Mastitis problem in East and Southern Africa

by

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Declaration

I, **Sserunkuma Phillip** declare that this dissertation has not been submitted to any other University than the University of KwaZulu-Natal and that it is my original work conducted under supervision of Prof I.V.Nsahlai. All the aid used in the production of this work and all references have been duly accredited.

Sserunkuma Phillip…….. **Date…….**

Approved as to style and content by:

Prof IV.Nsahlai…….. **Date……**

Abstract

Bovine mastitis is a disease caused by inflammation of the udder. It is one of the major causes of losses in dairy herds. Losses are caused by reduction of milk and milk with high SCC (Somatic cell count) has to be discarded because according to standards this milk is not supposed to be sold. The study was based on two broad objectives, to evaluate management factors that predispose animals to mastitis and the use of alternative therapies in treatment of mastitis. The alternative therapies referred to hear are the use of ethno veterinary medicine and trisodium citrate in treatment of mastitis.

Management factors that predispose animals to mastitis were evaluated in two countries that is in Uganda (Entebbe municipality) and South Africa (KwaZulu-Natal province) to find out how the mastitis problem. Structured questionnaires were used in both areas of study. Sixty percent of farmers had numerous cases of mastitis and 40% in Entebbe municipality in 2014. In KZN 86% of farmers had between 1-20 cases of clinical mastitis and 14% for cases between 21-70 cases in the month the survey was done in 2014. Fifty seven percent of farmers had mastitis in the dry season and 43% in wet season in Entebbe municipality. In KZN, 79% of farmers had mastitis in the wet season and 21% in the dry season. Dairy farmers who practiced washing teats had a tendency of associating (χ^2=3.21; P<0.07) with mastitis prevalence (MP). Also dairy farmers who practiced spraying regularly had a tendency associating (χ^2=3.21; P<0.08) with MP. In KwaZulu-Natal survey the practice of providing separate calving paddock had a tendency associating (χ^2=3.00; P<0.08) with MP. All the management factors on milking hygiene practices had no association (P>0.05) with MP. The practice of managing teat closure, nutrition and

other management factors in had no association (P>0.05) with MP. All dairy farmers in KZN showed that they practiced proper machine maintenance and functioning.

0n trisodium citrate study, the treatment groups were as follows T1=control, T2=15gm, T3=30gm and T4=45gm. Trail 1 was done in sept 2014 n=18 and Trial 2 July 2015 n=18. The main variable in the study was somatic cells and covariates were breed, lactation number and milk yield. The trails lasted 10 days and data was recorded from day 1, 3rd 7th and 10th day. There was a gradual decrease of growth rate of bacteria for 1-3 under treatment.

A decrease was noted for colonies 4-10 with good results recorded against most of the treatments. Treatment did not react against bacteria growth of >10 colonies.

Milk yield had a significant (P<0.05) on SCC on day 1. Breed and lactation number had no significant (P>0.05) from 1, 3, 7 and 10th post treatment. Generally treatment had no significant (P>0.05).

In ethno veterinary medicine study, Plant species were selected on the basis of their known antibacterial activity, use in ethno veterinary medicine and their ready availability for *in vitro* testing against a panel of bacterial species implicated in causing mastitis (both ATCC strains and clinical isolates). Water and acetone extracts were prepared from various plant parts of *Acacia nilotica*, *Tetradenia riparia, Aloe arborescens* and *Crassula multicava.* Antimicrobial activity was determined using a serial microdilution assay and cytotoxicity was evaluated against a mammalian kidney cell line using a tetrazolium-based colorimetric (MTT) assay.

Aloe arborescens and *Crassula multicava* were not generally active against tested bacteria. Acetone extracts of *A. nilotica* bark and *T. riparia* flower extracts were most active against Gram-positive bacteria. Activity against Gram-negative species, notably *Proteus vulgaris* and *Enterobacter aerogenes*, was also noted with MIC values as low as 0.0195 mg/ml. The best selectivity index (SI) value of 4.2205 was obtained by the *T. riparia* flower acetone extract against the field strain of *Streptococcus uberis.* Although toxicity of most of the extracts to mammalian cells was noted, good SI values indicate that activity was greater than toxicity for some extracts. These extracts, or purified active compounds derived from them, may prove useful in further investigations of alternative mastitis treatments.

Key words: Predisposing management factors ethnoveterinary medicine Somatic cell counts

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Dedication

To: The Almighty God who has given me strength and knowledge to accomplish this work. My mum, all my relatives and friends for motivation, encouragement, inspiration and support which has led to the success of this project.

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Chapter 1 General introduction

1.0 Back ground

Mastitis is defined as inflammation of the parenchyma of the mammary gland (IMI) (Souto *et al*., 2010) derived from Greek word "**Mastos**" which means breast or udder and suffix **itis** meaning inflammation (Kehrli *et al*., 1994). Inflammation is caused by physical or chemical irritants; Physical inflammation of the udder serves as a secondary route for mastitogenic pathogens. These mastitogenic pathogens go into the second line of defence that is after keratin which serves as the first line of defence in the teat canal (Martin and Andrew, 2004), colonise, multiply and produce toxins which affect the glandular tissue of the mammary epithelium due to fibrosis and indunation. This incidence can happen even without physical trauma to the udder as bacteria can easily move from one cow to another.

When this happens, polymorphonuclear neutrophils (PMN) leukocytes which are an integral part of the first line of innate immunity move from the bone marrow towards the invading bacteria in large numbers, this response is mediated by chemical messengers or chemotactic agents from damaged tissues. These masses of PMN then pass between milk producing cells into the lumen of the alveolus and this increases the somatic cell (SCC) damaging the secretory cells. Somatic cell count mainly consists of dead PMN and epithelial cells. Milk from healthy mammary glands should contain below 200,000 somatic cells/ml. A value of SCC above 300,000 is abnormal and is an indication of inflammation of the udder. There is plaethora of evidence that the dairy cow milk has a natural level of 100,000-150,000 somatic cells/ml and higher SCC indicates secretory disturbance (Hillerton, 1999). The most common mastitis causing pathogens are found either in the udder as contagious

pathogens or cows surroundings environmental pathogens such as beddings, manure and soil etc (Jones et al., 1984).

1.1 History of Gram stain

This procedure was developed by Christian Gram in 1884. In this procedure cells are stained with basic dye crystal violent, treated with potassium iodide mixture to fix the stain washed with acetone or alcohol and counter stained with a paler dye of different colour for example, Sufrain whereas most bacteria take up the initial violent stain only gram-positive retains it during subsequent steps. This empiric procedure was used for classifying bacteria for nearly 75 years before mechanism was traced to cell wall, the gram positive bacteria have a thicker cell wall which prevents elution dye-1_2complex (Claxton and Ryan, 1980). Gram Positive Bacteria include: *Staphylococcus spp*, *streptococcus spp* and *Mycoplasma* whereas Gram Negative bacteria includes *E.coli*, *Klebseilla pneumoniae*, *Enterobactor SPP, Serratia spp Pseudomonas spp* and *Proteus spp* (Agoston, 2010; Hogan and Hary 2003; Irene *et al*., 2011).

There are two basic mechanisms by which bacteria cause disease that is by invasion of tissues and production of toxins. Common contagious mastitogenic pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae mycoplasma*, *Staphylococcus chromogenes* and *cornye bacterium bovis* (Quinn *et al*., 1999). Enviromental pathogens include: *Streptococcus uberis*, *Streptococcus dygalactiae* (Santos and Fonseca., 2007; Bannerman and Hare., 2003; Jones *et al*., 1984; Khan and Khan 2006*) Enterobacter*, *Klebseilla spp, proteus spp* etc. Other pathogens include: Fungi and algae (Riberio *et al*., 2008).

Etiology

Streptococcus agalactiae*: S. agalactiae* is one of the most important gram positive mastitogenic pathogens and is transmitted directly among cows during milking. It affects the gland cistern and ducts of the mammary gland causing irritation leading to subclinical mastitis which may result into clinical mastitis (Erika *et al*., 2012).

Mycoplasma species: This mastitogenic species resides in the cow's respiratory and genital tract. *Mycoplasmas* damage the secretory tissue, induce fibrosis of glandular tissue and lymphatic nodules (Sandholm and Pyorala, 1990). This pathogen requires special culture media and is resistant to all treatments. According to Gonsalez and Wilson (2003) susceptibility to mycoplasma infection is high in the early stages of infection.

Staphylococcus aureus*:* S. aureus is one of the most important worldwide causes of mastitis (Martin and Andrew, 2004) and is the most commonly isolated udder pathogen. In South Africa *S. aureus* is the most common mastitogenic pathogen (Worobo *et al*., 1995).

Characteristics of *S. aureus*

- Ability to evade and influence the host immune system.
- Production of various kinds of enzymes and toxins which cause damage to the mammary tissue.

