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ASSESSMENT OF THE EFFECTS OF ACTIVATED LACTOPEROXIDASE SYSTEM ON MICROBIOLOGICAL QUALITY OF RAW COW MILK ON DIFFFERENT CLIMATIC ZONE OF ETHIOPIA

BY

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BISHOFTU, ETHIOPIA

ADDIS ABABA UNIVERSITY

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ASSESSMENT OF THE EFFECTS OF ACTIVATED LACTOPEROXIDASE SYSTEM ON MICROBIOLOGICAL QUALITY OF RAW COW MILK ON DIFFERENT CLIMATIC ZONE OF ETHIOPIA

A thesis submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Veterinary Microbiology

BY

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Assessment of the Effects of Activated Lactoperoxidase System On Microbiological Quality of Raw Cow Milk on different climatic zone of Ethiopia, Bishoftu, Ethiopia

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As members of the examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Hani Selemon Zewude**. Entitled: **Assessment of the Effects of Activated Lactoperoxidase System on Microbiological Quality of Raw Cow Milk on different climatic zone of Ethiopia.** And we recommend that it can be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Microbiology.

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LIST OF ABBREVIATIONS

CAC	Codex Alimentarius Commission
FAO	Food and Agriculture Organization of the United Nations
H_2O_2	Hydrogen peroxide
HOSCN	Hypothiocyanous acid
LP	Lactoperoxydase
LPS	Lactoperoxydase System
LSD	Least Significant Difference
NMSA	National meteorology service agency
OSCN	Hypothiocyanite
SCN	Thiocyanate
$(SCN)_2$	Thiocyanogen
SH	Sulphydry

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SUMMARY

The objective of this study was to assess the antimicrobial effect of the Lactoperoxidase system (LPS) in milk under different agro-climatic zones taking into account the variability of environmental temperatures toward improving milk quality and safety in Ethiopia. Experimental study design was employed to assess the antimicrobial effect of LPS on the keeping quality of raw cow milk. Milk samples were collected from Addis Ababa University Business enterprise Bishoftu fattening, dairy and poultry farm. A total of 12 liters of milk samples were collected and grouped into activated (treated with sodium thiocyante and Hydrogen peroxide) and non-activated (control). The activated and non-activated milk samples were subjected to four treatment groups based on temperature. The treatments were designed to represent the average temperatures of the four agro-ecology of the country namely cool to cold humid zone (14^oC), warm to cool, semi-humid zone (18°C), warm to hot, semi-arid zone (24^oC) and hot, arid zone (37^oC). The samples were incubated in adjustable incubator at each temperature. The effect of the system was evaluated using alcohol tests, total plate count and analysis of the composition of the milk every two hours. LPS activation has resulted in decrease of the number of microbial load significantly. Its effect tended to be more efficient as storage temperature decreases. There was significant reduction of microbial load in LPS activated milk samples as compared to the control group ($P<0.05$). The microbial load was decreased by 66.7%, 75%, 67.9% and 64.3% in activated milk samples kept at 37^0c, 24^0c, 18^0c and 14^0c, respectively. The activation of LPS can prolong the shelf life of milk ranging from 6 to 12 hours without deterioration. The study showed that LPS increased the shelf life of milk significantly at all temperatures. This study revealed that application of LPS had no significant impact on the nutritional composition of milk. In conclusion, activation of the LPS potentially decreases the microbial load and prolongs the shelf life of raw cow milk up to 6-12 hours based on the storage temperatures. Further studies should be done in different parts of the country under the specific temperatures especially in the high milk-shed areas of the country particularly by rural smallholder dairy farmers for practical usage of the system.

Key words: Lactoperoxidase system, antimicrobial effect, milk, temperature, microbial load, Bishoftu.

1. INTRODUCTION

Lactoperoxidase (LP) is a member of the peroxidase family, a group of natural enzymes, widely distributed in nature and found in plants and animals, including humans. Its primary function is to catalyze the oxidation of certain molecules, at the expense of hydrogen peroxide, in order to generate reactive products with a wide antimicrobial activity (Klaasand and Van Hooijdonk, 2000). Milk contains some essential antimicrobial factors such as lactoperoxidase, lysozyme, immunoglobulin and lactoferrin. Additionally, LP is released from mucosal glands and can be found in secretions like saliva or tears (Quinn *et al.,* 2011). The LP enzyme has a crucial role in the protection of the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganisms. LP consists of a single polypeptide chain containing 612 amino acid residues, whereas the molecular weight of bovine LP is about 80 kDa. It contains 15 half-cysteine residues and carbohydrate moieties that comprise about 10% of the molecular weight. The concentration of LP is about 30 mg/liter and varies according to lactation, the highest values being present 3 to 4 days post-partum, after which there is a gradual decline.

