese starter cultures may be different from that of cow milk.

The objective of this experiment was therefore to study the effect of the LP system on the activity of commercial mesophilic cheese starter cultures and to screen for LP-resistant cultures which could be used for cheesemaking from goat milk preserved by activation of the LP system.

3.2. Materials and methods

3.2.1. Starter cultures

The cultures used in this study were CHN11, CHN22, DCC240, CHN19, Flora Danica Normal (FDN), LL 50C, *Lactococcus lactis* subsp. *diacetylactis* NCDO 176, *Lactococcus lactis* subsp. *lactis* NCDO 605 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313. The first five were mixed strain cultures which were obtained from the supplier (Chr. Hansen, Denmark) in a freeze-dried form. All these five cultures were composed of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. The mixed strain culture LL 50C was obtained from Anchor Biotechnologies (Johannesburg, South Africa) in a freeze-dried form and it was composed of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. The single strain cultures *L. lactis* subsp. *lactis* and *L. lactis* subsp. *diacetylactis* were obtained from Irene Animal Nutrition and Animal Products Institute, of the Agricultural Research Council (Irene, South Africa). *Leuconostoc mesenteroides* subsp. *cremoris* was obtained from the South African Institute of Medical Research. The cultures were activated by growing in 10 % (w/v) sterile reconstituted skim milk powder at 22 °C for 16 h before use.

3.2.2. Source of milk samples

Saanen goat milk samples were obtained from the Onderstepoort Teaching Animal Unit of the Faculty of Veterinary Science, University of Pretoria. The goats were fed on a commercial ration composed of 23.8 % yellow maize meal, 19.84 % malt dust, 4.96 % sunflower oil cake, 7.93 % yeast, 29.76 % eragrostis, 11.9 % molasses, 0.5 % mono calcium phosphate, 0.5 % salt, 0.5 % limestone powder, 0.3 % premix, and 0.02 % Romensin. The milk samples were chilled and delivered to the laboratory for analysis within 1 h of milking. The average composition of the milk samples was: 3.1 % fat, 3.1 % protein and 11.4 % total solids. The milk samples had a thiocyanate content ranging from 1.27 mg/l to 4.67 mg/l with an average value of 2.78 mg/l. The thiocyanate content of each milk sample was determined and then the LP system was activated by addition of sodium thiocyanate (see 3.2.5).

3.2.3. Treatment of the milk samples

One percent (v/v) of each starter culture was inoculated into 400 ml of Saanen goat milk that had been pasteurised at 63 °C for 30 minutes in a thermostatically controlled water bath. The initial concentration of the cultures in the milk was determined immediately after addition of the starter cultures. The inoculated milk was then divided into two equal portions: one was used for the activation of the LP system whereas the other was used as untreated control. Both the treated and control milk samples were incubated at 30 °C for 8 h. The experiment was repeated five times for the starter cultures CHN 11, CHN 22, DCC 240 and Flora Danica Normal, four times for the cultures CHN 19 and LL 50C and three times for the single strain starter cultures *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*.

3.2.4. Growth and activity tests of the starter cultures

The inhibitory effect of the LP system on the starter cultures was measured by determining the number of colony forming units (cfu/ml) and lactic acid production at 2 h intervals over the 8 h incubation period. Prior to activation of the LP system, the thiocyanate content of each milk sample was determined and then the LP system was activated by addition of sodium thiocyanate (Saarchem, Midrand, South Africa) as a source of thiocyanate to a final concentration of 14 mg/l, as recommended by the International Dairy Federation (IDF, 1988). After 1 min thorough mixing, 30 mg/l sodium percarbonate (Sigma Aldrich Chemical Co., Johannesburg, South Africa) was added as a source of hydrogen peroxide (IDF, 1988). Lactic acid production was determined by titration with 0.1 N NaOH and expressed as percent lactic acid (Bradley, Arnold, Barbano, Semerad, Smith & Vines, 1993). For viable counts, suitable dilutions, made in quarter strength Ringer's solution, were plated in duplicate by the pour plate method using MRS agar (de Man, Rogosa & Sharpe, 1960). Plates were incubated at 30 °C for 48 h. In the case of the culture LL 50C, only the activity test (titratable acidity) was conducted, and in the case of the culture *Leuc. mesenteroides* subsp. *cremoris*, only the growth of the culture was measured.

3.2.5. Determination of chemical composition

Thiocyanate concentration in raw milk was determined spectrophotometrically as described by IDF (1988). Four ml of raw milk was mixed with 2 ml of 20 % (w/v) trichloroacetic acid solution. The mixture was blended well and then allowed to stand for 30 minutes. It was then filtered through Whatman No. 40 filter paper. The clear filtrate (1.5 ml) was then mixed with 1.5 ml of ferric nitrate reagent and the absorbance measured at 460 nm. The thiocyanate concentrations were calculated from a standard curve. The fat content of the milk samples was determined by the Gerber method as described by Bradley *et al.* (1993). The protein content of the milk samples was determined after measuring the total nitrogen content of the milk by the Dumas method (IDF, 2000) using a Leco FP-528 Protein/Nitrogen Analyser (Leco Corporation, Michigan, USA) and multiplying the total nitrogen by the factor 6.38. The total solids content of the milk samples was determined after measuring the water content of the milk samples using the forced draft oven method (Bradley *et al.*, 1993) and subtracting the values from 100.

3.2.6. Statistical analysis of the data

The differences in the growth and activity of the starter cultures in the treated and control milk samples at each incubation period were analysed by the Wilcoxon-Mann-Whitney test of the Statistical Analysis System (SAS, 1999). The mean, maximum and minimum values were calculated and the significance of these differences was calculated at the 5 % significance level.

3.3. Results and discussion

3.3.1. Mixed strain starter cultures

Figure 3.1 indicates the growth and acid production of various mixed starter cultures in LP-activated Saanen goat milk. The growth (\log_{10} cfu/ml) of most of the mixed starter cultures in LP-activated milk samples was less than their growth in the untreated control milk samples, throughout the incubation period. However, significant differences ($p < 0.05$) in growth were observed between LP-activated and control milk samples for the

cultures CHN22 (Figure 3.1b) and DCC240 (Figure 3.1c) only, at 6 h and 8 h of incubation.

Similarly, the lactic acid production of the mixed starter cultures used in this experiment was also affected by activation of the LP system. A significant difference ($p < 0.05$) in the level of acid production between LP-activated and control milk samples was observed for the cultures CHN11 (Figure 3.1a), CHN22 (Figure 3.1b), DCC240 (Figure 3.1c), CHN19 (Figure 3.1d) and FDN (Figure 3.1e) at 6 h and 8 h of incubation. However, the level of acid production in LP-activated and control milk samples throughout the incubation period was not significantly different for the starter culture LL 50C (Figure 3.1f).

For all cultures no significant increase in acid production was observed in the control milk samples until 4 h of incubation. Scott, Robinson and Wilbey (1998) reported that the increase in titratable acidity by a starter culture in cow milk at 30 °C is expected to reach an average value of 0.45 % after 4 h of incubation although it depends on the type of starter culture used. However, the activity of individual starter cultures varies to a considerable extent with the type of milk used (Dutta *et al.*, 1971; Cárcoba, Delgado & Rodríguez, 2000), an effect which may be associated with the difference in the composition of milk from different species. Freshly drawn cow milk has a bacteriostatic property which lasts for 1 to 2 hours (FAO, 1999). The delay in acid production in the control milk samples observed in this study suggests that goat milk might have a strong bacteriostatic property which lasts longer after milking. However, this merits further investigation.

The difference in acid production and growth in LP-activated milk samples between the cultures CHN11, CHN22, DCC240, CHN19, and FDN at 8 h of incubation is indicated in Table 3.1. For all, apart from one culture, the level of acid production was lower in the LP-activated milk than in the control. However, the decrease in acid production in LP-activated milk at 8 h among the cultures was different (Table 3.1). The level of acid production by the LL 50C culture in the LP-activated milk at 8 h was higher than that of the control (Table 3.1). This indicates that the starter cultures examined in this

experiment showed varying degrees of sensitivity to the LP system. This is in agreement with an earlier report by Guirguis and Hickey (1987). They reported that thermophilic starter strains were found to be sensitive to the LP system inhibition in cow milk, but varied in their susceptibility to inhibition by the system.

Table 3.1. The difference in the level of acid production and growth between mixed and single strain mesophilic cheese starter cultures in lactoperoxidase activated Saanen goat milk at 8 h of incubation at 30 °C

Starter culture	Acid production (% lactic acid)		Growth (Log ₁₀ cfu/ml)	
	% reduction ^e	% increase ^e	% reduction	% increase
	CHN-11 ^a	30.7		3.7
CHN-22 ^a	50.8		6.0	
DCC 240 ^a	42.9		4.4	
CHN-19 ^b	30.4		4.0	
FDN ^a	31.2		3.8	
LL 50C ^b		9.7	nd ^d	
<i>L. lactis</i> ^c	23.9			0.8
<i>L. diacetylactis</i> ^c	11.5			1.6
<i>Leuc. mesenteroides</i> ^c	nd			1.1

^aMean of 5 experiments; ^bMean of 4 experiments; ^cMean of 3 experiments

^dnd = not determined

^ePercent reduction or increase in acid production was calculated as follows: 100 x (acid production in the control milk at 8 h - acid production in the lactoperoxidase activated milk at 8 h) / (acid production in the control milk at 8 h).

In mixed strain cheese starters, inhibition, insensitivity or stimulation of activity by the lactoperoxidase system depends on the ratio of sensitive, insensitive and stimulated strains or variants within a starter (IDF, 1991b). The experiment on single strain cultures showed varying degrees of sensitivity to the LP system among the single strain cultures

used. The cultures *L. lactis* subsp. *diacetylactis* (Figure 3.2b) and *Leuc. mesenteroides* subsp. *cremoris* (Figure 3.2c) were insensitive to the LP system whereas the culture *L. lactis* subsp. *lactis* (Figure 3.2a) was inhibited by the LP system. Thus, the difference in sensitivity of the various mixed starter cultures examined in this study to the LP system might have been attributed to the differences in the proportions of the various strains between the mixed cultures used. The balance of species and/or strains within a mixed culture may alter and reduce the capacity of the culture to produce acid (Scott *et al.*, 1998). The strains can be slow or fast acid producers; hence, a shift in the proportion of the different strains can affect the activity of the mixed culture.

The activity of the mixed starter culture LL 50C was not affected by the LP system. The resistance of some starter cultures to the LP system could be associated with the presence of a 'reversal factor' as reported by Oram and Reiter (1966) and Reiter (1985b). This 'reversal factor' is an enzyme, NADH:OSCN oxidoreductase, that catalyses the reduction of the inhibitor OSCN, by NADH, to the inert thiocyanate. The starter strains in LL 50C culture might be naturally resistant to the inhibitory effect of the LP system and may possess mechanisms that counteract the oxidation product produced by the LP system.

The inhibitory effect of the LP system on the acid production by mixed starter cultures was greater than that on growth, as measured by colony count. This might have been attributed to the difference in composition of the single strain starter cultures used in the mixed cultures. The single strain culture *Leuc. mesenteroides* subsp. *cremoris* (Figure 3.2c) was found to be insensitive to the LP system; however, it produces insufficient acid in milk. In contrast *L. lactis* subsp. *lactis* which is the major lactic acid producer among the lactic starter cultures used for cheesemaking (Scott *et al.*, 1998) was sensitive to the LP system (Figure 3.2a). Thus, if a mixed starter culture has a high proportion of the resistant culture *Leuc. mesenteroides* subsp. *cremoris* and low proportions of the sensitive species *L. lactis* subsp. *lactis*, it can be expected that the acid production by the mixed culture will be inhibited more than their growth by the LP system.