- Can survive in keratin of teat canal of healthy cows and secrete a substance which under normal circumstances inhibits bacterial growth.
- Capability to resist phagocytosis from PMN for example some *S. aureus* strains binds (Fragment crystallisable zone) FC receptor portion of antibody molecules making the bacteria unrecognisable and even when phagocytised can still survive and multiply inside neutrophils (Martin & Andrew, 2004).
- *S. aureus* also produces a range of extracellular products which are classified alphatoxin and luecocidin. These toxins both have antiphagocytic activity which in sufficient levels is lethal.
- The Alpha-toxin induces lysosomal disruption within the neutrophil whereas leucocidin leads to external extrusion of lysosomal granules.
- Alpha–toxin also interacts with lipid especially in cell membrane and bind to vascular smooth muscle thereby creating contraction spasm and consequent ischaemia and anoxia (Bramley *et al*., 1989).
- Production of enzyme coagulase is another feature of S. aureus which causes plasma clot via activation of tissue factor thrombin which in the presence of thrombokinase reacts with active fibrinogen to form fibrin fibres. Experimental studies suggest the clumping of bacteria cells hindering phagocytosis. Due to aggregation between bacteria and leukocytes clots, this clotting factor blocks ducts and prevents complete removal of milk. Therefore, damage to epithelial cells and small ducts results in formation of scar tissue and sometimes permanent loss of function of portion of gland.

Environmental pathogens

These include mainly

- *S.uberis*, *S.dysgalactiae*, fungi and algae.
- Coliforms include; *E.coli*, *Klebsiella pneumoniae* and *Enterobactor aerogenes* (Santos and Fonseca, 2007; Joe and Harry, 2002; Bradley, 2002; Bannerman and Hare, 2003).

Streptococcus dysgalactiae: S. dysgalactiae is classified as environmental pathogen though it can also spread from cow to cow exhibiting some contagious features (Smith and Hogan, 2003). The pathogens go through the mammary gland for example during machine milking that is through teat cap liners. Vacuum fluctuations also lead to exposure to new infections (Christina *et al*., 2012). Therefore, these pathogens can persist in tissue and may be protected from antibiotic therapy since they can adhere to tissues without losing viability (Philpot and Nickerson, 1999).

S. uberis: Are gram positive bacteria and is one of the most isolated species from cases of mastitis. It is able to adhere to and taken up by epithelial cells and persist cellulary and is responsible for chronic infections (Tamilselvam *et al*., 2006). This pathogen spreads to infected cows through environmental contact and its importance is marked in early dry period (Christina *et al*., 2012).

Coagulase negative staphylococci (CNS): CNS are considered opportunistic gram positive mastitic pathogens resident colonisers (Hogan, 1997). CNS is a major cause of IMI (intramammary infection) in lactating cattle with subclinical and clinical mastitis (Hogan, 1997; Fox, 2009). The most commonly isolated species of CNS

from bovine mastitis are *staphylococcus epidemitis*, *staphylococcus chromogenes*, *staphylococcus lyicus* and *Staphylococcus stimulans* (Pitkala, 2004, Sampimon, 2009).

Coliforms: Coliform bacteria occupy several inhabitants of the cow's environment.

E.coli is normal habitant of the GIT of warm blooded animals. *Klebseilla spp* and *enterobactor spp* are found in soils, manure grains and git of animals as well. *Klebseilla spp* are non-motile and can utilize citrate as the source of carbon whereas enterobacter are motile and also utilize citrate. *E. coli* doesn't utilize citrate as carbon source and greater than 90% of strains are motile.

Psuedomonas spp and *proteus spp* infections are sources of contaminants by hose pipes used in washing of udders. This genera of coliforms is characterised by mobility and utilisation of citrate (Hogan, 1997).

Coliform bacteria do not colonise inside the mammary gland but multiply in the secretion without attachment to epithelial surfaces. The more rapidly coliforms can adjust metabolically to mammary secretion, the more rapidly bacterial numbers increase and disease can occur (Hogan *et al*., 1992). Virulence factors for coliforms also have ability to survive at near anaerobic conditions.

Mode of evasion of cells by coli forms

There are differences in susceptibility to phagocytosis among coliforms strains which is due to variability of surface exposure to antigens.

Capsules produced by *klebseilla pneumonia* isolated from bovine intra-mammary infections block depositions of complement and camouflage against antibody mediated opsonisation (Williams *et al*., 1988).

Most coliforms *pseudomonas spp*, *proteus spp* and *E.coli* produce capsular material associated with chronicity of disease.

E.coli strains with O Serotype 08 and 09 posses' antiphagocytic factors that are not related to capsule (Hill, 1983).

1.2 Justification

There has been increasing failure of chemotherapeutics, antibiotic resistance exhibited by pathogens (Shyamapada *et al*., 2011) and health risks posed by their residues to human and animals (Irene *et al*., 2011).This necessitates alternative prevention and treatment methods. Several associated risk factors tend to lower the resistance of the udder to infections and create conditions that predispose the mammary gland to pathogenic micro-organisms like tick bites, leaving the udder wet after washing, and improper use of milking machines (Aminu *et al*., 2012; Pankey *et a*l., 1982; Plozza *et al*., 2011). Therefore, there is great need to find out how these associated risks affect the prevalence of mastitis in dairy herds.

Nutrition affects immune system both specific and innate, therefore, deficiency in Vitamin E, Selenium, citrate and other anti-oxidants predisposes the animals to mastitis (Shook, 1991; Whitaker *et al*., 1997, Weiss *et al*., 1997). This leads to a great need to exploit nutrition therapy as an alternative to antibiotics. There is an increasing use of herbal plants as health promoters in both scientific and consumer circle (Hashemi and Davoodi, 2012; Treece *et al*., 1996). A number of physiological products, microbial products and herbs products have properties that boost the

immune system. Many herbs are being used by veterinarians fruitfully to treat a variety of conditions in animals as well as birds. Herbs provide potent anti-inflammatory, antibacterial, antiviral and antifungal benefits. Therefore, ethno veterinary medicine deals with people's knowledge, skills, methods practices and beliefs about care of their animals and to keep them healthy. This practical experience has been passed orally from generation to generation (Maria *et al*., 2011; Tichaczek, 1992). Natural medicine is more suitable for animal and human health care, with the advantage of low cost and total safety (Radostitis *et al*., 2007). In the era of emerging antibiotic resistance and residual effects in food products, these can play a wonderful role in safeguarding health of humans and animals.

1.3 Objectives

Specific objectives of the study were:

1. To evaluate the relationship between predisposing risk factors on the prevalence of mastitis.
2. The role of trisodium citrate in treatment of subclinical mastitis.
3. The reaction of specific herbs in-vitro with mastitic pathogens.

1.4 Hypotheses

The study will be guided by the following null hypotheses:

1. There is no significant relation between predisposing factors on prevalence of mastitis in dairy herds.
2. There is no significant effect of trisodium citrate on Subclinical mastitis.
3. There is no significant relationship between selected herbs and mastitic pathogens.

Chapter 2: Literature review

2.0 Introduction

Mastitis is very important to the dairy industry. Understanding the various form mastitis help the farmers to devise methods of treatment before it become adverse. This literature discusses, the relationship between somatic cell count and mastitis, predisposing factors to mastitis, mammary gland immunity, the relation between nutrition and immunity, management aspects that affect mastitis, bacteriocins, dry cow therapy and ethno veterinary medicine.

Forms of mastitis

Sub acute: This form of mastitis is present when there are obvious changes in the udder but examination of the milk reveals an increased somatic cell count and alterations in chemical properties of milk.

Acute: Acute mastitis is one of the most commonly treated and very common in high producing dairy cows. Animals suffering from acute mastitis have elevated body temperature above 39^0C, the mammary gland becomes painfully swollen followed by milk becoming microscopically abnormal (Rahalv and Kumar, 2009).

Per acute: This is a dangerous form of mastitis; it destroys extensive portions of the udder. The general wellbeing of the animal is affected and frequently leads to death of the animal. Symptoms of this form are characterised by pain, fever above 41^0C , swelling, redness, stock depression, shivering, dehydration and body weight loss are common in affected cows.

Chronic mastitis: This occurs when the quarter fails to respond to treatment over time, bacteria survive, show abnormal clinical changes like lumps and sometimes quarters are swollen (Claxton and Ryan, 1980).

2.1 Somatic cells

Somatic cells are mainly milk-secreting epithelial cells that have been shed from lining of the gland and white blood cells (leukocytes) that have entered the gland in response to injury or infection. These somatic cells include 75% dead leukocytes (neutrophils, macrophages, and lymphocytes) and 25% epithelial cells. Somatic cells are indicators of both resistance and susceptibility of cows to mastitis and can be used to monitor the level of occurrence of subclinical mastitis in herds or individual cows.

Somatic cell count (SCC) is a useful predictor of intra-mammary infection (IMI) and therefore, an important component in assessment of milk quality, hygiene and mastitis control. Epithelial cells or cells which produce milk are frequently found in udder secretions including the dry gland, at levels ranging from 0-7% of the cell population. Under normal udder conditions epithelial cells are shed, however, during infection these numbers increase. During inflammation of the udder the major increase in SCC is due to the influx of neutrophils into milk to fight infection. Normally milk from a healthy mammary gland is supposed to have a SCC 1×10^5 cell/ml while bacterial infections can cause it to increase to above 1×10^6 cells/ml (Bytyqui *et al*., 2010).

2.1.1 Factors affecting somatic cell count

Age and breed: A number of researchers have reported that SCC increases with increasing age (Beckley and Johnson, 1996; Blackburn, 1996). This increase is primarily due to increased prevalence of infection in older cows due to age. SCC variation has been noted between breeds of dairy animals (Sharma *et al*., 2011).

Parity, season and stress: The level of SCC has been reported to be influenced by parity (Blackburn, 1996). Somatic cell counts are generally lowest during the winter and highest during summer (Khate and Yadav, 2010). During summer the growth of environmental bacteria is increased in bedding material of housed stock due to favourable temperature and humidity.