The lactoperoxidase system (LPS) is natural antibacterial system. This method primarily used to prevent undue bacterial multiplication (Sisecioglu *et al.,* 2010). Milk is an ideal habitat for the growth and multiplication of microorganisms due to its nutritional constitution which it is the nutrient-rich liquid contains protein, carbohydrate, unsaturated fat, mineral and vitamins. All these components support the growth of many forms of bacteria (Omer *et al.*, 2008, Boulares *et al.*, 2011). The system has three primary components LP, hydrogen peroxide (H_2O_2), and thiocyanate (SCN). The biological significance of lactoperoxidase is its involvement in the natural host defense system against invading micro-organisms thereby protecting lactating mammary gland and the intestinal tract of newborn infants. The potential of LP to inhibit bacterial growth in milk has been recognized. LP catalyzes H_2O_2-dependent oxidation of thiocyanate (SCN-) to hypothiocyanite (OSCN-). The latter ion is a potent antimicrobial agent against gram-negative and gram-positive bacteria, fungi, and viruses (Koksal *et al.,* 2016). As mentioned, LPS has proven to be both bactericidal and bacteriostatic to a wide variety of microorganisms, while having no effect on the proteins and enzymes of the organisms producing

it (Reiter *et al.,* 1976, CAC/GL, 1991, Sisecioglu *et al.,* 2010), FAO, 2005). Moreover, it has, antiviral activity, degradation of various carcinogens and protection of animal cells against peroxidative effects (Klaasand and Van Hooijdonk, 2000).

Significant losses of milk occur at the farm level during post-harvest in form of spoilage in developing country like Ethiopia, Uganda, Tanzania and Kenya which account for about 1.3 to 6.4 percent of the value of available milk. The cost due to this spoilage was estimated to be 0.9–11million US dollars. Thus, LPS has been recognized as critical in the dairy industry for the preservation of raw milk to prevent the spoilage and associated financial loss. The system also serves as a processing aid in the production of other dairy products. (Lore *et al.,* 2005).

In Africa, application of the LPS was firstly tested in Kenya as a successful means of preserving milk at ambient temperatures and a strong effect of the LPS against Gram-negative psychrotrophs was demonstrated for improving the refrigerated storage of raw milk (Sulieman *et al.,* 2009). The most commonly used method to stop or retard the deterioration of milk on its way from the farmer to the dairy is cooling. 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 complicate 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 (Seifu *et al.,* 2005).

In many parts of Ethiopia milk is preserved by smoking milk vessels using wood splinters of *Oleaafricana* to impart desirable aroma to the milk. Smoking was also found to lower the microbial load of raw milk. Smoking of milk vessels is the major method that is traditionally used to preserve raw milk, under these circumstances; the chance of spoilage of milk is very high. A study which was done in Ethiopia showed that Activation of the LPS can maintain the quality of raw milk of cows for at least 7 hours during storage at ambient temperature by inhibiting the growth of the general flora. (Nigussie, 2007)

Bacteria's and other microbes, can only live and reproduce within a certain range of environmental conditions. Factors that can influence the growth and multiplication of microbes are temperature, pH, dissolved gases, osmotic pressure and water availability. Each species of microbe has its own, unique upper and lower limit of temperature for growth, which is a defining characteristic for that species There are merely few studies which were conducted in Ethiopia to evaluate the antimicrobial activity of LPS (Nigussie, 2007). However; the studies were not holistic as they focused only to specific geographical locations of the country. Thus, further study is needed to evaluate the practical usage of this technology in different temperatures so as to improve the shelf life of the milk by controlling the growth and multiplication of spoilage bacteria thereby improving the livelihood of dairy farmers across the country. Therefore, the objective of this study was:

- To assess the antimicrobial effect of the LPS in raw cow milk under different agro-climatic zones taking into account the variability of environmental temperatures toward improving milk quality and safety in Ethiopia.

2. LITRATURE REVIEW

2.1. Bacteriological Quality tests for milk

2.1.1. Total bacterial count

Milk quality is greatly influenced by the microbial load of the milk. When aseptically drawn, milk is sterile; however, it is contaminated during and after secretion and during the normal processes of production and processing. Infection of the mammary gland, udder and teat surfaces, milking equipment and storage tanks all have the potential to contaminate milk. One of the requirements of production of the high-quality milk is maintaining the bacteria count level of microorganisms in a product and to study the hygienic and sanitary conditions, under which milk was produced, handled, transported and processed (Murphy, 1997; FAO/WHO, 1992). Both temperature and storage time influence the multiplication of the micro-organisms, (Jayarao *et al*., 2004). The total bacteria count (TBC) measures the amount of bacterial contamination in milk. Alternative methods of Total Bacteria Determination is bactoscan method which is a technologically advanced method that uses epifluorescent microscopy to count bacterial cells that have been stained with acridine orange (Lachowsky *et al.,* 1997) and Coliform Counts which is indicative of the effectiveness of cow hygienic preparation procedures during milking and cleanliness of the cow's environment (Davidson *et al.,* 2004). In general, as long as the TBC of the milk is less than 100,000 colony forming units (cfu)/ml, it is within the regulatory limit. However, good quality milk has a TBC of < 10,000 cfu/ml (Gleeson *et al*., 2013).

2.1.2. Alcohol test

The test is quick, simple and is used as a screening test. It is based on instability of the proteins when the levels of acid is increased and acted upon by the alcohol. Also increased levels of albumen (colostrum milk) and salt concentrates (mastitis) results in a positive test. The test is done by mixing equal amounts of milk and 75% ethanol (usually 2ml) in a small bottle or test

tube. If the tested milk is of good quality, there will be no coagulation, clotting or precipitation upon shaking (O'Connor, 1995, Draaiyer *et al.*, 2009).