3.3.2. Single strain starter cultures

Acid production by *L. lactis* subsp. *lactis* (Figure 3.2a) in LP-activated milk was significantly ($p < 0.05$) lower than that in the control milk at 6 and 8 h of incubation. However, no significant differences in growth were observed between LP-activated and control milk samples for *L. lactis* subsp. *lactis* throughout the incubation period. This finding is consistent with that reported previously by Nakada, Dosako, Hirano, Oooka and Nakajima (1996). They found that the LP system suppressed the rate of acid production by, but not the rate of growth of, yoghurt cultures, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The greater inhibition of acid production than growth of *L. lactis* subsp. *lactis* by the LP system might have been attributed to inhibition of key metabolic enzymes by the LP system that are responsible for the production of lactic acid by the starter culture. It has been reported that the hypothiocyanite ion produced during activation of the LP system causes inhibition of specific glycolytic enzymes such as aldolase, hexokinase and glyceraldehydes-3-phosphate dehydrogenase (Condon, 1987).

Both the growth of, and acid production by the single strain culture *L. lactis* subsp. *diacetylactis* (Figure 3.2b) in LP-activated milk and in control milk did not differ significantly ($p > 0.05$) throughout the 8 h incubation period. The growth of *Leuconostoc mesenteroides* subsp. *cremoris* (Figure 3.2c) was not affected by the LP system. An attempt was made to measure the activity of *Leuc. mesenteroides* subsp. *cremoris*; however, it did not produce any measurable acidity in the milk samples during the 8 h incubation period. This trend is consistent with that of Frank and Hassan (1998) who reported that *Leuc. mesenteroides* does not produce sufficient acidity in milk to coagulate it.

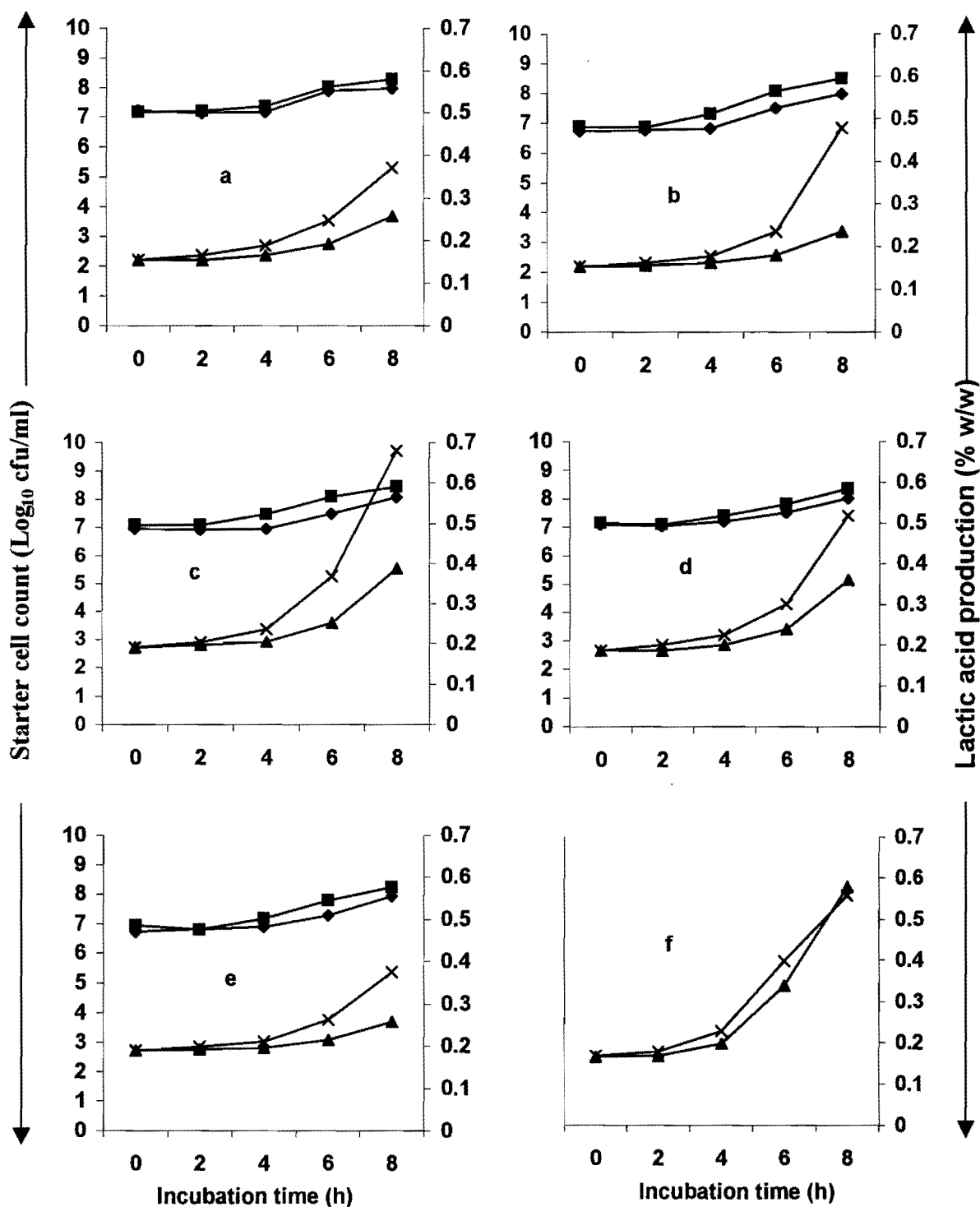


Figure 3.1. Starter cell growth (◆, ■) and acid production (▲, ✕) of mixed strain mesophilic cheese starter cultures CHN11 (a), CHN22 (b), DCC240 (c), CHN19 (d), FDN (e) and LL 50C (f) in lactoperoxidase (LP)-activated goats' milk (◆, ▲) and control milk (■, ✕) during 8 h of incubation at 30°C. ◆, Growth in LP activated milk; ■, Growth in control milk; ▲, Acid production in LP activated milk; ✕, Acid production in control milk. (n = 5 for the starter cultures CHN11, CHN22, DCC240 and FDN; n = 4 for the starter cultures CHN19 and LL 50C).

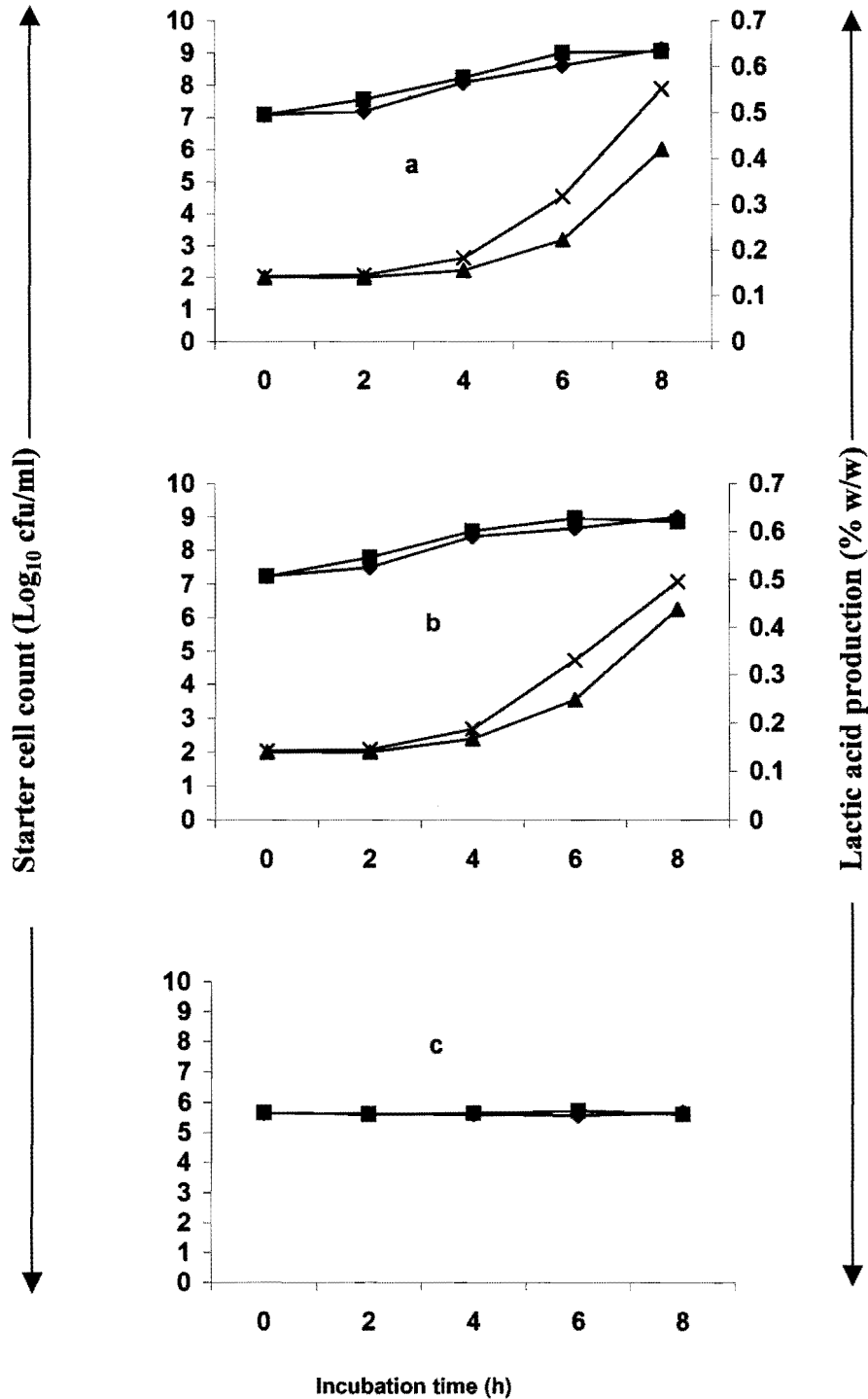


Figure 3.2. Starter cell growth (♦, ■) and acid production (▲, ✕) of single strain mesophilic cheese starter cultures *Lactococcus lactis* subsp. *lactis* (a), *Lactococcus lactis* subsp. *diacetylactis* (b) and *Leuconostoc mesenteroides* subsp. *cremoris* (c) in lactoperoxidase (LP)-activated goats' milk (♦, ▲) and control milk (■, ✕) during 8 h of incubation at 30°C. ♦, Growth in LP activated milk; ■, Growth in control milk; ▲, Acid production in LP activated milk; ✕, Acid production in control milk. (n = 3).

The difference in acid production between the single strain starter cultures *L. lactis* subsp. *lactis* and *L. lactis* subsp. *diacetylactis* at 8 h in LP-activated milk is indicated in Table 3.1. The percentage reduction in acid production by both cultures in LP-activated milk was lower than the values for mixed starter cultures (Table 3.1). In contrast to the trend for mixed starter cultures, the growth of the single strain starter cultures *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis* and *Leuc. mesenteroides* subsp. *cremoris*, in LP-activated milk at 8 h of incubation was higher than that in the control milk (Table 3.1).

Although the single strain starter cultures exhibited less sensitivity to the LP system compared to mixed strain cultures, differences in sensitivity to the LP system were observed among the single strain starter cultures. The growth and activity of mesophilic lactic starter cultures depends on their genetic properties among other factors (Pettersson, 1988). The variation in sensitivity between the single strain cultures to the LP system may be associated with differences in their genetic makeup. Roginski, Broome, Hungerford and Hickey (1984) reported that inhibition of starter cultures by the LP system is strain dependent. They showed that the growth of, and acid production by, *Lactococcus lactis* subsp. *cremoris* C1 were stimulated by the LP system in cow milk whereas the growth of *Lactococcus lactis* subsp. *cremoris* BK5 and *Lactococcus lactis* subsp. *lactis* C10 were inhibited by the LP system.

3.4. Conclusions

Most of the starter cultures examined were found to be sensitive to the LP system but varied in their susceptibility to inhibition by the system. Activation of the LP system resulted in greater inhibition of acid production than growth of the starter cultures. Of the mixed starter cultures evaluated in this study, only one culture (LL 50C) was found to be insensitive to the LP system and thus could be used for cheesemaking from goat milk preserved by the LP system. The results of this study indicate the need for routine selection and screening of starter cultures for their sensitivity to the LP system before using them for cheesemaking from goat milk preserved by the LP system.

Acknowledgements

This study was sponsored by the Agricultural Research and Training Project of Alemaya University (Ethiopia). Prof. B. H. Bester is highly appreciated for his encouragement and valuable comments during the inception of the experiment. The assistance of Dr. F. Kanfer and Dr. Mike van der Linde during the statistical analysis of the data is highly appreciated. Mr. Dirkie Weich of Anchor Bio-technologies is gratefully acknowledged for offering me the culture LL 50C. Thanks are also due to the Onderstepoort Teaching Animal Unit staff for their assistance in collecting the goat milk samples.