Diurnal variation: In general SCC is lowest just before milking increasing rapidly on stripping and may persist for up to 4 hours after milking then gradually declines up to 70-fold for individual quarters (White and Rattry, 1965). Day to day variations in cell counts could fluctuate to more than 40% without any of the circumstances described.

Milking interval: The length of milking interval can influence somatic cell counts to vary either by increasing or decreasing, however SCC tend to be higher following a shortened milking interval.

2.1.2 Predisposing factors of mastitis

There are several accessory and contributing factors which lower the resistance of the udder and create conditions that lead to invasion of mammary tissue by micro-organisms. These include traumata, tick bites, exposure of the udder to draughts, cold floors, incomplete and irregular milking, rough handling of the udder, the careless and improper use of the milking machine as wells as feeding to much concentrates.

Traumata: Apparent and in apparent injuries may provide portals of entry of bacteria and so cause mastitis. Studies by Christianen and Neilsen (1934) have shown that provided the teat was normal and sphincter not impaired, external infection could be minimised. The duct and sphincter of the teat may act as barrier to the passage of pathogens (Little, 1937). Under natural conditions the teat is liable to be injured by sharp objects like protruding nails, barbed wire thorns or by use of instruments like siphons and dilators in the hands of untrained persons. Mechanical injury may also result when cows are kept in improperly constructed stables, when the gutters are faulty, the stalls are narrow and partitions are not provided. Sometimes poking by other cows or vigorous sucking by strong calves may also cause injury to teats which often lead to mastitis.

Improper and incomplete milking: Research by Schalm and Mead (1943) has shown that improper milking lead to the development of mastitis. When a cow is incompletely or irregularly milked, too much milk is allowed to accumulate in udder thus distending it abnormally and making it more vulnerable. The physiology of the gland is disturbed as tissue becomes more susceptible to infections.

Milking machine: When the milking machine is not properly operated the teat orifice and duct are liable to be injured. This creates favourable conditions for entrance of pathogenic bacteria some of which may occur on the surface of the teat. The most common abuses that may lead to mastitis are excessive negative pressure in operating the machine, improper sterilization of teat cups and delayed removal of cups from teats. The mammary tissue is unduly strained, readily injured and therefore liable to become infected during drying-off period.

Improper feeding: A high protein deity tends to interfere with the normal metabolism and may cause an increased oedema and congestion of the udder.

Period of lactation: As a rule a cow appears to be most prone to infections after parturition, early lactation and during the dry period especially first two to three weeks probably due to increased oxidative stress and reduced antioxidant defence mechanisms (Sharma *et al*., 2011).

Hereditary factors: Cows in some families are more resistant and less liable to infection than females from other families. The conformation of the udder and shape of the teat are inheritable characteristics that may also lead to mastitis.

Stable milking hygiene: Unsanitary milking methods, wet milking and poor stable hygiene lead to the spreading of the infection. Before milking the teats of each cow should be washed thoroughly with clean water and a separate clean cloth must be used for each cow.

Age: It has been found out that occurrence of mastitis in quarter's increases with age (Emanuelson and Pearson, 1984).

Nutrition: The quality and plan of nutrition appears to be an important factor that influences clinical manifestation of mastitis in heifers and cows. Vitamin E is one of the important supplements in dairy feed to boost immune response of cows as it has been reported to enhance the neutrophil function as well as phagocytic properties of neutrophils after parturition. Vitamin E combined with selenium, acts as an antioxidant by preventing oxidative stress (Chamberlain and Wilkenson, 1996; Mustacich and Powis, 2000). Investigations have demonstrated that neutrophils of selenium supplemented cows are more effective at killing mastitis causing

microorganisms than those that were supplemented with selenium (Erskine *et al*., 1989; Chew, 1993). Beta carotene and vitamin A have also been effective in preventing the occurrence of mastitis, most probably due to their antioxidant and immune enhancing properties and contribution to mucosal surface integrity of mammary gland (Sordillo *et al*., 1997).

Weather and climate: The incidence of mastitis is greatly influenced by water conditions, prevailing climatic conditions, heat, humidity, cold and drought. A higher incidence rate of mastitis has been reported to occur in summer. Another study has reported a higher incidence of coliform mastitis during cold months (Jingar *et al*., 2014).

2.2 Mammary gland and immunity

The mammary gland is protected by two defence mechanisms: the innate and the specific immunity. The innate immunity is also known as non-specific or none immune responsiveness and is the first line of defence during early stages of infection. Therefore, if none specific defence mechanisms function adequately, most pathogens are readily eliminated within a short period of time and before the specific immune system is activated (Philpot and Nickerson, 1999).

The innate defences are mediated by the physical barrier of the teat, macrophages, neutrophils, basophils, eosinophils, natural killer cells and soluble factors.

When a pathogen evades the mammary gland and is not completely eliminated by the innate defence system the specific or acquired immunity is triggered. The host encountering the same antigen more than once leads to high level of reactivity which results to immunological memory compared to the first exposure to the bacterial antigen, hence a memory mediated response is normally faster. The immune system

can be able to distinguish self from non self and react only to foreign antigens through genetically diverse membrane bound proteins called major histocompatibility complex (MHC).

2.2.1 Antigen presentation

A specific immune response only occurs if antigens are combined with MHC molecules on the surface of certain cells this process is referred to as antigen presentation. Recognition of pathogenic factor is mediated by macrophages, lymphoid populations and immunoglobulin's (Ig) or antibodies. Therefore, both the acquired and innate mechanisms need to be highly interactive and co-ordinated in order to provide optimum protection from mastitis.

2.2.2 Anatomical defence system

The teat serves as the first line of defence and safe guards the mammary gland from invading pathogens. Sphincter muscles of the teat maintain tight closure between milking, hindering bacterial penetration. The teat canal is a lined with keratin stratified squamous epithelium which is crucial in maintenance of barrier function of teat end. This means that accumulation of keratin provides a physical obstruction to udder pathogens hence hindering their migration into gland cistern. Keratin can completely occlude the duct during non- lactating periods (Nickerson, 1987).

A number of antimicrobial agents have been identified in the teat keratin. These are esterified and none esterified fatty acids which include myristic acid, palmitoleic acid and linoleic acid. The cationic proteins associated with the keratin lining can bind electrostatically to mastitis pathogens after their cell walls and render them more susceptible to atmospheric pressure (Hogan *et al*., 1987).

As parturition approaches, considerable fluid accumulation occurs within the bovine mammary gland, causing increased intramammary pressure (Oliver and Sordillo, 1988). It has been observed that neutrophilic numbers are relatively low in healthy mammary gland (<10^5cells/ml). However, their number constitute greater than 90% of the total mammary leukocyte population and during IMI by mastitis (>10^6Cells/ml). These non-specific cells travel from the blood to the mammary gland in response to a variety of inflammatory mediators to phagocytise and kill bacteria pathogens.

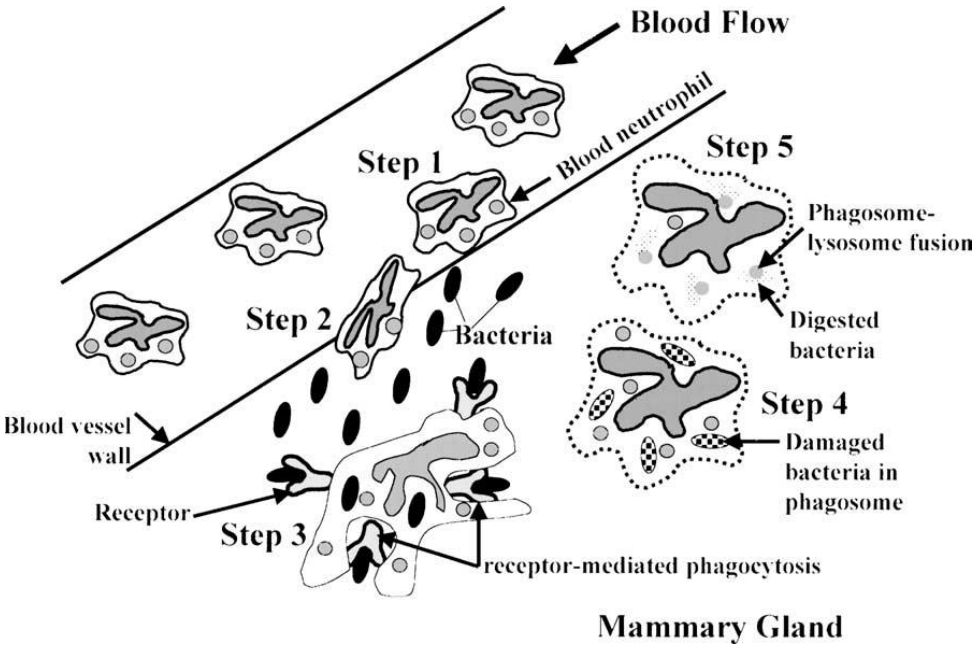
Neutrophils have bactericidal effects that are mediated through a respiratory burst that produces hydroxyl and oxygen radicles (Heumann *et al*., 1994). Previous studies (Selsted *et al*., 1993) have shown that neutrophils are also a source of small antimicrobial peptides defensins which kill a variety of mastitic pathogens. During the peripaturient period, a number of neutrophils functions are altered or impaired (Cai *et al*., 1994; Kelmanson *et al*., 2000; Shafter *et al*., 1996). At this time the number of immature neutrophils in the bovine blood increases while the number of immature neutrophils in the blood and mammary secretions are lowest. This impairs the activities of the neutrophils such as phagocytosis, respiratory burst activity, superoxide anion production, random cellular migration and chemo taxis.