2.1.3. Clot on Boiling

This is one of the oldest tests for abnormal acidity levels in milk, which is brought about by too much acid in milk (pH<5.8). The test is performed by boiling a small amount of milk in a spoon, test tube or any other suitable container. If there is coagulation or precipitation, the milk fails the test. The test is not sensitive to slightly sour milk (O'Connor, 1995; Draaiyer *et al.,* 2009).

2.1.4. Dye reduction test

The tests are based on the changes of certain dyes (put in milk) within a time frame due to oxidation reduction changes resulting from the metabolism of the organism present in the milk; it is an indirect measure of the number of microorganisms present in the milk. These tests include the resazurin and the methylene blue reduction tests. It is generally assumed that the greater the number of bacteria in milk, the quicker the oxygen will be consumed, and in turn the sooner the color will disappear. Thus, the time of reduction is taken as a measure of the number of organisms in milk (O'Connor, 1995; Draaiyer *et al.*, 2009).

2.2. Milk Composition Quality Test

Compositional characteristics are the features of raw milk related to natural composition that has special importance in processing e.g. fat content and total solids. Simple but time-consuming tests have been developed over the years to determine the composition of milk. These tests require laboratories with relatively costly equipment, materials and staff. More recently user-friendly, low-cost and rapid automatic milk analyzers have been developed and successfully

introduced for small-scale as well as large-sale applications. These units require minimal space and give virtually instant results (Draaiyer *et al*, 2009).

2.3. Lactoperoxidase System

The lactoperoxidase /thiocyanate/ hydrogen peroxide system is an indigenous antibacterial system in milk and human saliva. This method primarily used to prevent undue bacterial multiplication in raw milk. Bacteria may grow across a wide range of temperatures, from very cold to very hot. The inhibitory effect of the treatment is dependent on the temperature of the stored milk. LPS has been found to act for temperatures (30, 25, 20, 15^0C), (7-8, 11-12, 16-17, 24-26 period of time respectively in laboratory and field-experiments carried out in different countries with raw milk of an initial good hygienic standard (CAC/GL, 1991).

LPS is made up of three components: lactoperoxidase, thiocyanate, and hydrogen peroxide (Wolfson and Sumner, 1993). The activation of the system depends on the concentration of the two reactants, thiocyanate and hydrogen peroxide. In the presence of hydrogen peroxide, the system catalyzes the transformation of thiocyanate into hypothiocyanite, which has an antibacterial nature (Koksal *et al.,* 2016). The lactoperoxidase system can be activated in raw milk to give antibacterial effect by an addition of thiocyanate as sodium thiocyanate and hydrogen peroxide in the form of sodium percarbonate (CAC/GL, 1991). LP is normally found in sufficient amount in milk; however, thiocyanate and hydrogen peroxide are the limiting factors and need to be added from exogenous source to activate the LPS (Seifu *et al.,* 2004).

Lactoperoxidase is found in the mammary, salivary, and lachrymal glands of mammals and in their respective secretions; e.g., milk, saliva, and tears. In milk and saliva, lactoperoxidase exists in a soluble form, but within the cells of salivary and mammary glands, it is possible that the enzyme could be loosely bound to sub cellular particles (Wolfson and Sumner, 1993).

2.3.1. Thiocyanate (SCN)

The thiocyanate anion is widely distributed in animal tissues and secretions. Thiocyanate is largely a constituent of the extracellular fluid. It is, however, concentrated by certain cells of the body. Whereas the blood serum concentration of thiocyanate is 0.1-0.3%, the salivary concentration has been estimated at 1-27 % (Wolfson and Sumner, 1993). Thiocyanate occurs naturally at concentrations in milk which are adequate for inhibition (Bjorck *et al.,* 1975). The normal levels of thiocyanate in milk depend on the levels of thiocyanate and its precursors in the animals' diet. The concentrations vary between 2.3 and 3.5 milligrams/litter in milk from individual cows and to be around 8 milligrams/litter in bulked milk. Higher levels occur in colostrums and in mastitis milk (Perraudin, 2016).

Thiocyanate is also 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. The source is the anion itself, its esters and other precursors such as nitriles, isothiocyanate, and cyanide. High concentration of thiocyanate is found in yellow turnips and from brassica seeds, it can also have found in cassava, maize, bamboo shoots, sweet potatoes and lima beans (Gaitan, 2004). It is produced by the metabolism of sulfur amino acids and the detoxification of cyanide a well-recognized biological function common to man and animal (Bosch *et al.,* 2000).

2.3.2. Hydrogen peroxide

Hydrogen peroxide is the third component of the lactoperoxidase system. It may be formed endogenously. Many lactobacilli, lactococci, and streptococci produce sufficient H_2O_2 under aerobic conditions to activate the LP system. It may also be added or may be generated by the addition of one of a number of hydrogen peroxide generating systems (Wolfson and Sumner, 1993). H_2O_2 can also be provided exogenously, by addition to the system or in a bound form (e.g. sodium percarbonate, magnesium peroxide). The use of H_2O_2-producing systems such as glucose

oxidase/glucose and xanthine oxidase/hypoxanthine may provide a more effective antimicrobial effect than in the case of added H_2O_2 (Klaasand and Van Hooijdonk, 2000). H_2O_2 can be highly toxic to mammalian cells and bacteria; this effect of H_2O_2 is alleviated, however, in the presence of lactoperoxidase and thiocyanate (Carlsson *et al.,* 1984).