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CHAPTER 4

Quality Aspects of Gouda Cheese Made from Goat Milk Preserved by the Lactoperoxidase System

Submitted to the International Dairy Journal

Key words: Lactoperoxidase system; Gouda cheese; Goat milk.

Abstract

Gouda cheese was made from Saanen goat milk preserved by the lactoperoxidase (LP) system and the effect of the LP system on the biochemical, microbiological and sensory properties of Gouda cheese over a ripening period of 90 days was investigated. Cheese made from LP-activated Saanen goat milk had a significantly ($p < 0.05$) lower coliform and coagulase positive staphylococci as compared to cheese made from the untreated control milk. The LP-treatment did not affect the gross chemical composition of Gouda cheese made from Saanen goat milk. The level of proteolysis both in the control and the LP-treated Saanen goat milk cheeses was comparable. However, the level of lipolysis in Gouda cheese made from LP-activated Saanen goat milk was significantly ($p < 0.05$) lower than that made from the untreated control milk at the end of the ripening period. The lower lipolytic activity of cheese made from LP-activated Saanen goat milk suggests that LP-treatment of goat cheese milk might be of importance in reducing the strong flavour associated with goat milk cheeses. Significant differences ($p < 0.05$) in sensory attributes were observed between cheeses made from the untreated control and the LP-activated Saanen goat milk. Gouda cheese made from the LP-treated Saanen goat milk had a mild flavour as compared to the control. Thus, it can be concluded that preservation of Saanen goat milk by activation of the lactoperoxidase system can be used to improve the microbiological quality and flavour of Gouda cheese without any detrimental effect on the gross chemical composition of the cheese if an appropriate starter culture is used.

4.1. Introduction

Goats are important milk producers in several parts of the tropics and significantly contribute to human nutrition in many developing countries (Devendra, 1999). The production of cheese from goat milk has a very long history and is an important source of protein for people in several countries (Kalantzopoulos, 1993). Goat milk cheese is generally made in small artisanal units by a traditional technology (Lodi, Brasca, Carcano & Sangalli, 1996; Klinger & Rosenthal, 1997) and has a special taste and flavour, very different from that of cow milk cheese (Kalantzopoulos, 1993). Raw goat milk cheese represents a significant proportion of ripened cheeses in most Mediterranean countries (Trujiillo, Buffa, Casals, Fernández & Guamis, 2002). Cheeses made under these conditions may not have the minimum hygiene and sanitary guarantees necessary to obtain constant product quality (Emaldi, 1996). Most reports of processing of goat milk do not include pasteurisation (Loewenstein, Speck, Barnhart & Frank, 1980; Emaldi, 1996; Klinger & Rosenthal, 1997). The consumption of cheese made from unpasteurised goat milk has been identified as the cause of epidemics of brucellosis (Loewenstein *et al.*, 1980; Thapar & Young, 1986; Wallach, Miguel, Baldi, Guarnera, Goldbaum & Fossati, 1994), listeriosis (De Buyser, Dufour, Maire & Lafarge, 2001) and food poisoning due to enterotoxin production by staphylococci (De Buyser, Dilasser, Hummel & Bergdoll, 1987; Parente & Mazzatura, 1991; De Buyser *et al.*, 2001).

Milk production from goats in South Africa has increased over the last two decades and projects aimed at promoting goat milk production by householders and small-scale farmers are underway (Donkin, 1998). In South Africa, goat milk is produced by several small-scale milk producers and processed into various types of cheeses (USAID & ARC, 1998a). Gouda is one of the various types of cheeses made from goat milk by these producers. The high pH of Gouda cheese and the absence of enough lactic acid to suppress spoilage bacteria emphasize the importance of using milk of good bacteriological quality for making this cheese (Chapman & Sharpe, 1990).

Cheese milk is invariably subjected to a number of pre-treatments such as chilling and cold storage, thermization, bactofugation and pasteurisation prior to cheesemaking to

ensure the microbiological safety of cheese. Some of the pre-treatments are routinely practised during handling of milk at the production centre, whereas others are carried out in the cheese factory. The pre-treatments have a profound effect on the cheese manufacturing schedule, cheesemaking efficiency, physico-chemical, microbiological, and organoleptic characteristics of cheese as well as its shelf-life. These treatments of cheese milk may sometimes have a negative effect on cheese. Heat treatment of milk for instance can cause undesirable effects such as denaturation of serum proteins, which may lead to slow renneting, a weak curd, poor syneresis (Walstra, Noomen & Geurts, 1993), and development of a bitter flavour (Stadhouders & Hup, 1975; Walstra *et al.*, 1993). Cheeses made from pasteurised milk ripen more slowly and develop a less intense flavour than raw milk cheeses (Johnson, Nelson & Johnson, 1990; Grappin & Beuvier, 1997; Fox & McSweeney, 1998) due to the destruction of some desirable non-starter lactic acid bacteria present in milk.

The natural antibacterial system found in milk, the lactoperoxidase (LP) system, has been extensively used for preservation of raw cow milk especially in areas where it is not possible to use mechanical refrigeration for technical and/or economic reasons (IDF, 1988; FAO, 1999). The potential of the LP system to improve the safety of raw milk cheeses has also been proposed (Johnson *et al.*, 1990; Chapman & Sharpe, 1990). The possibility of making acceptable varieties of cheese from cow (Zall, Chen & Dzurec, 1983; Lara, Mendoza, de la Cruz & Garcia, 1987), sheep (Uceda, Guillen, Gaya, Medina & Nuñez, 1994; Santos, López-Díaz, García-Fernández, García-López & Otero, 1995) and buffalo (Abdou, Dawood, El-Hady & El-Nagar, 1996) milks preserved by the LP system has been reported. Activation of the LP system in Saanen and South African Indigenous goat milks showed bactericidal effect against *Listeria monocytogenes* and *Brucella melitensis* (see Chapter 2). However, to my knowledge no work has been reported on the cheesemaking properties of LP-treated goat milk. The composition of goat milk especially the various casein fractions are different from cow milk (Chandan, Attaie & Sahani, 1992; Park, 2001) and thus the cheesemaking characteristics of goat milk may also be different from cow milk. The objective of this study was therefore to assess the suitability of Saanen goat milk preserved by the LP system for the manufacture

of Gouda cheese and determine its effect on the biochemical, microbiological and organoleptic properties of Gouda cheese during the ripening period.

4.2. Materials and methods

4.2.1. Source and treatment of milk samples

Raw milk samples for the cheesemaking experiment were obtained from a herd of Saanen goats from the Faculty of Veterinary Science, University of Pretoria. The goats were milked by a milking machine according to standard procedures. Two ten-litre batches of milk samples were aseptically collected into sterile stainless steel milk cans. Both milk samples were delivered to the pilot plant of the Department of Food Science under cold storage within 1 h of milking. Prior to activation of the LP system, the thiocyanate content of the raw goat milk sample was measured (IDF, 1988). Based on the natural thiocyanate content of the raw milk, the LP system was activated in one of the batches by addition of sodium thiocyanate (Saarchem, Midrand, South Africa) to a final concentration of 14 mg/l as recommended by the International Dairy Federation (IDF, 1988). After thorough mixing of the milk sample, 30 mg/l sodium percarbonate (Sigma Aldrich, Chemical Co., Johannesburg, South Africa) was added as a source of hydrogen peroxide (IDF, 1988). Then the two milk samples were kept at room temperature (ca. 25 °C) for 2 h.

4.2.2. Cheesemaking

Gouda cheese was manufactured from Saanen goat milk in the pilot plant of the Department of Food Science, University of Pretoria, using the method described by Scott *et al.* (1998). Gouda cheese was made from the LP-activated milk (after 2 h of activation) and the untreated (control) milk in the same way. After heating the milk samples to 30 °C, 5 ml of (40 % (w/v)) food grade calcium chloride (Merck Laboratory Supplies Pty Ltd., Midrand, South Africa), 1 ml of diluted (1:10) annatto (Darleon, Johannesburg, South Africa) and 1 % (v/v) of starter culture (LL 50C) (Anchor Biotechnologies, Johannesburg, South Africa) were added to each of the milk samples. After 15 minutes, 0.6 ml of rennet (diluted 1: 10) (Darleon) was added to each milk sample. After addition

of the rennet, both milk samples were thoroughly stirred for five minutes and left undisturbed to coagulate. After 45-60 min at 30 °C, the coagulated milk was cut and after 15 min resting of the curd, the temperature of the curd-whey mixture was gradually increased from 30 °C to 36 °C (cooking temperature) over a period of 30-45 minutes. On reaching the cooking temperature, part of the whey (10 % of the volume of the milk) was removed and replaced by an equal volume of water of the same temperature. The curd and whey mixture was held for 1-1½ h at the cooking temperature. The whey was then drained, the curd put into rectangular wooden moulds and pressed for 1½ h. The next morning, the green cheese was cut into six equal small blocks (≈ 167 g) and put in a 20 % (w/w) brine solution (pH 5.0) at 15 °C for 4 h. After draining off the brine and drying the cheese blocks, each cheese block was vacuum packed in polyethylene plastic bags and put in a cheese curing room for three months at a temperature of 10 °C and a relative humidity of 85 %. The cheesemaking experiment was repeated four times. Samples for analyses were randomly taken from each batch at 1, 30, 60 and 90 days of ripening. A pilot study was conducted on South African Indigenous goat milk and because of limited supply of milk, only one batch of Gouda cheese was manufactured from the Indigenous goat milk in the same way as mentioned above for Saanen goat milk. Only the chemical composition of the Indigenous goat milk and cheese (one-day-old) was determined.

4.2.3. Sampling of cheese

From the six blocks of cheese of each batch, a block of cheese was randomly taken for analysis at each analysis time. The whole cheese block was aseptically grated by using a sterile cheese grater. From the grated cheese, samples for microbiological analysis were aseptically transferred into sterile universal bottles. The remaining samples were used for chemical analysis. Both chemical and microbiological tests were done on a monthly basis for three months. All tests were done in duplicate except for the pH measurements.

4.2.4. Rennet clotting time

The rennet clotting time of the milk samples was determined by modification of the method described by Björck (1978). Two hundred ml of raw Saanen goat milk sample was placed in a 400 ml beaker and LP-activated as stated above. After 30 minutes of LP

activation, the beaker with LP-activated milk sample was placed on a tripod and heated to 40 °C. A siphon containing a rubber tube was immersed into the beaker. After addition of 1 ml diluted (1: 9) rennet into the milk, the milk was allowed to drip slowly onto a glass slide which had been mounted at an angle for early observation of coagulation. The time from addition of the rennet to the first sign of coagulation of the milk was recorded. The coagulation time of a sample of untreated milk was used as the control.

4.2.5. Gross chemical composition

The pH of the cheese samples was measured with a penetration electrode (Sentron Integrated Sensor Technology, Sentron Inc., USA). Percent fat of the milk samples was determined by the Gerber method as described by Bradley, Arnold, Barbano, Semerad, Smith and Vines (1993) and the percent fat in cheese was determined by the method described by the British Standards Institution (BSI, 1969) using milk butyrometers. The salt content of the cheese samples was determined by the Volhard method as described by Bradley *et al.* (1993). The water content of the milk and the moisture content of the cheese samples were determined by drying the samples in a forced draft oven at 100 ± 2 °C for 3 h and 16.5 ± 0.5 h (Bradley *et al.*, 1993), respectively. The total solids content of the milk samples was determined by difference (100-percent moisture). The solids-not-fat content of the milk samples was calculated by subtracting the percent fat from percent total solids of the milk. Cheese yield after pressing was measured by weighing the cheese blocks on an analytical balance and expressed as kg dry matter per 100 litre of milk.