However, also chemotactic neutrophil migration from blood can be impaired further due to decreased portion of cells expressing the adhesion receptor CD 62(L-selection) which is responsible for penetration to sites of infection (Lee *et al*., 1998). In lactating mammary glands tissue macrophages are dominant cell type and during invasion by pathogens they serve as either innate or acquired immune response. Similarly to neutrophils the none specific functions of macrophages is to phagocytize pathogens and destroy them with proteases and reactive oxygen species.

2.2.3 Description of PMN (Polymorphonuclear Neutrophils) action

When migrating neutrophils detect infection in the underlying tissue, cells are activated to migrate through the blood vessel wall into the focus of infection. Migration activates neutrophils to undergo cell-mediated phagocytosis of the infecting pathogen, internalising the micro-organism in vesicles called phagosomes. Phagocytosis further activates the neutrophils, first to undergo respiratory burst. During degranulation, incoming phagosomes fuse with outgoing lysosomes to finish pathogen killing through oxygen-independent mechanisms. Since blood –derived neutrophils are first line of immunologic defence against mastitis causing bacteria in dairy cows they work rapidly to clear intra-mammary infections.

Figure 2. 1 Activity of Neutrophils



The presence of opsonic antibodies against particular antigens increases the phagocytic rate of these cells (Miller and Paape, 1985). The ability of bacteria to overcome the anatomical defence provided by the teat end leads to invasion of the antibacterial activities of mammary gland.

During inflammation macrophage numbers are lower and have fewer FC receptors, possibly decreasing their rate of phagocytosis compared to neutrophils. The most important role played by macrophages in none specific defence is to secrete substances that facilitate the migration and bactericidal activities of neutrophils than acting as professional. When macrophages are triggered they release postraglandlins and cytokines which greatly augment local inflammatory process. Macrophages also play a role in the development of specific immune response through antigen processing and presentation in association with MHC class II antigens (Miller and Paape, 1985; Philpot and Nickerson, 1999; Prasad *et al*., 2004). The total number and activity of mammary gland leukocyte population play early and vital roles in determining the severity and duration of mammary infections. This leukocytes are capable of mediating either the innate or acquired immune response. Bovine macrophage number is highest in the last week of gestation, the phagocytic capacity of these cells is decreased possibly due to lower opsonic activity in mammary secretions. This decrease in phagocytic activity is associated with low levels of IgM. In addition MHC expression by bovine macrophages during peripartum period is decreased which leads to poor antigen presentation, resulting into a week specific immune response from the mammary gland lymphocytes (Bernabucci *et al*., 2013; Fitzpatrick *et al*., 1999; Gitto *et al*., 2002).

2.2.4 Role lymphocytes

Lymphocytes are able to recognise antigens through specific membrane receptors which define the immunological characteristics of specificity, diversity, and self or none self-recognition. There are two main groups of lymphocytes T and B lymphocytes. T lymphocytes are classified α β T lymphocytes which are $CD4^+$ (T

helper), $CD8^+$ (T cytotoxic) or T suppressor lymphocytes and Y, *δ and* T cells. Lymphoid cell in both human and bovine milk also display a memory cell phenotype. Depending on the stage of lactation and tissue location, the percentage of lymphocytes can vary significantly and major shifts in trafficking patterns are correlated (Park and Davis, 1992; Vankampen and Mallard, 1977).

During mastitis invasion CD4+ T lymphocytes prevail and are activated in response to recognition of antigen MHC class II complexes on antigen presenting cells such as B cells or Macrophages. These cells function to activate lymphocytes and macrophages by their ability to secrete certain cytokines depending on the cytokines produced, the T helper cells response can trigger a cell mediated TH1type or humoral TH2 type immune response (Brown *et al*.,1998) .

Despite the fact that the initial TH1 VS the paradigm in moles now considered somewhat over simplified, interleukin (IL-2 and interferron IFN-y where characterised as the major cytokines produced during the response. However, it is known that IL10 dominates during TH2 response and can be produced by and regulate all subtypes of Th2.

According to Shafter *et al*. (1996) $CD4^+$ cells produce less IL-2 and IFN-y but more IL-4 and IL-10 during periparturient period as compared to later stages during lactation. The $CD4^+$ cells produce less IL-2 and IFN-y but more IL-4 and IL-10 during periparturient period as compared to latter stages during lactation. The $CD8^+$ T cells have either a cytotoxic or suppressor function, in that they either eliminate host cells expressing foreign antigen in association with MHC class 1 or the immune response by supressing the activation of these cells during mastitogenic infections. Suppressor

T lymphocytes are thought to control or modulate the immune response to bacterial infection.

An evaluation of lacteal secretions from mammary glands of dairy cows infected with *S. aureus* revealed a sub population of activated $CD8^+$ lymphocytes that are capable of altering or supressing the proliferation responses of $CD4^+$ lymphocytes (Shafter et al., 1996). Immunoregulatory role of $CD8^+$also greatly depends on lactation stage. Cells obtained from dairy cattle exhibited no cytotoxic activity and mainly expressed by 1L-4 (Shennan and Peaker, 2000). This data obtained by (Shennan and Peaker, 2000) suggest that a preferential trafficking of $CD8^+$ suppressor lymphocytes into mammary gland tissues and secretions may be responsible for the lower responsiveness of local lymphocyte populations compared with those from later stages.

2.2.5 Functions of Y, *δ* and T cells

They involve in cytotoxic response and provide a unique line of defence against bacterial infections. There are also indications that Y, *δ* and T lymphocytes like natural killer cells can mediate cytotoxicity in a none restricted way (Mackay and Hein, 1991). In relation to blood, both humans and ruminants express greater levels of Y, *δ* and T lymphocytes in mammary secretions and parenchyma (Richie *et al*., 1982).

2.2.6 The role of B lymphocytes

B lymphocytes produce antibodies against invading pathogens. However, unlike macrophages and neutrophils, B lymphocytes utilize their cells surface to recognize specific pathogens just as dendrites and macrophages. B lymphocytes function as antigen-presenting cells as they internalise, process and present antigen in context

of MHC class II molecules to T helper lymphocytes. Thus, after the presentation of processed antigen IL-2 is secreted by the T lymphocytes, proliferation and differentiation is induced the B lymphocytes into either plasma cells that produce antibody or memory cells.

Antibodies are known as Igs; the four classes of Igs that contribute to defence mechanisms of the mammary gland are IgG1, IgG2, IgA and IGM (Guidry and Miller, 1986). Generally Ig reaches its peak concentration in mammary secretions during colostrogenesis and during inflammation.

2.2.7 Description of Immunoglobulin's

The primary isotope found in healthy mammary secretions is IgG1 but IgG2 increases substantially during mammary gland inflammation. However, isotopes IgG1, IgG2 and IgM act as opsonins to enhance phagocytosis by neutrophils and macrophages. IgA is not involved in bacterial opsonisation, but functions instead in the agglutination of invading bacteria thus preventing the spread of bacterial disease in the mammary gland. Concentration of IgG in bovine serum is lower at parturition due to lack of IgG2 isotype which correlates to an increased incidence of mastitis (Mallard *et al*., 1997).

A study by Mallard *et al*. (1997) showed that all dairy cows experience a reduction in antibody responsiveness at parturition implying that animals do not develop this have a superior natural ability to produce antibodies.

2.2.8 The none specific bacterial static factors

Non-specific bacteriostatic factors work together with or independent of Ig. These include lactoferrin, which is an Iron binding glyco-protein produced by epithelial cells and leukocytes that functions to bind ferric ions in milk , thereby preventing the

growth of bacteria that depend on Fe as a nutrient for growth (Schanbacher et al., 1993). In ruminants, lactoferrin and IgG1 act synergistically to inhibit E.coli and *Klebseilla puemoniae*. Lactoferrin main role in the mammary gland is to provide protection against coliform infections, especially at involution.

2.2.9 Complement

This is a collection of proteins present in serum and milk that can impact both innate and acquired immunity. Proteins that comprise the complement system are synthesized mainly by hepatocytes. However, other sources include monocytes and macrophages. Biological activities of complements are mediated through receptors located on a variety of cells. These are the effector functions of complement: (1) Lysis of bacteria, (2) opsonisation, and (3) attraction of phagocytes to the site of complement activation. Research done by others (Riollet *et al*., 2000; Rainard and Poutrel, 1995) showed that the lowest concentration of complements is observed in healthy mammary glands of animals in milk during lactation and the highest in colostrum.

2.2.2.1 Lysosome

This is a bactericidal protein is also present in milk, these are the functions of lysosomes: They cleave to peptidoglycans from the cell wall of gram-positive bacteria as well as the outer membrane of gram- negative bacteria. Lysosomes enhance the binding of lactoferrin to the bacteria cell wall.

2.2.2.2 Lacto peroxidase

Enzyme lacto peroxidase in the presence of thiocynate and hydrogen peroxide is bacterio-static towards gram –negative and gram-positive bacteria. The lacto peroxidase thiocynate hydrogen peroxide complex exerts its anti-bacterial properties

through production of hypothiocynate which is a reactive metabolite formed from oxidation of thiocynate (Smith and Hogan, 2003).