2.3.3. Hypothiocyanite

Hypothiocyanite ion (OSCN-) is believed to be the major intermediary oxidation product of the LPS. It can be considered the hypohalite of thiocyanogen $(SCN)_2$, whose chemical characteristics are similar to those of other hypohalites, including stability in ionic form but instability as the acid (Wolfson and Sumner, 1993). Many factors affect the stability of hypothiocyanite. The decomposition of OSCN- is strongly dependent on the pH of the solution; OSCN- is more stable at pH 7.5 than pH 5.0. OSCN solutions are sensitive to light, yet they are very heat stable. Stability of OSCN- solutions decrease on addition of metal ions (Fe, Ni, Cu, Mn, etc.), glycerol, and ammonium sulfate, as well as the removal of LP (Wolfson and Sumner, 1993). The OSCN inhibits bacterial glyceraldehyde 3P dehydrogenase and thereby stops the bacterial production of acids from sugars (Carlsson *et al.,* 1984).

2.4. Relationship of LPS Components

LP are enzymes (proteins) that are part of the natural, non-immune defense systems in milk and in secretions of exocrine glands such as saliva, tears or intestinal secretions, they do not have any antimicrobial activity of their own, but in the presence of specific substrates (H_2O_2 and Thiocyanate) they constitute a powerful system of defense (CAC/GL, 1991). Thiocyanate ions are the substrate for LP and are normally added to milk at a level of approximately 14 milligrams/litter, the milk should then be mixed to ensure an even distribution of the SCN-. This is followed by addition 30 mg of sodium percarbonate per liter of milk (CAC/GL, 1991). Hydrogen peroxide would be added at a level of 1-10 milligram/litter. Hydrogen peroxide is

unstable and also reacts with proteins, although the latter is unlikely to cause problems at this concentration (FAO, 2005).

2.5. Principle and Mode of Action LPS

Many studies show that this system destroys several bacterial and fungal strains. The effects of different concentrations of SCN -H_2O_2 medium on several antibacterial and antifungal strains were studied to solve dairy industry issues (Welk *et al.,* 2009, Bafort *et al.,* 2014). They are capable of reducing bacterial growth by damaging the cell membranes and inhibiting activities of several cytoplasmic enzymes (Koksal *et al.,* 2016). The use of the LPS does not require the addition of further LP enzyme above the levels of the enzyme occurring in raw milk. As there is no change to the enzyme concentrations naturally present in milk, this component is not considered of toxicological significance (FAO, 2005).

The LP catalyzed reaction yields short-lived intermediary oxidation products called SCN- by using H_2O_2. This may be further oxidized to end-products such as sulfate, $C0_2$, and ammonia or may be reduced back to SCN-. Most researchers agree that the major intermediary oxidation product is OSCN-. Peroxidase-catalyzed oxidation of SCN- results in the accumulation of OSCN-. The hypothiocyanite ion can be produced by two different pathways. First oxidation of SCN- may yield thiocyanogen $(SCN)_2$, which hydrolyzes rapidly to yield hypothiocyanous acid (HOSCN), or OSCN or secondly SCN- may be oxidized directly to OSCN- (Wolfson and Sumner, 1993). These highly reactive intermediary oxidation products inhibit microorganisms by the oxidation of sulphydryl (–SH) groups in their enzyme systems and proteins. The structural damage of microbial cytoplasmic membranes by oxidation of –SH a group is reported as the principal reason that causes the death of microbial cells (Yener *et al.,* 2009).

The enzymatic reaction starts in milk when the hydrogen peroxide (sodium percarbonate) is added. It is completed within about 5 minutes from the addition of H_2O_2; thereafter, no hydrogen

peroxide is present in the milk. Hypothiocyanate has a very short half-life in milk, so that residual levels in milk treated with the LPS do not pose a toxicological risk (FAO, 2005).

The antimicrobial effects of the LP system differ from organism to organism. Rapid inhibition of metabolism and leakage of amino acids and potassium occurs in Gram-positive bacteria. Gram-positive, catalase negative bacteria like streptococci and lactobacilli are generally inhibited but not killed by the activated LPS. Gram-negative bacteria are more difficult to kill and inhibition is more dependent on temperature (between 5 and 20 ^{0}C) and pH (5.5-7). Gram- negative, catalase positive organisms such as pseudomonads, coliforms, salmonellae and shigella may be killed, provided H_2O_2 is supplied exogenously. This difference in sensitivity to the LPS can probably be explained by the differences in cell wall structure and their different barrier properties. Mammalian cells are not affected by oxidation products of SCN_2 and it is suggested that the LPS protect these cells against toxic effects of H_2O_2 (Klaasand and Van Hooijdonk, 2000).

The antimicrobial stability of the LPS depends on the stability of the oxidation products of SCN (Bosch *et a.l,* 2000). The oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins is considered to be the key to the antimicrobial action of the LPS. The structural damage of microbial cytoplasmic membranes by the oxidation of SH group results in leakage of potassium ions, amino acids and peptides into the medium and subsequently uptake of glucose, amino acids, purines, pyrimidines in the cell and synthesis of proteins, DNA and RNA is also inhibited (Kussendrager and Van Hooijdonk, 2000).