4.2.6. Measurement of nitrogen fractions

4.2.6.1. Total nitrogen (TN)

The total nitrogen content of milk and cheese samples ($n = 4$) was determined by measurement of the nitrogen converted into gas by combustion according to the Dumas principle (IDF, 2000) using a Leco FP-528 Nitrogen/Protein Analyser (Leco Corporation, Michigan, USA). The percent protein content of the milk and cheese samples was determined by multiplying the TN by the factor 6.38. Degree of proteolysis in the cheese samples was monitored by measuring the water soluble nitrogen, trichloroacetic acid soluble nitrogen and phosphotungstic acid soluble nitrogen at 30, 60 and 90 days of

ripening. The percentages of these nitrogen fractions over the TN were used as indices of proteolysis.

4.2.6.2. Water soluble nitrogen (WSN)

The WSN of the cheese samples ($n = 4$) was determined by the method described by Kuchroo and Fox (1982). Twenty grams of grated cheese was mixed with 40 ml of distilled water and homogenized by using a Stomacher (Lab-Blender 400, Seward Ltd, UK) at ≈ 20 °C for 10 min. The homogenate was held for 1 h at 40 °C in a water bath. The samples were then centrifuged using Medifriger-BL centrifuge (J.P. Selecta S.A., Barcelona, Spain) at 3000 g for 30 min at 20 °C and then cooled to 4 °C. The suspension was finally filtered through Whatman No. 40 filter paper (Merck). The nitrogen content of the filtrate was determined in duplicate by the Dumas method (IDF, 2000) using a Leco Nitrogen Analyser.

4.2.6.3. Trichloroacetic acid (12 %) soluble nitrogen (TCASN)

The TCASN ($n = 4$) was determined by the method described by Bütikofer, Rüegg and Ardö, (1993). Ten ml of WSN extract and 10 ml of 24 % (w/v) trichloroacetic acid (Sigma-Aldrich Chemical Co., South Africa) were mixed and the suspension was held at room temperature for 2 h and then filtered through Whatman No. 40 filter paper (Merck). The nitrogen content of the filtrate was determined in duplicate by the Dumas method (IDF, 2000) using a Leco Nitrogen Analyser.

4.2.6.4. Phosphotungstic acid soluble (5 %) nitrogen (PTASN)

The PTASN ($n = 4$) was determined according to the method described by Bütikofer *et al.* (1993). Ten ml of WSN was added to 7 ml of 3.95 mol/L sulphuric acid solution and 3 ml of 33 % (w/v) phosphotungstic acid (Sigma-Aldrich Chemical Co., South Africa) solution. The mixture was equilibrated overnight at 4 °C and then filtered through Whatman No. 40 filter paper (Merck). The nitrogen content of the filtrate was determined in duplicate by the Dumas method (IDF, 2000) using a Leco nitrogen analyser.

4.2.7. Free fatty acid (FFA) analysis

The total FFA content of the cheese samples ($n = 4$) was determined by the method described by Nuñez, García-Aser, Rodríguez-Martin, Medina and Gaya (1986). Ten grams of grated cheese sample was macerated with 6 g anhydrous sodium sulphate in a mortar and transferred with 60 ml diethyl ether into a 100 ml screw capped bottle. The homogenate was stirred for 1 h, with Ultra Turrax T25 (IKA-Labortechnik, Germany) for 30 s at 15 min intervals, decanted and the supernatant filtered through Whatman No. 1 filter paper (Merck). The precipitate in the bottle was resuspended in three successive 20 ml portions of diethyl ether, decanted and filtered. The total solvent was titrated with 0.1 N ethanolic potassium hydroxide (KOH) solution to the phenolphthalein end point. After titration, the solvent was evaporated in a vacuum oven at 30 °C for 24 h and fat was weighed. Free fatty acid in cheese was expressed as milliequivalents per 100 g fat. All analyses were done in duplicate.

4.2.8. Microbiological analyses

Tenfold serial dilutions of cheese samples ($n = 4$) were made by aseptically transferring 11 g sample of cheese into 99 ml of sterile 2 % (w/w) trisodium citrate solution which was heated to 40 °C to give a 10^{-1} dilution (White, Bishop & Morgan, 1993). Samples were then homogenized for 2 minutes using a Stomacher 400 laboratory blender. Further 10-fold dilutions of up to 10^{-8} were made by transferring 11 ml of successive serial dilutions into universal bottles containing 99 ml of sterile quarter-strength Ringer's solution. Aerobic plate count (APC) in milk and cheese samples was determined using plate count agar (Merck). The inoculated plates were incubated at 30 °C for 48 h. Coliforms in milk and cheese samples were determined using Violet Red Bile Mug agar (Merck) after incubation at 30 °C for 24 h. Lactic acid bacteria (LAB) in cheese samples were determined using MRS agar (de Man, Rogosa & Sharpe, 1960) after incubation at 30 °C for 48 h. Coagulase positive staphylococci (CPS) in cheese samples were enumerated on Baird Parker Medium (Oxoid, Hampshire, England) after incubation at 37 °C for 48 h. Mould counts in cheese samples were evaluated using Chloramphenicol agar (Merck) after incubation at 25 °C for five days.

4.2.9. Sensory analysis

Sensory difference tests were performed at the end of the ripening period (90 days) using the triangle test. Each of the four batches of cheese was judged by thirty untrained panellists. The total number of panellists participated in the sensory sessions was 120. The panellists were given three coded samples, two of which were similar and one different and asked to indicate the sample which was different from the other two samples. They assessed the overall sensory attribute (taste, odour and appearance) of the samples. The cheese samples were grated and presented in aluminium foil cups at room temperature. The sensory session was conducted in an air-conditioned room under white light in separated booths. Correct responses were counted and significant differences were determined by consulting probability tables for the triangle test (Roessler, Pangborn, Sidel & Stone, 1978).

4.2.10. Statistical analysis

The data for chemical composition and microbiological count were analysed by the Wilcoxon-Mann-Whitney test (SAS, 1999). The mean values of each variable of cheese made from goat milk preserved by the LP system were compared with the respective mean values of the control cheese at each analysis time. Significant differences were calculated at a 5 % significance level.

4.3. Results and discussion

4.3.1. The composition of Saanen and South African Indigenous goat milk

Table 4.1 indicates the composition of Saanen and South African Indigenous goat milk used for the cheesemaking experiment. The fat, protein, total solids (TS), solids-not-fat (SNF) contents of South African Indigenous goat milk were higher than the corresponding values for Saanen goat milk (Table 4.1). These differences in composition between Saanen and South African Indigenous goat milk have been reflected in Gouda cheese made from these two types of milk. Gouda cheese made from South African Indigenous goat milk had higher fat, fat-in-dry-matter and lower moisture and protein contents (Table 4.3) than that made from Saanen goat milk (Table 4.4). The fat and

protein contents of Saanen and South African Indigenous goat milk observed in this study (Table 4.1) are consistent with the work of Habteyohannes (2001). He reported a mean fat content of 3.4 % and a mean protein content of 2.75 % for Saanen goat milk and a mean fat content of 4.96 % and a mean protein content of 4.2 % for South African Indigenous goat milk. The average composition of Gouda cheese made from Saanen goat milk observed in this study (Table 4.4) is similar to the composition of Gouda cheese made from cow milk in South Africa (Smit, Schönfeldt, de Beer and Smith, 2001). Smit *et al.* (2001) reported that Gouda cheese made from cow milk in South Africa had a protein content ranging from 22.65-25.59 % with a mean value of 23.93 %, a fat content ranging from 30.48-32.92 % with a mean value of 31.40 % and a moisture content ranging from 39.81-42.31 % with a mean value of 40.55 %.

The most interesting difference observed between Gouda cheeses made from South African Indigenous goat milk and that made from Saanen goat milk was with respect to yield of cheese. The yield of Gouda cheese made from South African Indigenous goat milk (19.6 kg per 100 litre of milk) was higher than the yield of Gouda cheese made from Saanen goat milk (10.1 kg per 100 litre of milk). This could be of significant importance for small-scale cheese producers. Moreover, during the cheesemaking process, South African Indigenous goat milk took a shorter time (40 min) to coagulate as compared to Saanen goat milk (60 min) and resulted in a firmer curd during cutting. This could be associated with the higher TS (17.58 %), SNF (12.66 %), fat (4.92 %) and protein (4.22 %) contents in the South African Indigenous goat milk. Clark and Sherbon (2000) reported that goat milk containing high TS, SNF, fat and protein coagulates quickly and forms firmer curd than milk containing low levels of these components. Although it is not possible to make statistical comparisons and draw conclusions from these results due to the limited number of data in the case of South African Indigenous goat milk cheese, it can be noted that these differences in composition between the two milk types can have a profound effect in the cheesemaking process and have implications for those who are planning to or engaged in goat milk cheesemaking in South Africa.

Table 4.1. Chemical composition of Saanen and South African Indigenous goat milk used for the cheesemaking experiment

Parameter	Saanen goat milk ^a	South African Indigenous goat milk ^b
Fat (%)	3.83 ± 0.23	4.92
Protein (%)	2.73 ± 0.18	4.22
TS (%)	11.97 ± 0.08	17.58
SNF (%)	8.15 ± 0.09	12.66

^aMean ± standard deviation of four trials; ^bMean of duplicate samples.

SNF = Solids not fat; TS = Total solids.

4.3.2. Effect of the lactoperoxidase system on the clotting time and microbiological properties of the Saanen goat milk used for cheesemaking.

The effect of the LP system on the microbiological quality and clotting time of the Saanen goat milk used for the cheesemaking experiment is presented in Table 4.2. The APC and coliform counts of LP-activated Saanen goat milk were significantly ($p < 0.05$) lower than their respective values in the control milk. After 2 h of LP activation, the APC and coliform counts decreased by 1.12 log units and by 1.14 log units, respectively in the LP-treated milk samples when compared to their respective values in the untreated control milk (Table 4.2). This is in agreement with the findings of Girgis, Ismail, El-Dieb and Zaky (2001) who reported that activation of the LP system in both cow and buffalo milks resulted in a significant decrease in coliform and total bacterial counts during storage of the milks prior to cheesemaking. This may be of significant importance for small-scale cheese producers who in most cases are obliged to collect their goat milk over a few days to get a sufficient amount for cheesemaking.

The presence of thiocyanate (SCN^-) ion in milk inhibits rennet-casein interaction since SCN^- binds to cationic regions of casein resulting in a longer coagulation time of milk with rennet (Bringe & Kinsella, 1986). No significant differences in clotting time were observed between the LP-activated and the untreated control Saanen goat milks (Table 4.2). During the cheesemaking experiment, the time for setting the curd following

renneting for both the LP-treated and control milks was also similar. This agrees with the absence of significant differences in clotting time between the LP-treated and the control milks.

Table 4.2. Effect of the lactoperoxidase system on clotting time, coliform and aerobic plate counts (Log_{10} cfu/ml) of Saanen goat milk used for the cheesemaking experiment ($n = 4 \pm \text{SD}$)

Parameter	Lactoperoxidase treated	Control
Clotting time (seconds)	108 ± 16^a	97 ± 11^a
Aerobic plate count	4.86 ± 0.04^a	5.98 ± 0.16^b
Coliform count	1.37 ± 0.52^a	2.51 ± 0.68^b

Superscripts in the same row with different letters were significantly different ($p < 0.05$)
SD = Standard deviation.

4.3.3. Effect of the lactoperoxidase system on the microflora of Gouda cheese.

The results of the microbiological count data of Gouda cheese made from Saanen goat milk over the ripening period of 90 days are illustrated in Figure 4.1. The APC (Figure 4.1a) and LAB count (Figure 4.1b) of cheese made from LP- activated Saanen goat milk (LP cheese) and the control cheese slightly decreased over the ripening period. The APC in the control and the LP cheeses were comparable. The LAB counts in the control and the LP cheeses were also comparable. The absence of significant differences in the APC and LAB counts between the LP cheese and the control cheese might be associated with the use of the LP-resistant starter culture LL 50C (see Chapter 3). This finding is consistent with the report of Atamer, Koçak, Çimer, Odabasi, Tamuçay and Yamaner (1999) who found no significant differences in APC in Kaşer cheese made from cow milk preserved by activation of the LP system and the control.