2.2.2.2 Myelo peroxidase

This is produced by neutrophils and catalyses peroxidase reactive compounds and additionally oxidation of chloride.

2.2.2.4 Cytokines

The cytokines group consist of IL (Interleukin), colony-stimulating factors (CSF), interferons (IFN) and tumour necrosis factor, TNF (Kherli *et al*., 1999; Riollet *et al*., 2000; Rai, 2006). The term inter-leukin means cell- free soluble factors that function as communicator molecules between leukocytes.

Many of well characterised cytokines are designated as IL; IL2 is the most extensively characterised of all bovine cytokines. IL-2 was originally described as T cell growth factor IL-2 and is produced by T lymphocytes immune response and is responsible for establishing immunologic memory following mitogenic or antigen stimulation. Another important function of IL-2 is to provide B lymphocyte growth, differentiation, thymocyte proliferation enhancement, activation of NK (natural killer) cells and induction of cytotoxic T cell growth activation.

2.2.2.5 CSF (colony stimulating factors)

These are a group of cytokines required for the proliferation and differentiation of a variety of hematopoletic stem cells. They bind to cells by a common receptor and are produced by a variety of cells such as fibroblasts, endothelial cells, macrophages and T cells. Each CSF tends to target a specific cellular lineage to expand or activate its function.

Granulocyte G-CSF has a pronounced influence on phagocytosis of cell populations. Previous studies (Cotter and Ross, 2005) have shown that recombinant human G-CSF administered substantially to cows in doses ranging from 1 to 5µg per day resulted into increase in PMN after 3-5 days of injection.

Granulocyte-macrophage Gm-CSF in dairy cows is important for inducing the growth of these cell types but also affects a variety of functions of granulocytes. Treatment of bovine peripheral blood and mammary gland with RbGm-CSF significantly increased the chemotactic and bactericidal capabilities of these cells.

2.2.2.6 Interferons

Interferons are closely related proteins; they consists of two major classes (1) IFN which consists of IFNα, IFNβand IFN *ω*. The types IFNα and IFNβ are produced by a variety of all types in response to several inducers, including viral infections, bacterial and tumour cells. In bovine IFN *ω* genes codes for proteins produced by the early embryonic trophoblast and these are referred to as IFN-t.

The second class of IFN-y consists of a single protein and is a cytokine derived from T lymphocyte that is often produced in response to stimulation by antigens or mitogens. Others (Sordillo and Babuik, 1991) suggest that IFN-y could elicit functional changes in in phagocytosis cells in the mammary gland that could make it effective in the control of bovine mastitis. In-vitro treatment of mammary gland neutrophils with IFN-y was shown to reverse the suppressive effect of mammary gland secretions and significantly increase functional capabilities of these cells against S. aureus. TNFα is produced during the early stages of infections and causes endotoxin shock during peracute coliform mastitis.

2.3 Nutrition and the immune system

There have been recent developments on the role played by nutrition to increase cow's defence against infections. Vitamins and minerals such as Vitamin E, Selenium, Copper and Zinc when properly supplemented enhances the animals immunity against diseases such as mastitis, and by decreasing severity of this infections when they occur (Goff, 2006; Smith *et al*., 1985; Stephen, 2013). The nutritive condition of a cow is affected by the feeding system, forage combinations quality and feed volume in each herd. Therefore, malnutrition alters the immune response and puts the animal at a higher risk of contracting infectious diseases (Najera *et al*., 2004; Suskind *et al*., 1980). Important free radicles present in the biological system are superoxide, hydrogen peroxide, hydroxyl radicle and fatty acid radicles (Smith *et al*., 1984). These are very toxic elements and therefore there is need of antioxidant system to remove them from the system of the animal. Micro minerals Se, Cu, and Zn are essential component of glutathione peroxidase GSH-PX and superoxide SOD which removes these free radicles from the animals system.

2.3.1 Vitamins and minerals useful in udder health

Zinc: Zinc maintains health and integrity of skin due to its role in cellular repair, replacement and increasing the speed of wound healing. This mineral is also an essential component of several enzymes involved in the synthesis of DNA and RNA. It is also part of a group of elements that induces the synthesis of metallothionin which binds to free radicles (Quadri *et al*., 1994).

Zinc also plays an important role in keratin formation which is the first line of defence of the innate immune system. As a component of the enzyme superoxide

dismutase, it stabilizes cell membrane structures (Quadri *et al*., 1994; Reneé and Todorov, 2010). It has been observed that levels of zinc decreased at parturition , transfer of zinc to colostrum and increased stress at this time. Supplementation of Zinc methionine to dairy cattle has reduced somatic cell count (Kellog *et al*., 1990; Spain, 1993).

Copper: Copper is a component of enzyme cerulo plasmin, which is synthesized in the liver and assist in iron absorption as well as transport (Broadely and Hoover, 1989). This acute phase protein is important in scavenging superoxide radicles and component of superoxide dismutase that protects the cell from toxic effects of oxygen metabolites released during phagocytosis (Halliwell and Gutteridge, 1999). These functions are important in reducing the incidence of mastitis during the periparturient period. Heifer supplementation with copper 60d pre-calving decreased severity *E.coli* mastitis (Scalleti *et al.,* 2003).

Chromium: This element is able to enhance action of insulin. However, studies have indicated that chromium supplementation may affect health and improves immunity in stressed calves (Spears, 2000).

Magnesium: This element is known to activate 300 different enzymes and is essential in energy metabolism, transmission of genetic code, membrane transport and nerve impulse transmission.

Potassium: Potassium plays an important role in acid-base balance, muscle contraction, nerve impulse and enzymatic reactions.

Selenium: Selenium and vitamin E supplementation reduce SCC in milk and prevents subclinical mastitis (Erskine *et al*., 1989; Weiss *et al*., 1990). It is also a

cofactor of glutathione peroxidase enzyme and also activates thioredoxin reductase which prevents oxidative stress (Mustacich and Powis, 2000). Selenium enhances lymphocyte proliferation, cytotoxicity and antibody production (Erskine *et al*., 1989; Kiremidjian *et al*., 1990; Kiremidjian and Stotzky, 1987; Rumke *et al*., 1971). Selenium supplementation of cows consistently improved neutrophil function and effectively killed mastitogenic bacteria relative to unsupplemented cows. As suggested by Baulez and Dussert (2011) yeast selenium is superior in terms of benefit to immune system than sodium selenite.

Vitamins

Vitamins originate primarily from plant tissue and are present in animal tissue only as a consequence of feeding on plant because ruminants have resident microorganisms that synthesize them.Vitamin A and D differ from the other two fat soluble Vitamins E and K in that they occur in plant tissue as pro vitamin.

Riboflavin: Riboflavin in the form of mononucleotide (FMN) and Flavin adenine (FAD) functions as a co-enzyme in diverse enzymatic reactions. Riboflavin enhances innate host defence mechanism against a variety of infections in mice (Kevin *et al*., 2012) by stimulating the generation of neutrophils and their functioning.

Vitamin A: Vitamin A exists in plants as precursor (carotenoid) is converted into Vitamin A by a specific enzyme located in the intestinal wall of the animal. Beta carotene is effective in preventing the occurrence of mastitis in dairy herds due to anti-oxidant properties and contribution to the mucosal surface of the mammary gland (Chew, 1993; Sordillo *et al*., 1997).

Vitamin D: This mineral is essential for bone growth and maintenance and is directly involved in Ca absorption as well as Phosphorus from the kidney. Vitamin D also plays an important role in modulating the innate and adaptive immune system. Another key role is regulation of gene expression in immune cells and enhancement of their ability to kill pathogens (Nazke, 1981).

Serum level of activated form of Vitamin D (125($_{OH2}$) D3) di hydroxy vitamin D_3 have been correlated with efficacy of human macrophages to kill mycobacteria tuberculosis pathogens (Lui *et al*., 2006). It is also important in the phosphorylation of carbohydrate which is part of energy metabolism process.

α Tocopherol (Vitamin E): Vitamin E serves as inter and intracellular antioxidant and is involved in the structural formation of biological membranes (Lacy, 1998; Weiss and Spears, 2006). Supplementation of vitamin E and selenium in diets of animals reduce mastitis than using one alone. It is an important lipid soluble antioxidant that protects cells against free radicle lipid peroxidation (Halliwel and Gutteridge, 1999).

2.3.2 Nutritional deficiencies in relation to mastitis

Hypocalcaemia: Animals with milk fever have shown slow closure of teat sphincter which leads to entry of pathogenic bacteria (Schonewille *et al*., 1999). Calcium homeostasis is maintained by parathyroid hormone and di hydroxyl vitamin D_3 via increased bone reabsorption and intestinal absorption. During early lactation this process cannot fully meet the calcium need which leads to negative calcium balance. Calcium is important in activation of T cell therefore, lower levels of calcium reduced activation of T cell (Kimura and Reinhardt, 2001).