2.6. Practical Application of the Method

The practical application of the antimicrobial activity of the LPS can be used in wide varieties of areas such as in dairy industry to prevent milk spoilage, in human patients to prevent dental caries and plaque accumulation, breast cancer, viral infections, gastritis due to Helicobacter Pylori and cystic fibrosis (Sharma *et al.,* 2013, Bafort *et al.,* 2014, FAO, 2005).

The antimicrobial activity of the LPS against a wide variety of milk spoilage and pathogenic microorganisms including bacteria, viruses, moulds, yeasts, mycoplasma and protozoa has been well documented in both laboratory and practical settings. The overall activity is primarily bacteriostatic, the extent of which is dependent on the initial total bacterial load, species and strains of contaminating bacteria and the temperature of milk. While its effectiveness against well-known milk spoilage and pathogenic microorganisms is well established, it was concluded that further studies would be useful on the efficacy of the LPS against milk-borne viruses and emerging pathogenic microorganisms (FAO, 2005). Antibody conjugates of glucose oxidase and to LP have been found to be effective in killing tumor cells in vitro (Stanislawski *et al.,* 1989). In addition, macrophages exposed to LP are stimulated to kill cancer cells (Lefkowitz *et al.,* 1990). Peroxidase-generated hypothiocyanite inhibits herpes simplex virus (Mikola *et al.,* 1995) and human immunodeficiency virus (Pourtois *et al.,* 1991).

Due to its broad spectrum antibacterial properties, LPS was explored for its potential role as an agent to preserve foods and milk, and its use in medicine. Initially, several reports established LPS as a feasible procedure for controlling the growth of bacteria in raw milk at refrigeration temperatures as well as pasteurized milk. It was shown that LPS can be successfully utilized to increase the shelf life of milk in the tropics (CAC/GL, 1991, Yener *et al.,* 2009, Bafort *et al.,* 2014).

LPS was also effective in the prevention of dental caries and plaque accumulation. It was also shown that toothpaste containing LPS reduced the incidence of cryogenic bacteria in children. LPS was also found to inhibit several strains of clinically active gastric pathogen, *Helicobacter Pylori*. LPS was also found to be effective in the bacterial clearance of the airways, indicating a possible application in patients suffering from cystic fibrosis (Sharma *et al.,* 2013). A combination of LP, glucose, glucose oxidase, iodide and thiocyanate are claimed to be effective in the preservations of cosmetics (Galley *et al.,* 1997).

Temperature is one of the most important factors influencing microbial growth. The role of refrigeration and the cold chain in maintaining the quality and safety of both raw and pasteurized milk is well recognized but there are also bacteria's which can grow well in cold temperatures.

Many bacteria are mesophilic, growing best at temperatures of 30ºC to 40ºC. However, psychrotrophic and psychrophilic bacteria can grow at low temperatures, with some strains capable of surviving and growing at temperatures down to 0ºC. *Listeria monocytogenes* is an example of a pathogenic bacterium that can grow at very low temperatures; however, in products such as milk that has a diverse micro flora, it would normally be outgrown by the psychrotrophic spoilage bacteria, such as members of the genera, Pseudomonas, Bacillus and Micrococcus (FAO, 2005).

The most widely recommended industrial application of the LPS in food production is 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 LPS 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 (Seifu *et al.,* 2005).

2.7. Application of Lactoperoxidase system in Improving Milk Quality and Safety

Milk ranks high among other foods and is considered as the most perfect food for human having good sensory properties and all nutrients required for the body for rapid growth and also it prevent or reduce risks of many nutritional deficiency diseases (Zeinhom and Abdel-Latef, 2014). At the same time, it is also an ideal habitat for the growth and multiplication of microorganisms due to its nutritional constitution; therefore milk spoilage is a major problem of the dairy sector in tropical countries due to its nutritional value that support growth and multiplication of bacteria. The high temperature coupled with absence of cooling facilities and lack of adequate transportation means accelerate the spoilage of the milk produced in this area (O'Mahoney and Peters, 1987, Tsadkan and Amaniel, 2016).

Raw milk is considered as a vehicle for the transmission of potentially pathogenic bacteria because of the fact that it is consumed directly and indirectly via consumption of milk products

such as cheeses by large number of people both developed and developing world with greater risk in rural settings. This emphasizes the need for the use of cost effective technologies especially in resource limited countries (Zeinhom and Abdel-Latef, 2014). To overcome this problem as an alternative technology LPS is developed and recommended by FAO /WHO to improve the storage condition of milk in high ambient temperature conditions in tropical and sub-tropical countries (FAO, 2015).

In many parts of the world, the LPS have been used to protect dairy products, particularly in remote areas where farmers are not in close proximity to the market. It is a natural defense system against microbial contamination. All the components of the LPS system occur naturally in human and animal liquid secretions and present no new exposures to the human body (Perraudin, 2016).