The coliform (Figure 4.1c) and CPS counts (Figure 4.1d) were significantly lower ($p < 0.05$) in the LP cheese than in the control cheese throughout the ripening period. Both in

the control and the LP cheeses, the coliform (Figure 4.1c) and CPS counts (Figure 4.1d) decreased throughout the ripening period. After 90 days of ripening, no coliforms (Figure 4.1c) and no CPS (Figure 4.1d) were detected in the LP cheese. Large numbers of coliforms in cheese milk cause early blowing of cheese resulting in a characteristic defect with pinholes throughout the body of the cheese (Chapman & Sharpe, 1990). The lower coliform count observed in the LP cheese as compared to the control is in agreement with the report by Lara *et al.* (1987) who found a similar reduction in coliform count in a fresh type cheese made from LP-activated cow milk. High levels of CPS in cheese milk can result in enterotoxin production during cheesemaking (Chapman & Sharpe, 1990). At normal acidity, it is thought that at least 2.8×10^7 staphylococci g^{-1} of cheese must occur for enterotoxin to develop (IDF, 1980b; Chapman & Sharpe, 1990). Tham, Hajdu and Danielsson-Tham (1990) reported that coliforms and CPS might be the most useful parameters when examining the hygienic quality of on-farm manufactured goat milk cheeses. The decrease in coliform and CPS in cheeses made from goat milk preserved by activation of the LP system suggests that activation of the LP system in goat milk prior to cheesemaking could be of practical importance especially for small-scale cheese producers who in most instances produce cheese from unpasteurised milk.

No significant differences in mould counts were observed between the control and the LP cheeses throughout the ripening period (Figure 4.1e). Fresh milk only rarely contains yeasts and moulds (Burgess *et al.*, 1994); however, spores of moulds are widely distributed in the atmosphere, particularly in the air and dust (Kosikowski & Mistry, 1999) and spoil a number of dairy products. Hence, from the current study it seems that activation of the LP system in goat cheese milk has little effect on the growth of moulds which usually grow on cheese surfaces weeks after manufacture of the cheese.

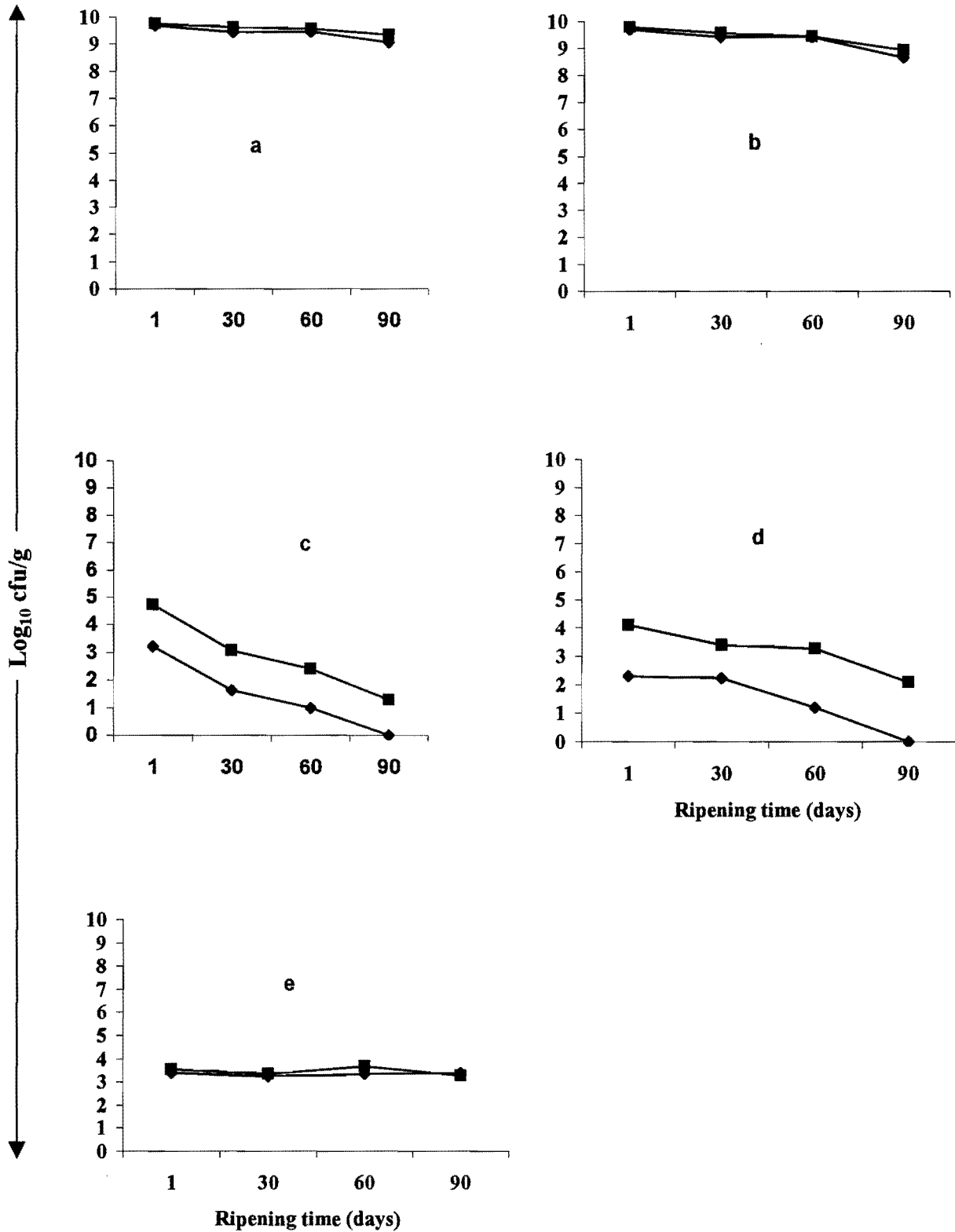


Figure 4.1. Changes in the aerobic plate (a), lactic acid bacteria (b), coliform (c), coagulase positive staphylococci (d) and mould (e) counts in Gouda cheese made from lactoperoxidase activated (♦) and control (■) Saanen goat milk during a ripening period of 90 days. (n = 4).

4.3.4. Effect of the lactoperoxidase system on the yield and gross chemical composition of Gouda cheese

Table 4.4 indicates the gross chemical composition and yield of Gouda cheese made from LP-activated and control Saanen goat milk. No significant differences in the gross chemical composition (fat, protein, salt and moisture) were observed between the control and the LP cheeses. This is in line with previous reports. Santos *et al.* (1995) did not find significant differences in compositional parameters (dry matter, protein and fat) between Villalón cheeses made from ewe milk preserved by activation of the LP system and the untreated control milk. Similarly, Abdou *et al.* (1996) reported that treatment of raw buffalo milk with the LP system had no effect on the salt, fat and total nitrogen contents of the cheese. An increase in percent fat, salt and protein contents was observed at the end of the ripening period (Table 4.4). This increase may have been due to the decrease in moisture content of the cheese samples (Table 4.4) and a concomitant increase in the percentage of these components. No significant differences in pH were observed between the control and the LP cheeses (Table 4.4). This might have been attributed to the use of the LP-resistant starter culture (LL 50C) during the cheesemaking experiment.

Table 4.3. Yield and gross chemical composition of one-day-old Gouda cheese made from lactoperoxidase activated and control South African Indigenous goat milk^a

Parameter	Cheese type	
	Control	Lactoperoxidase treated
Fat (%)	41.95	39.8
Fat in dry matter (%)	63.76	63.12
Moisture (%)	37.17	36.94
Protein (%)	21.41	21.41
pH	5.05	5.12
Yield ^b	19.6	19.7

^aMean of duplicate samples; ^bYield expressed as kg dry matter per 100 litre milk

No significant differences in yield were observed between the experimental and the control cheeses (Table 4.4). The comparable yield of the LP-treated and control cheeses

observed in this study is contrary to a previous report by Hefnawy, Ewais and Abd El-Salam (1986). They reported that pickled soft cheese made from cow and buffalo milks preserved by activation of the LP system had lower yield as compared to cheese made from the control milk.

Table 4.4. Yield and gross chemical composition of Gouda cheese made from lactoperoxidase activated and control Saanen goat milk during a ripening period of 90 days (n = 4 ± SD)^a

Parameter	Cheese type	Ripening time (days)			
		1	30	60	90
Moisture (%)	LP	40.8 ± 1.03	40.6 ± 0.57	40.7 ± 0.48	40.3 ± 0.57
	C	41.2 ± 0.31	41.0 ± 0.41	40.9 ± 0.42	40.7 ± 0.0.32
pH	LP	5.16 ± 0.11	5.08 ± 0.72	5.18 ± 0.03	5.14 ± 0.07
	C	5.11 ± 0.06	4.99 ± 0.07	5.09 ± 0.06	5.09 ± 0.08
Protein (%)	LP	23.8 ± 1.06	23.9 ± 0.72	24.3 ± 0.71	24.2 ± 0.63
	C	24.1 ± 1.11	24.3 ± 0.97	24.7 ± 1.10	24.1 ± 1.14
Fat (%)	LP	32.4 ± 2.3			34.1 ± 2.2
	C	32.5 ± 2.7			34.2 ± 2.1
Fat in dry matter (%)	LP	56.7 ± 5.4			57.1 ± 2.95
	C	55.1 ± 4.2			57.8 ± 3.7
Salt (%)	LP	2.1 ± 0.29			2.5 ± 0.58
	C	2.0 ± 0.09			2.6 ± 0.20
Salt in moisture (%)	LP	5.3 ± 0.61			5.0 ± 1.62
	C	5.1 ± 0.14			5.1 ± 1.67
Yield ^b	LP	9.9 ± 0.74			
	C	10.1 ± 0.55			

^aNo significant differences were observed between the experimental and the control cheeses for all the parameters; ^bYield expressed as kg dry matter per 100 litre milk; LP = Lactoperoxidase; C= Control; SD = Standard deviation.

4.3.5. Effect of the lactoperoxidase system on the level of proteolysis and lipolysis in Gouda cheese.

Table 4.5 shows the change in the proteolytic and lipolytic patterns during the ripening of Gouda cheese made from LP-activated and control Saanen goat milk. The levels of WSN, TCASN and PTASN in both the control and the LP cheeses increased over the ripening period of 90 days (Table 4.5). The level of proteolysis in the LP cheese as measured by the WSN, TCASN and PTASN was comparable to that of the control cheese. Atamer *et al.* (1999) found no significant differences in the TN and soluble nitrogen contents in Kaşer cheese made from LP-activated and control cow milk. Formation of TCASN in Manchego cheese was not slowed down by activation of the LP system in ewe milk (Uceda *et al.*, 1994). Proteolysis in cheese is brought about by proteinases and peptidases derived from the starter cultures, non-starter cultures, the rennet and the milk used (McSweeney & Fox, 1997; Sousa, Ardö & McSweeney, 2001; Park, 2001). During early stages of cheese ripening, rennet is responsible for the breakdown of casein resulting in the formation of large peptides. However, as ripening advances, proteolytic enzymes of starter cultures particularly the peptidases are responsible for the production of small peptides and amino acids (Law, 1987; Park, 2001). In Dutch-type cheeses such as Gouda, proteolysis is brought about mainly by the action of starter enzymes (Venema, Herstel & Elenbaas, 1987). Since the starter culture used in the present study was resistant to the LP system, this in part explains the reason for the absence of significant differences in the level of proteolysis between the experimental and the control cheeses.