Negative energy balance: Negative energy balance is common during the peri-paturient period this causes body fat to be modified into blood stream in form of None Sterified Fatty Acid (NEFA) These NEFAs cannot be used to make glucose but are important fuel source for body tissues such as muscle and are incorporated into milk fat. However, excessive secretion of NEFAs into the blood stream and into the liver leads to malfunctioning of liver. This occurs because NEFAS in the liver exist as triglycerides (Overton and Waldron, 2000; Overton and Walden, 2004)**.**

Fatty liver: As saturated fatty acids increase in blood NEFA, other fatty acids such as mono- and polyunsaturated fatty acids decrease. Some of these fatty acids include arachidonic acid (an Omega-6 fatty acid), eicosapentanoic acid (EPA), and docosahexanoic (DHA; both omega-3 fatty acids). These fatty acids are essential for immune function because their products affect different steps of the inflammatory process. Reduction in the availability of these necessary fatty acids may promote immune dysfunction in dairy cows.

2.3.3 The buffer system of the udder

Mastitic milk is significantly low in citrate and alkaline (Oshima and Fuse, 1981; Dhillon and Singh, 2009). The normal udder pH of milk is 6.5 a level which doesn't appear to be congenial for growth of commonly isolated mastitic organisms (Cruickshank and Swain, 1970). Citrate the main constituent of the udder buffer system and is responsible for the pH 6.5. Citrate regulates the homeostasis between ca^{2+} and H^{+} ions and plays a major role in the fluidity of milk through its effects on casein micelles.

Therefore, it can be concluded that the initial lesion in the pathogenesis of mastitis is caused by disturbed homeostasis of citrate and Ca^{2+} in the udder (Harmon, 1994). For example low levels of citrate and free Ca^{2+} are responsible for this injury in the udder and alkaline pH is due to seepage of bicarbonate from blood into the udder providing most conducive condition for establishing environmental and contagious pathogens.

2.3.4 Trisodium citrate and udder health

Based on literature on mastitis and biosynthesis of milk, citrate is important in the lactogenesis and maintenance of udder through ionic equilibrium (Hyvonen and Pyrola, 2010). It has been hypothesized that a protective benefit would be derived by replenishing citrate deficient animals with tri sodium citrate. Citrate is in important in boosting both innate and specific immune system (Zhao and Laccase, 2007).

2.3.5 Enzyme Glutathione peroxidase (GSH-Px) protective role as part of the antioxidant defence system.

GSH-PX Glutathione peroxidase is a common resident in most of mammalian cells, this includes; Erythrocytes and Leukocytes. GSH-Px catalyses this reduction into alcohols (R-OH) and water where glutathione GSH is the source of reducing equivalents .This peroxides that undergo reduction are produced by phagocytic cells as they attack foreign antigens and are also by-products of cellular oxygen consumption. The presence of peroxides in cells limits availability of $NADP^{+}$ for reduction of O_2 and H_2O_2 hence their deficiency leads to decreased activity of PMN to kill ingested pathogens in Se deficient animals. The reduction of peroxidase also prevents lipid peroxidation and cellular membrane damage.

Bovine tissues with high GSH-PX activity include spleen, myocardium, erythrocytes, brain thymus, adipose tissue and stratified muscle. GSH-PX activity can be used as indicator to determine ability to produce reactive oxygen species during oxidative burst in phagocytosis by neutrophils and macrophages. Increased deit of Se resulted into increased activities and of GSH-PX (Erskine *et al*., 1989).

2.4 Management aspects and mastitis

Milking procedure: Proper milking hygiene and milking practices have an effect on cases of mastitis in dairy herds This includes practices like maintaining clean dry environment for cows, checking the udder for signs of clinical mastitis, fore stripping, washing teats with warm sterilizing solutions and drying teats with 30-45 seconds after pre milking.

Milking machine and Mastitis: Mature keratin cells of about 10-20% lining of the teat canal are lost during a single milking and mechanical reaming of the teat canal removes up to 80% keratin. These two incidences above would increase new mastitis infection rate, relative to milking with normal pulsation. During normal pulsation at a single milking up to 40% of keratin cells are lost. Therefore, a desirable amount of Keratin is supposed to be removed by the milking machine. Thus, a depletion of more than 80% keratin leads to exposure of immature cells. Less removal of keratin cells by 10-20% slows the rate turnover of keratin. Thickening of skin around the teat in response to physical trauma creates a condition called hyper keratosis which predisposes the animal to infection (Neijenhus *et al*., 2001; *Mein et al*., 2003).

Under milking and mastitis: This refers to an excessive amount of residual milk in the quarter after cluster removal leading to inadequate flushing of udder. Therefore, low levels of bacterial contaminants remain in the cistern. Under milking is a problem associated with out breaks of clinical mastitis in early lactation. An excessively low or high vacuum leads to under milking predisposing the animal to infections. Old stretched liners slow milk output due to altered pulsation, these should be replaced at least every 1200 milking.

Pulsation ratio is supposed to be at 60:40 through an exact setting which depends on vacuum liner type. A storm in the teat cap exposes the animal mainly to contagious as well environmental pathogens due to increased changes in teat end condition. Storm in the teat caps also creates congestion and oedema in teat wall which slows the closure of teat canal and exposes the animal to infections (Hamman *et al*., 1994). A slow rate and removal of teat canal keratin also creates storm in the teat cap (Lacy, 1998; Woolford, 1997). The cluster should line with the backbone of the cow. Twisting of the cluster often results in uneven milk out and leads to under milking.

2.5 Bacteriocins (Antimicrobial peptides)

These are ribosomally synthesized antimicrobial peptides produced by bacteria that are active against other bacteria. Bacteriocins are natural antimicrobial peptides or protein synthesized by various groups of bacteria. Lactic acid bacteria form an ecologically heterogeneous group of gram-positive bacteria; they are none spore forming, immobile , catalase negative that excretes lactic acid as a major end product and are generally regarded as safe (GRAS) . They are selected as probiotic

and are able to promote health and prevent infections against pathogenic bacteria (Fernadez *et al*., 2003). The term “Probiotics” means live organisms that when ingested in adequate amounts exert health benefit (Eamonn, 2010). Bacteria of genera LAB (Lactic Acid Bacteria) include*: Lactococcus, oenococcus*, *lactobacillus*, *lueconostoc*, *pediococcus*, *streptococcus*, *Aerococcus*, *carnobacterium*, *Enterococcus*, *oenococcus*, *sporolactobacillus, Teragerococcus*, *Vagococcus* and *Weissella* (Eamonn, 2010). LAB strains are able to inhibit food borne pathogens such as S.aureus, salmonella typhimurium, E.coli and listeria monocytes (Darsnaki *et al*., 2012, Jamuna and Jeevanatum, 2004). In addition LAB are efficient in inhibiting mycotoxicogenic fungi *penicillium expansum*, *Botrytis* cinerea, *Aspergillis niger, Aspergillus falvus* and *fusarium graminarum*. These important strains if produced in enough quantities can be a safe way to treat mastitis

2.5.1 Bacteriocins and immune system

Normal micro flora influences the structure of host mucosa, its function and the development through an increase in duodenal IgA plasmocyte (Monreu *et al*, 1987). It is also known that micro flora of the gut stimulate the proliferation of epithelial cells and increases whole intestinal surface (Montville and Bruno, 1994). The application of probiotics can influence micro flora composition by increasing the number of lactobacilli and other anaerobes. Dietary supply of probiotic bacteria stimulates IgA immune response (Kaila *et al*., 1992) and the transport of target antigens through payer’s patches (Isolauri *et al*., 1993) which are the primary sites where specific immune responses are performed.

LAB induces the production of cytokine secretion released after presentation to T lymphocyte or cytokine production from direct interaction between LAB and immune component (LeFrancois, 1994; Taguchi *et al*., 1991). The presence of specific receptors for peptidoglycan, a compound on LAB cell, was manifested on lymphocytes and macrophages (Bhakadi *et al*., 1991; Dziarski, 1991; Tufano *et al*., 1991; Heumann *et al*., 1994). This peptidoglycan has the ability to induce secretion of IL-1, IL-6 and TNα by monocytes.

2.5.2 The effect of Lab on the innate immune system

LAB enhanced activity of peritoneal and pulmonary macrophages and blood leukocytes (Silvia *et al*., 1995; Paubert *et al*., 1995). The work by (Balasubaramanya *et al*., 1995) showed increased production of reactive oxygen, nitrogen radicles and monokines of phagocytic cells. In-vivo studies (Pendigon *et al*., 1999) enhanced clearance of colloidal carbon as an indicator of phagocytic ability of monocyte macrophage system.

2.6 Ethno Veterinary medicine

There is an increasing use of herbal plants as health promoters in both scientific and consumer circle (Hashemi and Davoodi, 2012). A number of physiological products, microbial products and herbal products have immunodulation properties. Many herbs are being used fruitfully to treat a variety of conditions in animals as well as birds. Herbs provide potent anti-inflammatory, antibacterial, antiviral and antifungal benefits. Therefore, ethno veterinary medicine deals with people's knowledge, skills, methods practices and beliefs in caring for and keeping animals healthy. These practical experiences have been passed orally from generation to generation (Maria

et al., 2011; Toyang *et al*., 2007). Natural medicine is more suitable for animal and human health care, with the advantage of low cost and total safety (Rahal and Kumar, 2009). In the era of emerging antibiotic resistance and residual effects in food products, these can play a wonderful role in safeguarding health of human and animals.

2.6.1 Description of herbs and their antimicrobial properties

2.6.2 Tetradenia riparia

This is commonly known as plume brush, water salie in africanas and ibozane (Zulu). *T. riparia* is a soft wooded deciduous shrub which flower in late autumn and winter. It grows best in frost free areas (Khuzwayo, 2011). The species of *T. riparia* are found in wooded hillsides and stream banks of coastal KwaZul-Natal, Mpumalanga and Northern Province of South Africa. It grows rapidly to a height of 2m and is slightly stick when touched.