LP is the most common enzyme in milk and it is also commonly present in whey, which is the liquid remaining after milk has been curdled and strained. Each LP enzyme contains an iron molecule. The conformation of the protein is stabilized by a chelated calcium ion. The antibacterial effect of the system on milk is based on the oxidation of SCN^- ions catalyzed by the LP enzyme in the presence of H_2O_2. The short-lived components, OSCN- ions, generated in this oxidation reaction have bacteriostatic effect (Koksal *et al.,* 2016). Activity of the LPS has been shown to inhibit the growth of many bacterial species such as *Escherichia coli*, *Salmonella Typhimurium*, *Campylobacter jejuni*, *Staphylococcus aureus, Listeria monocytogenes, Yersinia enterocolitica, Brucella melitensis* (FAO, 2005) in cow milk. However, milk from different species responds differently towards activation of the LPS due to inherent difference in composition of the milk (Seifu *et al.,* 2004).

The dairy industry is the most widely recommended industry for application of the LPS for the preservation of raw milk during storage and/or transportation to processing plants (Seifu *et al.,* 2005). In 2004, the total world milk output was 613 million metric tons of which 263 million metric tons was produced by developing countries – contributing about 30% share of the total world milk production, with small dairy farmers contributing about 70% of the total (FAO,

2005). A rapid assessment of milk which is conducted by FAO in 2003 post-harvest losses in five countries, including the Near East and Eastern Africa shows, in Kenya, for example, the study found that a total of 15.4% of milk was lost at the farm and market level. The total national loss was estimated at 95 million litters, valued at about US$ 22.4 million. The losses at farm level are equivalent to US$15.4 million. Viewed against the poverty level where almost 60% of the populations survive on less than US$ 1 a day, the loss at farm level alone is equivalent to the annual salary for 32,000 rural wage earners on US$ 40 per month (FAO, 2005).

It has been shown that LPS is more cost effective than cooling in areas where milk quantities are large or there is irregular or no power supply because without any additional cost this system can easily transported from place to place without spoiling. This is also the best way to improve the flow of milk from the farm to markets thereby creating additional income for dairy households (FAO, 2005).

In Ethiopia practical use of the LPS is not implemented for controlling the growth of microorganisms in the country. Instead of using LPS, smoking of milk vessels is the major traditional method used to preserve raw milk (Nigussie, 2007).

2.8. Effect of LPS on nutritional composition of milk

Jooyandeh *et al*., (2011) said that Observations from laboratory and field studies indicated that the LPS does not induce any significant adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products. Musa and Hamid, (2013) also revealed that chemical composition of the milk activated with LPS and the control values protein, fat, total solid, and density had no significant difference in all milk samples.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Ada'a district, Bishoftu town, East shoa zone, Ethiopia. The area lies between latitudes 8° 45' 8.10" N and longitude 38° 58' 42.46" E. Bishoftu is located at a distance of 47.9 kilometers southeast of Addis Ababa. According to 2007 national census report, the total population for Bishoftu was estimated 99,928, of which 47,860 were men and 52,068 were women. Topographically, the city is located in tepid to cool sub-moist mid highland at an altitude of about 1920 meters above sea level. The mean annual rainfall of the area is 866 ml with mean minimum and maximum temperatures of 14^{o}C and 26^{o}C, respectively and means relative humidity of 61.3% (NMSA, 2003).

3.2. Experimental design

The study employed an experimental design. The experimental design was based on the previous works (Kassa *et al.,* 2013, Nigussie, 2007, Musa and Hamid, 2013, Marks *et al*. 2001 and Dajanta *et al*., 2008). Milk samples were collected immediately after milking from Addis Ababa University Business enterprise Bishoftu fattening, dairy and poultry farm and kept in icebox containing icepacks and transported to Addis Ababa University College of veterinary medicine and Agriculture, Veterinary Public Health laboratory of the College. Before starting the experiment, the sample was tested for freshness using alcohol test and bacterial load was determined using total bacterial count (TBC) technique to know the initial microbial load. Thereafter, the sample was grouped into two: LPS treated (activated) and non-LPS treated (control). The milk sample from each group was subdivided into sub groups containing 0.5L milk samples and kept at an average temperature representing the four agro-ecology of the country namely, an average temperature for hot, arid zone (37^{o}C), warm to hot, semi-arid zone (24^{o}C), warm to cool, semi-humid zone (18°C) and cool to cold humid zone (14^{o}C) (Ecotravel Word, 2017). The four agro-ecological temperatures were simulated using adjustable incubators. The time of milking, start of the experiment and duration of the storage during the experiment

was carefully recorded. All experiments were repeated three times on different days to have estimated average value of the outcome.

3.3. Activation of the LPS

The LPS was activated by addition of 7mg of sodium thiocyanate and 10mg hydrogen peroxide in 500ml of milk samples followed by thorough mixing as recommended dose by CAC/GL, (1991). In this experiment in order to get 7 mg of NaSCN 0.1g NaSCN was added into 100ml distilled water and then 7ml of the solution was taken. Whereas for H_2O_2 3.3ml of 30% H_2O_2 was added to 96.7 ml of distilled water to get 100ml of H_2O_2 having 1% concentration from which 0.5ml of 1% H_2O_2 was taken for LPS activation per 0.5L of milk.