The total FFA levels of both the control and the LP cheeses made from Saanen goat milk increased throughout the ripening period (Table 4.5). The FFA level of the LP cheese was lower than that of the control cheese; however, a statistically significant difference ($p < 0.05$) was observed only at day 90. Free fatty acid generation and resulting characteristic flavour of goat milk products is due to the distribution of lipoprotein lipase in various components of the milk system (Chilliard, Selselet-Attou, Bas & Morand-Fehr, 1984; Juárez & Ramos, 1986). Ahrné & Björck (1985) reported that activation of the LP system in cow milk inhibited the activity of lipoprotein lipase and resulted in lower levels of FFA and as a result a marked reduction in lipolysis in the milk. Thus, the lower FFA

level (lipolysis) observed in the LP cheese might have been attributed to the inhibition of lipoprotein lipase by the oxidation product of the LP system. This finding is consistent with the report by Abdou *et al.* (1996). These authors found that the total volatile fatty acid content in Domiati cheese made from cow and buffalo milks preserved by activation of the LP system was lower than that made from the untreated control milk. The strong

Table 4.5. Proteolytic and lipolytic changes during ripening of Gouda cheese made from lactoperoxidase activated and control Saanen goat milk (n = 4 ± SD)

Parameter	Cheese type	Ripening time (days)		
		30	60	90
WSN (% TN)	LP	7.31 ± 0.07 ^a	7.69 ± 0.03 ^a	9.56 ± 0.03 ^a
	Control	7.51 ± 0.07 ^a	8.02 ± 0.02 ^a	10.01 ± 0.03 ^a
TCASN (% TN)	LP	0.92 ± 0.02 ^a	1.52 ± 0.02 ^a	2.12 ± 0.01 ^a
	Control	0.99 ± 0.03 ^a	1.80 ± 0.01 ^a	2.24 ± 0.01 ^a
PTASN (% TN)	LP	-0.84 ± 0.03 ^a	-0.26 ± 0.02 ^a	0.28 ± 0.01 ^a
	Control	-0.75 ± 0.03 ^a	-0.21 ± 0.01 ^a	0.23 ± 0.01 ^a
FFA ^c	LP	6.98 ± 1.12 ^a	8.62 ± 1.61 ^a	10.19 ± 1.03 ^a
	Control	8.21 ± 0.62 ^a	9.70 ± 0.95 ^a	12.27 ± 1.06 ^b

Superscripts in the same column within a parameter with different letters indicate significant difference ($p < 0.05$). ^cFree fatty acid expressed as milliequivalent 100 g⁻¹ fat.

TN = Total nitrogen; WSN = Water soluble nitrogen; TCASN = Trichloroacetic acid soluble nitrogen; PTASN = Phosphotungstic acid soluble nitrogen; LP = Lactoperoxidase

flavour of goat milk cheeses is due to the release of FFA during the ripening period of the cheese (Loewenstein *et al.*, 1980; Juárez and Ramos, 1986; Chandan *et al.*, 1992). Even though some consumers like this strong “goaty” flavour, most people do not like it (Loewenstein *et al.*, 1980). Thus, activation of the LP system in goat cheese milk might be of significant importance in reducing the strong flavour associated with goat milk cheeses.

4.3.6. Effect of the lactoperoxidase system on the sensory properties of Gouda cheese.

The results of the sensory evaluation revealed significant differences ($p < 0.05$) in the overall sensory attributes between the experimental and the control cheeses made from Saanen goat milk (Table 4.6). The significant difference in sensory properties observed between the experimental and the control cheeses might have been attributed to the difference in the level of lipolysis (FFA level) in the two cheese types. Furthermore, 29 % of the 52 assessors who correctly detected differences between the two cheese types commented that Gouda cheese made from LP-activated goat milk had a mild flavour as compared to Gouda cheese made from the untreated control milk. This is also consistent with the lower FFA content observed in cheese made from goat milk preserved by the LP system. The sensory analysis result of the present study is in agreement with the finding of Girgis *et al.* (2001) who reported that Domiati and Kariesh cheeses manufactured from LP-activated buffalo milk had better organoleptic properties than the control. Thus, activation of the LP system in goat milk may be used to improve the flavour of goat milk cheeses by lowering the extent of lipolysis during the ripening of the cheese.

Table 4.6. The sensory difference test between Gouda cheeses made from lactoperoxidase activated and control Saanen goat milk^a

Total number of assessors	Correct responses	P value
120	52	0.014

^aSignificant differences calculated at 5 % significance level.

4.4. Conclusions

From the results of the current study it can be concluded that preservation of Saanen goat milk by the LP system can be used to improve the microbiological quality and flavour of Gouda cheese manufactured from Saanen goat milk without any detrimental effect on the gross chemical composition of the cheese if an appropriate starter culture is used. Since most goat milk cheeses are manufactured from raw milk without heat treatment,

preservation of goat milk by the LP system may be used to increase the safety margin of cheeses made from raw goat milk.

Acknowledgements

This study was sponsored by the Agricultural Research and Training Project of Alemaya University (Ethiopia). Prof. B. H. Bester is highly appreciated for his encouragement and valuable comments during the inception of the experiment. I am also grateful to Dr. Riëtte de Kock and Mrs. Marise Kinnear for their assistance during the sensory evaluation of the cheese samples.

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CHAPTER 5

General Discussion, Conclusions and Recommendations

5.1. General Discussion

Goat milk and milk products are important sources of protein for humans in many developing countries especially in areas where climatic conditions and tradition are not conducive for raising dairy cattle (Klinger & Rosenthal, 1997). The demand for goat milk and milk products is increasing in many countries due to the image of health foods attached to goat milk and milk products (Sutmoller, 1997). However, unlike cow milk, there are no legalised standards for the production and distribution of goat milk in several countries (Klinger & Rosenthal, 1997). In many countries goat milk is produced by small-scale farmers and it is commonly used for an on-farm manufacture of cheese. Most goat milk cheeses are manufactured from raw goat milk with or without thermal treatment (Klinger & Rosenthal, 1997). These cheese varieties are mostly 'artisan-type' and recipes and manufacturing methods are passed from generation to generation without a standardised technology or regulatory definition (Sutmoller, 1997). Difficulties in managing the sanitary quality of goat milk and milk products arise from a series of factors which include the low level of production per head, the milking system, adverse climatic conditions and the spread of production over a wide geographic area (Klinger & Rosenthal, 1997).

Thus, it is imperative to look for processing alternatives which are safe, simple and affordable and which can be used under situations where conventional preservation methods such as cooling and pasteurisation could not be used, and yet can ensure the safety and shelf-life of goat milk and milk products. Activation of the naturally occurring LP system in goat milk may therefore be used to safeguard the microbiological quality of goat milk and end products made from it. The present study addresses the effect of the LP system on: the growth and survival of selected food-borne pathogens in goat milk, the activity of commercially available mesophilic lactic cheese starter cultures in goat milk and the quality of Gouda cheese manufactured from goat milk preserved by the LP system.

The ~~experiment on~~ experiment on pathogens was aimed at determining the antibacterial effect of the lactoperoxidase system on the growth and survival of *Escherichia coli*, *Staphylococcus*

aureus, *Listeria monocytogenes* and *Brucella melitensis* in the milk of Saanen and South African Indigenous goats at an ambient temperature of 30 °C. The results of this study revealed differences in sensitivity to the inhibitory effect of the LP system among the various pathogens tested in the milk of the two goat breeds. Different groups of bacteria show a varying degree of sensitivity to the LP system in cow milk and depending on the bacterial species and strain of the same bacterium the effect can be bacteriostatic or bactericidal (Korhonen, 1980).

Activation of the LP system both in Saanen and Indigenous goat milks was found to be bacteriostatic against *E. coli*. The LP system was found to be bactericidal against *E. coli* in cow milk and the inhibitory effect was related to the incubation temperature and the initial level of inoculum used (Reiter *et al.*, 1976; Farrag *et al.*, 1992b). At higher temperature and when a high initial inoculum of bacteria is used, the inhibitory effect of the LP system becomes lower and vice versa. Thus, the lower inhibitory effect of the LP system against *E. coli* observed in the present study might be related to the high incubation temperature (30 °C) and the high level of inoculum (10^6 - 10^7 cfu/ml) used.

Activation of the LP system in Saanen goat milk was found to be bactericidal against *S. aureus*; however, it was only bacteriostatic against *S. aureus* in the Indigenous goat milk. Saanen goat milk had higher LP activity (0.79 U/ml) than Indigenous goat milk (0.26 U/ml). It has been reported that the variability of the bactericidal properties of milk is caused by variations of the quantities of peroxidases contained in different milk samples (Pruitt & Kamau, 1991). Thus, the strong inhibitory effect of the LP system against *S. aureus* in Saanen goat milk might be associated to the higher LP activity in Saanen goat milk. It seems that *S. aureus* needs a minimum inhibitory concentration of LP before being inhibited by the LP system.

The levels of *E. coli* and *S. aureus* found in goat milk under normal conditions are low. Foschino *et al.* (2002) reported a mean *E. coli* level of 2.9 cfu/ml and a mean *S. aureus* count of 1.2×10^3 cfu/ml in raw goat milk. Since the antibacterial activity of the LP system is greater when the initial number of bacteria is low, the bacteriostatic effect of

the LP system against *E. coli* and *S. aureus* observed at the inoculum level used in the present study (10^6 - 10^7 cfu/ml) could be of significant importance in controlling the multiplication of these organisms in raw goat milk under practical conditions.

Activation of the LP system both in Saanen and Indigenous goat milks resulted in a bactericidal effect against *L. monocytogenes*. The LP system in bovine milk was found to be bactericidal against *L. monocytogenes* during the initial period at 35 °C (Kamau *et al.*, 1990a). *Listeria monocytogenes* is a pathogen of major concern to the dairy industry as food-borne listeriosis has been related to consumption of contaminated milk and milk products (Griffiths, 1989). A recent report (Morgan *et al.*, 2001) indicated that *L. monocytogenes* was able to survive in soft lactic cheese made from raw goat milk. The inhibitory effect of the LP system against *L. monocytogenes* observed in the present study suggests that activation of the LP system may help in controlling the growth of *L. monocytogenes* during collection and transportation of goat milk at ambient temperatures, as is the case in many developing countries.

Activation of the LP system exhibited a bactericidal effect against *Br. melitensis* both in Saanen and Indigenous goat milks. *Brucella melitensis* is highly pathogenic to humans (Garin-Bastuji & Verger, 1994; Ryser, 1998) causing one of the most serious zoonoses in the world. Goats are the primary reservoir of *Br. melitensis* and consumption of raw goat milk and goat milk cheese has been reported to be the probable means of brucellosis infection (Ryser, 1998). Outbreaks of human brucellosis caused by *Br. melitensis* due to consumption of unpasteurised goat milk cheese have been reported (Thapar & Young, 1986; Wallach *et al.*, 1994). The bactericidal effect of the LP system against *Br. melitensis* observed in this study is the first information on the species and suggests that activation of the LP system in goat milk could be of practical importance in controlling the proliferation of *Br. melitensis* in goat milk.

The same pathogen showed different degrees of sensitivity to the LP system in the milk of the two goat breeds probably because of the differences in the LP system components in the two milk types. The thiocyanate (SCN⁻) concentration and LP activity were

different in Saanen and Indigenous goat milks. Saanen goat milk had a mean SCN⁻ concentration of 2.78 ± 1.21 ppm and a mean LP activity of 0.79 ± 0.18 U/ml; whereas Indigenous goat milk had a mean SCN⁻ concentration of 4.58 ± 1.92 ppm and a mean LP activity of 0.26 ± 0.10 U/ml. These variations in LP activities and SCN⁻ concentrations between the milks of the two goat breeds support breed variations in LP system components observed by Zapico *et al.* (1991). They found a mean SCN⁻ content of 5.76 ppm and 3.20 ppm and a mean LP activity of 0.95 U/ml and 2.15 U/ml, for milk from the Verata and Murciano-Granadina breeds of goats, respectively.

The International Dairy Federation (IDF, 1988) recommended addition of 14 mg/l sodium thiocyanate and 30 mg/l of sodium percarbonate to activate the LP system in cow and buffalo milks. However, earlier reports indicated that the optimum levels of SCN⁻ and hydrogen peroxide (H₂O₂) needed to activate the LP system varies for different types of milk. Gupta, Patel, Patil, Singh and Mathur (1986) reported that buffalo milk required higher levels of SCN⁻ and H₂O₂ to exhibit the same preservative effect as compared to cow milk. Thus, the amount of SCN⁻ and H₂O₂ needed to activate the LP system in raw goat milk needs to be optimised. Activation of the LP system using different levels of the LP system components may help to determine the optimum level of SCN⁻ and H₂O₂ needed for activation of the LP system in goat milk and this needs further investigation. The results of the experiment on pathogens suggest the potential of the LP system in controlling the growth of pathogenic bacteria in raw milk obtained from Saanen and South African Indigenous goats.