Phytochemicals: Studies of *T. riparia* have indicated presence of 7α hydroxyroyleanone sandanacopimanadiene 7α18- diol, α pyrones tetradenolited and diteperiodiol 3 were found to display good antimicrobial and antiparistic activity. Leaf extracts of T. riparia 80% ethanol tested for antimicrobial and antiviral activity inhibited the growth of *S.aureus*, *candida albicans*, *Mycobacterium smegmentis*, *microsporum canis*, *Trichophyton menthe graphytes* and *bacillus subtilis* (Khuzwayo, 2011).

2.6.3 Acacia nilotica

This is known as Gum Arabica, Babul Egyptian horn or prickly acacia and is a multipurpose nitrogen fixing tree legume. Zulu names include Umbombo, ubobe, amnqawe and unqawe. This specie thrives best at sea level to over 2000m and has

potential to withstand extreme weather conditions, high temperatures and frost. It widely spreads in Sub tropical and tropical Africa from Egypt to Mauritania down southwards to South Africa. The secondary metabolites include amines, alkaloids, cynogenic glycosides cyclitols, fatty acid, seed oils. Fluroacetate, gums, none protein amino acids, and terpenes are other metabolites. Essential oils are diterperpenes, pytostenol, triterpene geninis, saponins, hydrolysable tannins, flavonoids and condensed tannins (Seigler, 2003).

Medicinal properties: It is an anti-cancer, antioxidant, antiplasmodial, and used for intestinal pains, dysentery, haemorrhages opthalmia diarrhoea and sclerosis (Sapna *et al*., 2011). Crude alkaloids of A. nilotica have higher inhibitory potential against bacteria (Verpoorte, 1998). Pods have been found to be active against (Methicillin resistant staphylococcus aureus) MRSA, E. coli, beta lactamase positive E. coli and Klebseilla Spp (Saba *et al*., 2011).

2.6.4 Aloe arborescens

Aloe aborescens is also known as Krantz aloe in English, Kranjaalwyn Africans, Ikalane in Xhosa and Ikalane in Zulu. This plant belongs to the Asphodelacae family (Vanwyk and Smith, 2004; Smith *et al*., 2008). *Aloe arborescens* develops into multiheaded shrub 2-3 high with striking grey green leaves in attractive rosettes. The large colourful flower spikes are borne in profusion during cold winter months (May-July). The inflorescence is usually unbranched. *Aloe arborescens* is the third widely aloe specie through KwaZulu-Natal, Mpumalanga, Limpopo province and further into Mozambique, Zimbabwe and Malawi.

Medicinal properties: The hypoglycaemic activity of *Aloe arborescens* leaf components have been evaluated and confirmed in animal model of diabetes (Beppu

et al., 2006) are attributed to the presence of polysaccharides, arboran A and B in the leaves. *Aloe arborescens* have been found to be a commonly used herb in communal poultry management, mostly in treatment of diarrhoea infection (Okitoi *et al*., 2007). The sap is used to prevent illness in poultry (Jaarsveld, 2002).

2.6.5 Crassula multicava

Crassula multicava has common names fairy crassula, Fairy crassula, Skaduplakkie (Afrikaans) and Umadinsane (Zulu). Crassula is a succulent small plant. This plant has a uniformity, compactness and low-growing appearance. As with members of this genus, the glossy, oval to round leaves are formed in opposite pairs. Its leaves contain water secreting pores which serve for rapid absorption from the leaf surface. *C. multicava* occurs in forests, river and stream banks and in coastal subtropical thickets from Mpumalanga, KwaZulu-Natal to the Eastern and Southern cape. Plants show preference for well composted deep soils and clay soils. This plant is found sheltered in frost free areas.

Medicinal properties: Decotation of the whole plant is used as strong emetic (Hutching, 1996). Research (Okem, 2011) indicates that whole plant of *Crassula multicava* showed good antibacterial activity.

2.7 Dry Cow Therapy

The non lactating dry phase of a dairy cow is a period between two lactating phases when the mammary gland changes dynamically both in structure and function. According to Bramley et al. (1989) the dry Cow therapy is effective in controlling IMI due to *Streptococcus agalactiae* and against *S.aureus* (Nazke, 1981). The exposure of mammary gland to contagious pathogens during the dry period is minimal due to the absence of regular milking which means therapy at drying off tend to control

pathogens more effectively. Exposure to contagious pathogens is concentrated at the beginning of dry period and becomes less intense as the period progresses. The high concentration of lactoferrin to low citrate ratio makes the involuted udder naturally resistant to gram-negative mastatic pathogens. Therefore, dry cow therapy would benefit in treatment of *strep uberis*, *strep dysgalactiae* and *Arcanobacterium.*

2.8 Conclusion

There is a relationship between nutrition and udder health that is the boosting of both innate and specific immune system. For example Se plays a key role in boosting enzyme glutathione peroxidase which reduces peroxides (free radicle) that are produced by PMN in respiratory burst during phagocytosis. Since the initial lesion in the udder during the pathogenesis of mastitis is caused by disturbed homeostasis between citrate and Ca^{2+}, it is very important to monitor citrate levels in the animals. Proper milking hygiene and usage of the milking machine are important minimising mastitis. With increased resistance to antibiotic treatment, there is a big urgency to explore alternative medicine in the natural form because of their lowers costs and their efficiency in clearing resistant mastitis strains like the Methicillin resistant *S. aureus* (MRSA) and Vancomycin resistant *S. aureus* (VRSA). Therefore, herbs and bacteriocins (antimicrobial peptides) would be useful in treatment of mastitis. In the era of emerging antibiotic resistance and residual effects in food products, these can play a wonderful role in safeguarding health of humans and animals.

Chapter 3: The role of management factors on prevalence of mastitis in KwaZulu-Natal Province and Entebbe municipality

Abstract

Bovine mastitis causes losses to dairy farmers at clinical level and even higher at subclinical level. The objective of this study was to find out the role of management factors on prevalence of mastitis in herds of large scale farmers at KwaZulu-Natal province, South Africa and small holder farmers in Entebbe Municipality in Uganda. Structured questionnaires were used in both areas of study. Sixty percent of farmers had numerous cases of mastitis and 40% in Entebbe municipality in 2014. In KZN 86% of farmers had between 1-20 cases of clinical mastitis and 14% for cases between 21-70 cases in the month the survey was done in 2014. Fifty seven percent of farmers had mastitis in the dry season and 43% in wet season in Entebbe municipality. In KZN, 79% of farmers had mastitis in the wet season and 21% in the dry season. Dairy farmers who practiced washing teats had a tendency of associating (χ^2=3.21; P<0.07) with mastitis prevalence (MP). Also dairy farmers who practiced spraying regularly had a tendency associating (χ^2=3.21; P<0.08) with MP. In KwaZulu-Natal survey the practice of providing separate calving paddock had a tendency associating (χ^2=3.00; P<0.08) with MP. All the management factors on milking hygiene practices had no association (P>0.05) with MP. The practice of managing teat closure, nutrition and other management factors in had no association (P>0.05) with MP. All dairy farmers in KZN showed that they practiced proper machine maintenance and functioning.

Key words: Management factors, prevalence of mastitis, season

3.1 Introduction

Mastitis is one of the major diseases in the dairy industry across the world (Wallenberg et al., 2002; Seegers., 2003). It is economically an important disease accounting for about 38% of total direct losses (Albenzio *et al*., 2002; Nooruddin *et al*., 1997). Clinical mastitis is accompanied by Physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Samad, 2008). Sub-clinical mastitis can be known after laboratory examination as there are no gross inflammatory changes in the udder tissue. This form of mastitis causes even greater losses due to elevated somatic cell counts and reduction of milk. The elevated somatic cell counts means that milk cannot be sold according standards. The increased somatic cell counts for both forms of mastitis is due to increased leukocyte levels as they get involved in the removal of infectious pathogens and epithelial cells (Bagnicka *et al*., 2011).

Selenium and vitamin E supplementation enhance phagocytic activity of neutrophils and have been associated with decreased clinical mastitis (Heinriches, 2009). Several studies have shown that susceptibility to intramammary infections can be influenced by level of vitamin A, E and minerals such as Se, Cu, and Zn in the diet. Metabolic disorders like milk fever, ketosis and fatty liver diseases have been linked with mastitis (Vacek et al., 2009). This evidences clearly link mastitis with nutrition hence great need to evaluate farmers on nutrition management aspect. Mastitis has also been linked with stage of lactation (Olde *et al*., 2007; Suriyasathaporn, 2000) and therefore, fore knowledge of this factor is essential to a dairy farmer in that it

helps in devising management strategies to minimise mastitis. Mugabe *et al*. (2004) reported significant association between clinical mastitis and cows with at least 8 parities and in the fourth month of lactation period.

Temperature, humidity and season have also been found to have an effect on clinical mastitis (Morse *et al*., 1988). Therefore, the effect of season and mastitis requires evaluation because it helps farmer devise measures to minimise mastitis in the season its more prevalent. According to Rahman *et al*. (2009) mastitis was more prevalent in the wet season than in dry season. California milk test is a very important method of determining somatic cell counts at quarter level per individual cow (Schroeder, 2012). If this this management factor is followed strictly by farmers mastitis can be identified and treated earlier before it becomes critical hence minimising heavy losses. The practice of not buying replacement heifers is sometimes neglected by farmers. However, it prevents farmers from acquiring heifers which have been infected with mastitis. Findings by Osteras (2009) showed that this management aspect is important in the dairy industry.