3.4. Evaluation of the experiment

3.4.1. Alcohol test

Milk freshness was tested by using alcohol test. For this test, 75% Ethanol was mixed with equal volume of milk as recommended by O'Connor (1994) and used by several researchers elsewhere (Musa and Hamid, 2013; Nigussie, 2007; Kassa *et al.,* 2013; Dajanta *et al.,* 2008). The principle of the test is milk coagulation which indicates milk quality deterioration and it was considered to be a break point for shelf life of milk.

3.4.2. Analysis of milk composition

The nutritional constituents of milk (fat, protein, lactose and water) of the activated milk samples were examined using milk analyzer Lactoscan according to the manufacturer instructions.

3.4.3. Determination of bacterial load

Total microbial count for milk was estimated using the standard plate count (SPC) method. Each milk sample (1 ml) was transferred into sterilized tubes containing 9 ml of sterilized distilled water and properly mixed, forming 10^{-1} dilution. Serial dilutions were made up to $1{:}10^{-8}$ dilution level. About 20 ml of plate agar (sterilized and cooled to 45 ^{o}C) was added to each Petri dish and then, 0.1 ml of solution was spread in Petri dishes after the agar was set (Richardson, 1985). The Petri dishes were placed in an incubator at 37^{o}C for 48 hours. The estimated colony count was computed by the formula described by IDF (1991).The resulting colonies were counted and expressed as colony forming units per milliliter (cfu/mL^{-1}) of sample, and then the microbial counts were expressed as their logarithms.

3.5. Statistical Analysis

Statistical analysis was performed using Genstat Statistical software (15th edition). The comparison of the effects of LPS between the experimental groups was evaluated based on one-way ANOVA analysis result by comparing the mean value of bacterial load. The effect of the system on the shelf life of milk storage without spoilage was analyzed using descriptive statistics based on the result of alcohol test.

4. RESULTS

4.1. Effect of LPS on microbial load in milk

This study investigated and compared the microbiological load of LPS treated (activated) and LPS untreated (control) raw milk at different time intervals kept at an average temperature representing the four agro-ecology of the country. The activity of the LPS was assessed every two hours during the storage time (Table 1). The effect of the LP system varied with temperature difference. This study indicated that LPS significantly decreased ($p<0.05$) bacterial multiplication in all temperatures.

Table 1: Mean total bacterial counts (TBC) in control and LPS activated raw cow's milk stored at different temperatures

Temp	Group	Storage period (hr)						
		0	**2**	**4**	**6**	**8**	**10**	**12**
37°c	CONT	9.929±0.101	10.073±0.101	10.196±0.101	10.244±0.102	10.299±0.101	10.336±0.101	10.401±0.101
	ACT	9.929±0.101	9.996 ±0.101	9.755 ±0.101	9.697 ±0.101	9.761 ±0.101	9.859±0.101	9.933 ±0.101
24°c	CONT	9.851±0.101	9.970±0.101	10.010 ±0.101	10.164±0.101	10.260±0.101	10.314±0.101	10.355±0.101
	ACT	9.851±0.101	9.899 ±0.101	9.723 ±0.101	9.680 ±0.101	9.632±0.101	9.689 ±0.101	9.755 ±0.101
18°c	CONT	9.842±0.101	9.955±0.101	10.019±0.101	10.082±0.101	10.139±0.101	10.197 ±0.101	10.297 ±0.101
	ACT	9.842±0.101	9.951±0.101	9.874±0.101	9.822 ±0.101	9.784 ±0.101	9.780±0.101	9.756 ±0.101
14°c	CONT	9.925±0.101	9.988±0.101	10.046±0.101	10.078±0.101	10.150±0.101	10.240±0.101	10.277±0.101
	ACT	9.925±0.101	9.980 ±0.101	9.888±0.101	9.859 ±0.101	9.823 ±0.101	9.802 ±0.101	9.772 ±0.101

CONT= Control, ACT= Activated

There was a significant reduction ($P<0.001$) in bacterial load when milk was activated by LPS between the means. This reduction in microbial load occurred within the first 3-4 hr. The use of LPS in milk which stored at 37^0c, 24^0c, 18^0c and 14^0c had reduced the microbial load by 66.7%, 75%, 67.9% and 64.3% after 6, 8 and 12hrs respectively. At temperatures of 18^0c and 14^0c there was no rise in microbial load until 12 hrs.

Table 2: Microbial cell count (cfu mL^{-1}) of raw milk samples after final reduction at different temperatures

Temperature	**status**	**Initial microbial count**	**Activated LPS**	**Percent reduction***
37^0c(6hr)	Control	9.53×10^9	1.58×10^{10}	66.7%
	Activated	9.53×10^9	5.26×10^9	
24^0c(8hr)	Control	7.55×10^9	1.84×10^{10}	75%
	Activated	7.55×10^9	4.45×10^9	
18^0c(12hr)	control	7.16×10^9	1.987×10^{10}	67.9%
	activated	7.16×10^9	6.867×10^9	
14^0c(12hr)	Control	9.71×10^9	1.96×10^{10}	64.3%
	Activated	9.71×10^9	7×10^9	

*Percent reduction in viable count was calculated as follows: 100 x (viable count in the control milk at the final reduction hr in activated milk – viable count in the LP activated milk at the final reduction hr / (viable count in the control milk at final reduction hr in activated milk).