If activation of the LP system in goat milk exhibits bactericidal effect against pathogens artificially inoculated into raw goat milk, then it can be expected that preservation of raw goat milk intended for cheesemaking by the LP system may also be effective against the raw milk flora or pathogens present in the milk during storage of the milk prior to cheesemaking and thus render cheese of good quality. Thus, the cheesemaking experiment was aimed at investigating the suitability of goat milk preserved by the LP system for cheesemaking. For the manufacture of hard cheese varieties, the use of starter cultures is crucial. However, one of the concerns regarding the use of the LP system for

preservation of milk intended for further processing is the possible effect it may have on the activities of the starter cultures used. Earlier reports indicated the inhibitory effect of the LP system on the activity of thermophilic lactic starter cultures in cow milk (de Valdez *et al.*, 1988). Thus, the experiment on starter cultures was aimed at determining the effect of the LP system on the activity of commercially available mesophilic cheese starter cultures and selecting a starter culture which is resistant to the LP system and which could be used for cheesemaking from goat milk preserved by activation of the LP system. The effect of the LP system on the activity of commercially available mesophilic cheese starter cultures was investigated by measuring their growth and acid production over 8 h incubation period at 30 °C in Saanen goat milk. Most of the starter cultures examined were found to be sensitive to the LP system, but varied in their susceptibility to inhibition. The activity of the mixed starter cultures CHN11, CHN22, CHN19, DCC240 and Flora Danica Normal was strongly inhibited by the LP system. However, the mixed starter culture LL 50C showed resistance to the LP system.

In mixed strain cheese starters, inhibition, insensitivity or stimulation of activity by the lactoperoxidase system depends on the ratio of sensitive, insensitive and stimulated strains or variants within a starter (IDF, 1991b). All the mixed strain starter cultures used in this study except LL 50C were composed of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. The mixed culture LL 50C was composed of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. The experiment on single strain starter cultures revealed variations in sensitivity to the LP system between the single strain cultures tested. The activity of the culture *Lactococcus lactis* subsp. *lactis* NCDO 605 was inhibited by the LP system; however, the cultures *Lactococcus lactis* subsp. *diacetylactis* NCDO 176 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313 were insensitive to the LP system. Thus, the difference in sensitivity of the various mixed starter cultures examined in this study to the LP system might have been attributed to the differences in the proportions of the various strains between the mixed cultures used. The balance of species and/or strains within a mixed starter culture may alter and reduce the capacity of the culture to produce acid (Scott *et*

al., 1998). The strains can be slow or fast acid producers; hence, a shift in the proportion of the different strains can affect the activity of the cultures.

The mixed starter culture LL 50C exhibited resistance to the LP system. The resistance of some starter cultures to the LP system could be associated with the presence of a 'reversal factor' as reported by Oram and Reiter (1966) and Reiter (1985b). This 'reversal factor' is an enzyme, NADH:OSCN oxidoreductase, that catalyses the reduction of the inhibitor OSCN, by NADH, to the inert thiocyanate. The starter strains in the LL 50C culture might be naturally resistant to the inhibitory effect of the LP system and may possess mechanisms that counteract the oxidation product produced by the LP system.

The inhibitory effect of the LP system on the acid production by the mixed starter cultures was greater than that on their growth, as measured by colony count. This might have been attributed to the difference in composition of the single strain starter cultures used in the mixed cultures. The single strain culture *Leuc. mesenteroides* subsp. *cremoris* was found to be insensitive to the LP system; however, it produces insufficient acid in milk; whereas the culture *L. lactis* subsp. *lactis* was sensitive to the LP system. *Lactococcus lactis* subsp. *lactis* is the major lactic acid producer among the lactic starter cultures used for cheesemaking (Scott *et al.*, 1998). Thus, if a mixed starter culture is composed of higher proportions of the resistant culture *Leuc. mesenteroides* subsp. *cremoris* but lower proportions of the sensitive species *L. lactis* subsp. *lactis*, it can be expected that the acid production of the mixed culture will be inhibited more than their growth. This finding is consistent with that reported previously by Nakada *et al.* (1996). They found that the LP system suppressed the rate of acid production by, but not the rate of growth of, yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). The greater inhibition of acid production than growth of the starter cultures might also have been attributed to inhibition of key metabolic enzymes by the LP system that are responsible for the production of lactic acid by the starter cultures. It has been reported that the hypothiocyanite ion produced during activation of the LP system causes inhibition of specific glycolytic enzymes such as aldolase, hexokinase and glyceraldehydes-3-phosphate dehydrogenase (Condon, 1987).

Differences in sensitivity to the LP system were observed between the single strain starter cultures. The variation in sensitivity between the single strain cultures to the LP system may be associated to differences in their genetic makeup. Roginski *et al.* (1984) reported differences in sensitivity of various starter strains to the LP system in cow milk. They showed that the growth of, and acid production by, *Lactococcus lactis* subsp. *cremoris* C1 were stimulated by the LP system in cow milk; whereas the growth of *Lactococcus lactis* subsp. *cremoris* BK5 and *Lactococcus lactis* subsp. *lactis* C10 were inhibited by the LP system indicating that inhibition of starter cultures by the LP system, is strain dependent.

The results of this study indicate the need for routine selection and screening of starter cultures to determine their sensitivity to the LP system before using them for cheesemaking from goat milk preserved by activation of the LP system. Such studies would help to facilitate the adoption of the LP system for preservation of goat milk by small-scale goat milk and/or cheese processors. The culture LL 50C, which was resistant to the LP system, could be used for cheesemaking from LP-activated goat milk.

The experiment on cheese was aimed at assessing the suitability of goat milk preserved by the LP system for the manufacture of Gouda cheese and to determine its effect on the biochemical, microbiological and organoleptic properties of Gouda cheese over a ripening period of 90 days. Activation of the LP system in raw Saanen goat milk did not affect the yield and gross chemical composition of Gouda cheese. No significant differences in pH, fat, salt, protein and moisture contents were observed between Gouda cheeses made from LP-treated Saanen goat milk and that made from the untreated control milk throughout the ripening period. This finding is in line with previous reports. Santos *et al.* (1995) found no significant differences in the chemical composition between cheeses made from ewe milk preserved by the activation of the LP system and the control. Abdou *et al.* (1996) also reported the absence of significant differences in composition between cheeses made from raw buffalo milk treated by the LP system and the control. The fat, protein and salt contents of both the experimental and the control

cheeses increased at the end of the ripening period; whereas the moisture content of both the LP-treated and the control cheeses decreased throughout the ripening period. The increase in percent fat, percent protein and percent salt contents at the end of the ripening period may be due to the decrease in the moisture content of the cheese samples and a concomitant increase in the percentage of these components. Kumar and Mathur (1989c) reported a lower rate of acid production by starter cultures when LP-activated buffalo milk was used either for the manufacture of yoghurt or Mozzarella cheese. However, in the present study, no significant differences in pH were observed between cheeses made from LP-activated and control Saanen goat milks. This might have been attributed to the use of the LP-resistant starter culture (LL 50C) during the cheesemaking process. Comparable cheese yields were obtained from both the LP-treated and the control Saanen goat milks. Thus, it can be concluded that Gouda cheese of satisfactory composition and yield can be manufactured from Saanen goat milk preserved by activation of the LP system.

One of the concerns in using LP-treated milk for cheesemaking is the possible effect of the LP system on the coagulation of the milk during cheesemaking. The presence of thiocyanate (SCN^-) ion in milk inhibits rennet-casein interaction since SCN^- binds to cationic regions of casein resulting in a longer coagulation time of milk with rennet (Bringe & Kinsella, 1986). However, in the present study, no significant differences in clotting time were observed between the LP-treated and the control Saanen goat milks. During the cheesemaking experiment, the time for setting the curd following renneting for both the LP-treated and the control milks was similar. This agrees with the absence of significant differences in clotting time between the LP-treated and the control milks.

Activation of the LP system in Saanen goat milk prior to cheesemaking resulted in a significant ($p < 0.05$) reduction of coliform and aerobic plates counts. It is in agreement with the findings of Girgis *et al.* (2001) who reported that activation of the LP system both in cow and buffalo milks caused a significant decrease in coliform and total bacterial counts during storage of the milks prior to cheesemaking. This may be of significant

importance for small-scale cheese producers who in most cases are obliged to collect their goat milk over a few days to get a sufficient quantity for cheesemaking.

Gouda cheese made from LP-activated Saanen goat milk (LP cheese) had significantly lower ($p < 0.05$) coliform and coagulase positive staphylococci (CPS) as compared to Gouda cheese made from the untreated control milk throughout the ripening period. After 90 days of ripening, no coliforms and no CPS were detected in the LP cheese. However, no significant differences in the total aerobic, lactic acid bacteria and mould counts were observed between the control and the LP cheeses. The manufacture of cheese from raw goat milk presents public health hazards (Tham *et al.*, 1990). Large numbers of coliforms in cheese milk cause early blowing of cheese resulting in a characteristic defect with pinholes throughout the body of the cheese (Chapman & Sharpe, 1990). The lower coliform count observed in cheese made from LP-activated Saanen goat milk as compared to the control is in agreement with the report by Lara *et al.* (1987) who found a similar reduction in coliform count in fresh type cheese made from LP-activated cow milk. High levels of CPS in cheese milk can result in enterotoxin production during cheesemaking (Chapman & Sharpe, 1990). At normal acidity, it is thought that at least 2.8×10^7 staphylococci g^{-1} of cheese must occur for enterotoxin to develop (IDF, 1980b; Chapman & Sharpe, 1990). Tham *et al.* (1990) reported that coliforms and CPS might be the most useful parameters when examining the hygienic quality of on-farm manufactured goat cheeses. The decrease in coliform and CPS in cheeses made from goat milk preserved by activation of the LP system suggests that activation of the LP system in goat milk prior to cheesemaking could be of practical importance especially for small-scale cheese producers who in most instances produce cheese from unpasteurised milk.

The level of proteolysis in Gouda cheese made from LP-activated Saanen goat milk as measured by the water soluble nitrogen (WSN), trichloroacetic acid soluble nitrogen (TACSN) and phosphotungstic acid soluble nitrogen (PTASN) was comparable to that of the control cheese. An increase in the levels of WSN, TCASN and PTASN were observed with increase in ripening time. This is in agreement with the reports of Atamer *et al.* (1999) and Uceda *et al.* (1994). Proteolysis in cheese is brought about by

proteinases and peptidases derived from the starter cultures, non-starter cultures, the rennet and the milk used (McSweeney & Fox, 1997; Sousa *et al.*, 2001). During early stages of cheese ripening, rennet is responsible for the breakdown of casein resulting in the formation of large peptides. However, as ripening advances, proteolytic enzymes of starter cultures particularly the peptidases are responsible for the production of small peptides and amino acids (Law, 1987). In Dutch-type cheeses such as Gouda, proteolysis is brought about mainly by the action of starter enzymes (Venema *et al.*, 1987). Since the starter culture used in the present study is resistant to the LP system, this in part explains the reason for the absence of significant differences in the level of proteolysis between cheeses made from LP-activated and control Saanen goat milk.

The total FFA levels both in the experimental and the control cheeses increased throughout the ripening period. The FFA level of cheese made from LP-activated Saanen goat milk was lower than that of the control cheese; however, a statistically significant difference ($p < 0.05$) was observed at day 90. Free fatty acid generation and resulting characteristic flavour of goat milk products is due to the distribution of lipoprotein lipase in various components of the milk system (Chilliard *et al.*, 1984). Lipoprotein lipase activity in goat milk is significantly correlated with spontaneous lipolysis and it plays a major role in flavour development in milk stored at 4 °C (Juárez & Ramos, 1986). Thus, the lower FFA level (lipolysis) observed in cheese made from LP-activated Saanen goat milk might have been attributed to the inhibition of lipoprotein lipase by the oxidation product of the LP system. This finding is consistent with the report by Abdou *et al.* (1996). These authors found that the total volatile fatty acid in Domiati cheese made from cow and buffalo milks preserved by activation of the LP system was lower than that made from the untreated control milk. The strong flavour of goat milk cheeses is due to the release of FFA during the ripening period of the cheese (Loewenstein *et al.*, 1980; Juárez & Ramos, 1986; Chandan *et al.*, 1992). Even though some consumers like this strong “goaty” flavour, most people do not like it (Loewenstein *et al.*, 1980). Thus, activation of the LP system in goat cheese milk might be of significant importance in reducing the strong flavour associated with goat milk cheeses.