4.2. Effect of LPS on keeping milk quality on different storage temperatures

Effect of LPS on milk freshness was assessed by alcohol test and the result showed that the use of LPS treatment has improved significantly ($p<0.05$) the shelf life of milk as compared to the LPS untreated milk. The LPS treated milk stored at temperatures of 37^0c, 24^0c, 18^0c and 14^0c had additional 4, 6, 8 and 8 hours shelf life respectively as compared to the LPS untreated milk stored in similar condition.

4.3. Effect of LPS activation on milk composition

Activation of milk with LPS system has no significant effect on milk composition (fat, protein, Lactose and water).

Table 3: Mean composition of activated milk at different storage periods (hrs)

	Storage period (hrs)						
Milk Composition	0	2	4	6	8	10	12
Fat	6.41± 0.793	4.750± 1.121	1.987± 1.121	5.773± 1.121	5.787± 1.121	4.867± 1.121	4.327± 1.121
Lactose	3.73± 0.363	3.693± 0.514	0.950± 0.514	3.653± 0.514	3.647± 0.514	3.770± 0.514	3.933± 0.514
Protein	3.22± 0.491	3.297± 0.694	1.423± 0.694	3.240± 0.694	3.240± 0.694	3.317± 0.694	3.493± 0.694
Water	0.087± 2.042	6.520± 2.887	1.023± 2.887	6.540± 2.887	6.577± 2.887	4.937± 2.887	2.820± 2.887

5. DISCUSSION

This study investigated and compared the microbiological quality of inactivated control and LPS activated raw milk at different time intervals in different environmental temperatures of Ethiopia. The results of the study showed that activation of row cow milk with LPS reduces the growth of microorganisms and treatment enhanced the shelf life of row cow milk. The result revealed that activated milk has statistically significant effect ($p<0.05$) on the growth of microorganisms as well as extending the shelf life of milk. In the study bacteria load in LPS activated milk were decreased in count as compared to the control raw milk. LPS has the ability to catalyze the oxidation of SCN by H_2O_2 with the production of antibacterial hypothiocyanate (OSCN-) (Reiter, 1985; Ozdemir *et al.,* 2002; Sisecioglu *et al.,* 2009, 2010). The decrease (0.232 log units) in total bacterial count observed in LPS-activated milk at 6 h of storage as compared to the initial count suggests that activation of the LPS can maintain the quality of raw milk of cows for at least 6 h when stored at 37^0c by inhibiting the growth of the general flora. Valdez *et al.,* (1988) found LPS was inhibitory against microorganisms up to 8 hours in sample of bovine milk stored at 30^oC. Similarly, Masud *et al*., (2004) found shelf-life of 6 hours for control buffalo milk and 8 hours for LPS activated sample kept at 35^o C. Hence it can be said at higher temperature, the effect of LPS in microbial load is low. According to FAO/WHO (2005), the shelf-life of hygienic raw milk is increased by 4-7 hours at 31-35^{o}C by the activation of LPS and the efficacy of the LPS persists for a limited period of time, which decreases as the ambient temperature increases. At the same time when stored at 24^0c, 18^0c and 14^0c there was a decrease with 0.222, 0.062 and 0.122 log units at 8, 12 and 12 hr of storages respectively as compared to the initial microbial load. Thus, LPS treatment could be more effective as temperature decreases. The result in this study was in agreement with findings of Ponce *et al*., (2005) and Kassa *et al.,* (2013).

According to Barrett *et al*., (1999), the alcohol stability can be used as a good indicator of milk freshness due to its reliable and consistent results. The result indicated that the storage period had significant ($P < 0.05$) effect on alcohol test of the milk samples of the different treatments. As the storage period progressed, the milk freshness also increased when compared to the non-treated control milk samples. Similarly the qualities of the milk samples were not deteriorated at

temperatures of 37^0c, 24^0c, 18^0c and 14^0c until 6, 8, 12 and 12 hours respectively. This result showed that introduction of the LPS can extend milk freshness similar to the reports by Marks *et al*., (2001) and Dajanta *et al.,* (2008).

The effect of LPS on raw cow milk composition showed that no significant difference with milk composition of all milk samples stored in all temperatures, this implies that activation of milk with LPS has no impact on milk composition. These results were in line with the findings of Kumar and Mathur (1989), FAO/WHO (2005), Boulares *et al.,* (2011) and Seifu *et al.,* (2004).

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that activation of raw cow milk with LPS potentially decreases the microbial load and extends the shelf life of the milk ranging from 6 to12 hours depending on the storage temperatures. The reduction in microbial load started within the first 3-4 hr of activation of LPS. The study also showed that activation of LPS has no effect on nutritional composition of milk. Hence, based on the findings of the current study the following recommendations were given:

- It is recommended that the concerned bodies should raise awareness on the importance of the system and extends the result of this study in the four agro-ecology zones of the country for improving the preservation and reducing milk loss due to microbial spoilage.
- Further studies should be done in different parts of the country under the specific temperatures especially in the milk-shed areas particularly by rural smallholder dairy farmers to establish the shelf life of milk as a result of the activation for practical usage of the system.
- Further study should also be done on the cost and benefit of LPS application.

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