The sensory analysis showed significant differences ($p < 0.05$) in the overall sensory attributes between the experimental and the control cheeses. The significant difference in sensory properties observed between the experimental and the control cheeses might have been attributed to the difference in the extent of lipolysis (FFA level) in the two cheese types. Furthermore, the panellists commented that Gouda cheese made from LP-activated goat milk had a mild flavour as compared to Gouda cheese made from the untreated control milk. Thus, activation of the LP system in goat milk may be used to improve the flavour of goat milk cheeses by lowering the extent of lipolysis during the ripening of the cheese.

Milk intended for cheesemaking is usually pasteurised in order to kill spoilage and pathogenic bacteria. However, pasteurisation of milk can cause undesirable effects such as denaturation of serum proteins, which may lead to slow renneting, a weak curd, poor syneresis (Walstra, *et al.*, 1993), and development of a bitter flavour (Stadhouders & Hup, 1975; Walstra *et al.*, 1993). Furthermore cheeses made from pasteurised milk ripen more slowly and develop a less intense flavour than raw milk cheeses (Johnson *et al.*, 1990; Grappin & Beuvier, 1997; Fox & McSweeney, 1998) due to the destruction of some desirable non-starter lactic acid bacteria present in milk. Moreover, cheeses whose traditional flavour result in part from native milk enzymes and microflora (e.g., Swiss and Italian types) would also be affected if milk pasteurisation for cheesemaking were mandatory (Johnson *et al.*, 1990). Thus, preservation of raw goat cheese milk by the LP system could be a suitable alternative to pasteurisation which impairs protein functionality and may not be feasible to apply in most rural areas due to technical and/or economic reasons. The majority of goat milk cheeses are manufactured from raw goat milk (Lodi *et al.*, 1996). Thus, the use of the LP system may increase the margin of safety of cheeses made from raw goat milk.

Due to lack of enough amounts of milk, only one batch of Gouda cheese was made from South African Indigenous goat milk. This pilot trial indicated differences in composition between Saanen and Indigenous goat milk and cheese. The most important difference between Gouda cheeses made from Indigenous goat milk and Saanen goat milk was with

respect to yield of cheese. The yield of Gouda cheese made from Indigenous goat milk was 19.6 kg per 100 litres of milk whereas the yield of Gouda cheese made from Saanen goat milk was 10.1 kg per 100 litres of milk. Moreover, during the cheesemaking process, Indigenous goat milk took shorter time (40 min) to coagulate as compared to Saanen goat milk (60 min) and resulted in firmer curd during cutting. This could be associated with the higher total solids (17.58 %), solids-not-fat (12.66 %), fat (4.92 %) and protein (4.22 %) contents in the milk of Indigenous goats as compared to the lower fat (3.83 %), protein (2.73 %), total solids (11.97 %) and solids-not-fat (8.15 %) contents in Saanen goat milk. Clark and Sherbon (2000) reported that goat milk containing high TS, SNF, fat and protein coagulates quickly and forms firmer curd than milk containing low levels of these components. From this pilot trial it can be noted that Indigenous goats have genetic potential which could have a significant importance especially for small-scale cheese producers. Thus, breeding programmes aimed at selection of South African Indigenous goats or crossbreeding them with dairy type goats may be of paramount importance for cheese producers.

In summary, the experiment on cheese revealed the suitability of goat milk preserved by activation of the LP system for further processing into cheese. Lactoperoxidase-treatment of Saanen goat cheese milk had no detrimental effect on the physico-chemical properties of Gouda cheese and supports the hypothesis that LP-treatment of goat cheese milk may not affect the chemical composition of Gouda cheese made from LP-treated goat milk. Gouda cheese made from LP-activated Saanen goat milk had significantly lower ($p < 0.05$) coliform and coagulase positive staphylococci compared to Gouda cheese made from the untreated control Saanen goat milk. This also supports the hypothesis that LP-treatment of goat cheese milk may improve the microbiological quality of Gouda cheese made from such milk. LP-treatment of Saanen goat milk significantly ($p < 0.05$) reduced the level of lipolysis in Gouda cheese at the end of the ripening period. This suggests that LP-treatment of goat cheese milk could be of paramount importance in reducing the strong flavour associated with goat milk cheeses. Yet all these were possible due to the identification and use of a starter culture which was resistant to the LP system. The importance of use of LP-resistant cultures for cheesemaking from LP-activated goat milk

was reflected in the absence of significant differences in pH, total lactic acid bacteria count and proteolysis between the experimental and the control cheeses.

In general, the results of this study indicates the potential for the use of the LP system for preservation of goat milk and for the improvement of the qualities of cheese manufactured from such milk. This could be of significant importance to small-scale goat milk and/or cheese producers especially under situations where heat treatment and refrigeration of milk cannot be easily applied. The use of the LP system for preservation of goat milk can prevent the loss of large volumes of goat milk due to spoilage by microorganisms. The generation of surplus milk would increase the income of small-scale goat milk and/or cheese producers and may significantly contribute to household food security. One of the major problems of small-scale goat milk producers in rural areas is accessing distant markets where there is a huge demand for fresh, good quality milk as a result of lack of well-developed transportation systems. Consequently, milk is usually transported long distances during which time it gets spoiled. Thus, activation of the LP system would enable the delivery of goat milk to processing plants and distant markets in a safe condition. This would result in the development of small-scale goat milk producers and processors.

Although the use of the LP system has not been legally approved in some countries due to the addition of chemicals into milk to activate the LP system, any side effect of adding small amounts of chemicals to activate the LP system should be carefully weighed against the loss of large amounts of milk which could otherwise contribute significantly to the improvement of the nutrition of the rural poor who usually suffer from under nutrition or malnutrition.

5.2. Conclusions and Recommendations

The lactoperoxidase (LP) system of Saanen and South African Indigenous goat milk is bactericidal against *Listeria monocytogenes* and *Brucella melitensis* at 30 °C. The lactoperoxidase system of Saanen goat milk is bactericidal against *Staphylococcus aureus*; however, the LP system in Indigenous goat milk is only bacteriostatic against *S. aureus*. On the other hand, the LP system is only bacteriostatic against *Escherichia coli* both in Saanen and Indigenous goat milks. Saanen and Indigenous goat milks exhibit different degrees of inhibition against pathogens probably because of the difference in the LP system components between the two milk types. The mean lactoperoxidase activity and mean thiocyanate content of Saanen goat milk are 0.79 ± 0.18 U/ml and 2.78 ± 1.21 ppm, respectively. However, the mean lactoperoxidase activity and mean thiocyanate content of Indigenous goat milk are 0.26 ± 0.10 U/ml and 4.58 ± 1.92 ppm, respectively.

Most commercially available mesophilic cheese starter cultures are sensitive to the LP system in Saanen goat milk. The activity of the mixed starter cultures CHN11, CHN22, CHN19, DCC240 and Flora Danica Normal is strongly inhibited by the LP system in Saanen goat milk. However, the mixed starter culture LL 50C is resistant to the LP system in Saanen goat milk. The activity of the single strain starter culture *Lactococcus lactis* subsp. *lactis* NCDO 605 is inhibited by the LP system. However, the cultures *Lactococcus lactis* subsp. *diacetylactis* NCDO 176 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313 are insensitive to the LP system in Saanen goat milk.

Gouda cheese of acceptable quality can be manufactured from Saanen goat milk preserved by activation of the lactoperoxidase system. The LP-treatment does not affect the physico-chemical properties of Gouda cheese made from Saanen goat milk. The LP-treatment of Saanen goat milk also does not affect the yield of Gouda cheese and the coagulation of the milk during the cheesemaking process. The level of proteolysis in Gouda cheese manufactured from Saanen goat milk preserved by the LP system is not affected during the ripening of the cheese. However, LP-treatment of Saanen goat milk significantly ($p < 0.05$) reduces the level of lipolysis at the end of the ripening period of Gouda cheese. This could be of paramount importance in reducing the strong flavour

associated with goat milk cheeses. The lactoperoxidase-treatment significantly ($p < 0.05$) reduces the levels of coliform and coagulase positive staphylococci in Gouda cheese manufactured from Saanen goat milk. Differences in sensory attributes are observed between Gouda cheeses made from the LP-treated and the untreated control Saanen goat milks. Lactoperoxidase-treatment of Saanen goat milk results in a Gouda cheese with a mild flavour.

Future research needs

During the course of the study, it was observed that LP-treatment of goat cheese milk resulted in a lower level of free fatty acids at the end of the ripening period. The strong flavour of goat milk cheeses is due to the release of free fatty acids during the ripening of the cheese (Chandan *et al.*, 1992). Thus, detailed studies aimed at investigating the potential of the LP system in reducing the strong 'goaty' flavour associated with goat milk cheeses and identifying the target fatty acids inhibited by the LP system is recommended. Such studies may help to avoid the negative image of the general public on goat milk or cheese.

Activation of the LP system in raw goat milk resulted in inhibition of pathogenic bacteria which were artificially inoculated into raw goat milk. Similar studies in cheese will give more information on the potential of the LP system of inhibiting such pathogens in cheese. Thus, challenge studies by artificially inoculating pathogens in LP-activated goat cheese milk would help to determine the survival of these pathogens during ripening of cheese made from LP-treated goat milk. Besides, isolation of lactoperoxidase from goat milk and investigating its effectiveness against a range of pathogens in other food systems either alone or in synergy with other preservation methods needs attention.

The International Dairy Federation has published a guideline for activation of the LP system in cow and buffalo milks (IDF, 1988). However, to date there is no published method for activation of the LP system in goat milk. The present study indicated the differences in the concentrations of the LP system components between Saanen and

South African Indigenous goat milks. Similar studies in the milk of other goat breeds may help to determine the optimum levels of thiocyanate and hydrogen peroxide needed to activate the LP system in goat milk.

Genetic improvement of starter cultures with respect to industrially important traits such as bacteriophage resistance and their ability to produce bacteriocins efficiently has been possible by the application of modern biotechnology techniques (Ross, Stanton, Hill, Fitzgerald & Coffey, 2000). The present study on starter cultures indicated that most of the commercially available lactic cheese starter cultures were significantly inhibited by the LP system in goat milk and variations in sensitivity to the LP system was observed among the starter cultures. Thus, genetic improvement of starter cultures with respect to LP resistance may help to solve this problem and needs further study.

The present study indicated the potential of the LP system for preservation of raw goat milk and the possibility of manufacturing Gouda cheese of acceptable quality from goat milk preserved by the LP system. Thus, application of the LP system is recommended for preservation of goat milk under situations where pasteurisation and refrigeration cannot be easily applied for technical, economic and/or practical reasons and it would have a significant beneficial effect particularly for small-scale goat milk or cheese producers living in rural areas.

6. References

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7. Publications

SEIFU E., BUYS, E. M., DONKIN, E. F. & PETZER, I-M. Antibacterial activity of the lactoperoxidase system against food-borne pathogens in Saanen and South African Indigenous goat milk. Manuscript in press [**Food Control**]

SEIFU, E., BUYS, E. M. & DONKIN, E. F. 2003. Effect of the lactoperoxidase system on the activity of mesophilic cheese starter cultures in goat milk. Published: **International Dairy Journal**, 13, 953-959.

SEIFU, E., BUYS, E. M. & DONKIN, E. F. Quality aspects of Gouda cheese made from goat milk preserved by the lactoperoxidase system. Manuscript submitted to the **International Dairy Journal (June, 2003)**

Presentations

SEIFU, E., DONKIN, E. F., BESTER, B. H. & BUYS, E. M., 2002. New recognition of an old enzyme: the antibacterial effect of the lactoperoxidase system in goat milk. Poster presented at the 26th IDF World Dairy Congress, September 24-27, 2002, Paris, France.

SEIFU, E., BUYS, E. M. & DONKIN, E. F. 2003. Application of the lactoperoxidase system to improve the quality and safety of goat milk and goat milk cheese. Presentation at the South African Association of Food Science and Technology Symposium, September 1-4, 2003, Pretoria, South Africa.

SEIFU, E., BUYS, E. M. & DONKIN, E. F. 2003. Effect of the lactoperoxidase system on the safety of goat milk. Presentation at the IDF World Summit, September 8-12, 2003. Bruges, Belgium.