Environmental mastitis is mainly caused by moisture, mud and manure in cow housing areas. Cleanliness of animal housing has a major influence on the rate of clinical and subclinical mastitis. The risk of clinical mastitis was increased when cows housing were not cleaned (Elbers *et al*., 1998; Peeler *et al*., 2000; Schukken *et al*., 1990; Schukken *et al*., 1991. There is great need to assess the issue of housing (bedding) as a management aspect. Sometimes farmers are reluctant in the practices of separating heifers from lactating cows, if this practice is not adhered to

pathogens can be spread from lactating cows to hiefers. According Barkema *et al*. (1998) dirty milking parlours were responsible for sub-clinical and clinical mastitis in dairy herds. Damien *et al*. (2009) reported that sub-clinical mastitis was prevalent in unhygienic milking parlours. An evaluation of this practice helps farmers know how they are performing hence providing room for improvement. Pre dipping is an essential management practice in the control of mastitis. Therefore, there is great need to find out if this practice is followed by dairy farmers. Galton *et al*. (1986) noted that this practice reduced coliform bacteria. However, it does the same with contagious bacteria like *Staphylococcus aureus*. Fore stripping is also essential in the management of mastitis because it shows the abnormality of milk. This helps in detecting early stages of mastitis before it becomes severe, necessitating early intervention and minimising greater losses.

Drying teats is also an important practice in the dairy industry. Moxely *et al*. (1978) reports that dried teats had bulk tank SCC values of 44000 cells/ml. A follow up of farmers on this practice will help them minimise mastitis. The spread of contagious mastitis from one udder to another is due to contaminated milkers' hands, cloth and towels used to wash more than one animal's udder (Sharif, 2009). Therefore, there is great need to wear gloves, wash hands and use of separate towels. An evaluation of these practices is of great benefit to the farmers as they can improve, if there performance is not good. Post milking teat disinfection is another important practice in the dairy industry. This practice reduced SCC values by 70,300cell /ml in Quebec dairy herds (Moxely *et al*., 1978).

The dress code in the dairy industry is also part of hygienic practices. Therefore, it is important to assess whether this practice is taken heed of in the dairy industry. Horn flies are very common in the summer and in hot season for tropical countries. This fly acts vector for transmission of *S. aureus* mastitis and this makes spraying and dipping an important practice. If proper use of milking machine is not adhered to, it can cause mastitis in numerous ways from failure of disinfection of teat caps to incorrect vacuum pressure (Jones, 2009). Therefore, there is great need to evaluate practices concerning proper use of milking machine in relation to mastitis. The objective of this study is to evaluate the effect of management factors on prevalence of mastitis in herds of large scale and small holder dairy farmers. It has been hypothesized that poor hygiene practices lead to mastitis.

3.2 Materials and methods

3.2.1 Study site 1

The survey was carried out on dairy farms in KwaZulu- Natal, South Africa from Jan to May 2014. KwaZulu-Natal is located 29.00000S, 31.0000^0 E 29.0000° S, 31.0000°.

Data was collected on lactating cows from farmers cross-sectionally to investigating the association of management risk factors and mastitis prevalence (MP) using structured questionnaires. These were sent out electronically to dairy farmers in the region via newsletter by the Milk Producing Organisation of South Africa (MPO). Three hundred (300) farmers were reached via newsletter, emails and phone. However, due to unknown reasons only 19 questionnaires were returned. Lack of funds for transport and other essential items made it impossible to reach on them.

Questions on management factors that predispose dairy animal to mastitis were categorised into two classes in the questionnaire: (1) factors associated with the dairy cow. These were mainly stage of lactation and period of the year in which MP is high. (2) Factors related to management, mainly milking hygiene practices, maintenance of milking machine, nutrition and other associated factors.

Data analysis: All data from the returned questionnaires was entered into Microsoft excel (XL) spread sheet. Descriptive statistics was used to analyse the magnitude of mastitis and losses caused by mastitis. Prevalence of mastitis (MP) as independent variable was associated with dependent variables, milking hygiene practices, proper use of milking machine, nutrition aspects of management, somatic cell count management, and money lost per year, vet check-ups were all measured using Proc Freq. All these analysis were done using SAS 9.8 (2011).

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3.2.2 Study site 2

The study was carried out in Entebbe Municipality Uganda from Feb- March 2015. The coordinates of Entebbe are: 0°03'00.0"N, 32°27'36.0"E. Entebbe experiences tropical rain forest climate. Dry spells are from July through September, and wettest months are April and May with roughly 250mm of rain in these months.

Data collection and analysis in Entebbe Municipality Uganda: A questionnaire was designed to evaluate the incidence of mastitis and management associated factors that lead MP in dairy herds of Entebbe municipality farmers. Questionnaires were distributed to 30 random dairy farmers in the region out of a total of 55 (vet extension worker). Lack of funds was a stumbling block to the survey because dairy farmers from the other zone could not be reached. Questions asked were on the scale of the

problem, milking hygiene practices, season, herd size nutrition and dress code. Questions on other management aspects, under milking, record keeping, visitations of vet officer and dairy farmers associations were included. Prevalence of mastitis was used as independent variable with an aim of finding out if the above factors (dependent variables) influenced the incidence mastitis. This was done by associating this independent (MP) variable with dependent variables, milking hygiene procedures, nutrition, herd size, season and other management variables.

Data analysis: All data from returned questionnaires were entered into Microsoft excel spread sheet XL. Descriptive statistics was used to analyse the scale of the problem. The GLM SAS 9.8 (2008) was used to analyse herd size using the model: $yij = \mu + Gi + eij$, where μ is the overall mean, G is gender and eij is error term. Logistic regression was used to analyse odds ratio on which gender (male or female managed zero grazing unit) had more incidence of mastitis than the other and season of the year in which mastitis was more prevalent. Mastitis prevalence for year 2014 was associated with dependent variables milking hygiene practices, dress code of farmer's, nutrition management, and other management aspects such as ways to detect under milking, record keeping, and being in a dairy association was analysed using proc freq All these analysis were done using SAS 9.8 (2011).

3.3: Results

3.3.1 KwaZulu-Natal, South Africa

Results from KZN survey indicate that 86 % of dairy farmers had 1-20 clinical cases of mastitis while 14% had 21-70 cases.

Table *3. 1*: Association between cow attribute, season of year, milking hygiene practices, proper use of milking machine with prevalence of mastitis KZN

		Prevalence of mastitis			
		High	low	χ^2	P-Value
Cow attribute					
Lactation stage	Early	32	37	1.7	0.43
	Mid	0	5		
	Late	5	21		
Period of year					
	Wet	37	42	2.96	0.09
	Dry	0	21		
Milking hygiene practices					
Pre-stripping	Yes	32	53	0.2	0.90
	No	5	10		
Washing hands	Yes	32	47	0.3	0.6
	No	5	16		
Washing teats	Yes	5.26	5.26	0.2	0.7
	No	32	57		
Clean parlour	Yes	32	63	1.8	0.20
	No	5.26	0		
Separate calving paddock	Yes	21	58	3.2	0.08
	No	16	5		
Post milking disinfection	yes	31.6	63	1.8	0.20
	No	5	0		
Drying teats	Yes	11	16	0.03	0.90
	No	26	47		
Providing shed	Yes	11	16	0.03	0.90
	No	26	47		
Frequency of spraying	Regularly	32	32	0.30	0.58
	Seldom	5	31		
Milking machine practices					
Replacing parts regularly	Monthly	5	16	0.31	0.59
	Annually	32	47		
Water at mouth of teat cap	yes	32	63	1.81	0.12
	No	5	0		

Seventy nine percent of dairy farmers had mastitis in the wet season while 21% in dry season with a tendency of associating (χ^2=2.96; P<0.08) with MP Sixty seven percent of cows were in early lactation, 5% in mid lactation and 26% in late lactation; without any association (P>0.05) between stage of lactation and MP.

Farmers who pre-stripped were 85% while those that did not were 15%. Famers who washed teats were 10.5% while those that did not were 89.5%. Close to 95 % of dairy farmers kept parlours clean and 5% did not; neither of the above variables was associated (P>0.05) with MP. Close to 79% kept animals in separate calving paddock while 21% did not; with a tendency of associating (χ^2=3.00; P<0.08) with MP.

Dairy farmers who practiced post milking teat disinfection constituted 94.6% while those that did not were just 5.3%; 26% of famers dried teats while 74% did not; neither of these variables was associated (P>0.05) with MP. Close to 27% of farmers provided shed for dairy cows while 73% did not without any association with MP. About 64% of farmers sprayed regularly while 36% seldom sprayed without any association (P>0.05) with MP.

On the maintenance of milking machine, farmers who replaced parts regularly were 21% while those that did not were79%; 95% of farmers prevented water from accumulating at the mouth of teat caps while 5% did not; neither of these was associated (P>0.05) with MP.

Table 3. 2: Association between practice that lead to teat closure, nutrition, money lost and other management factors with cases of clinical mastitis per year in KZN

		Mastitis prevalence			
		High	Low	χ^2	P-Value
Is feed provided	Yes	21	21	1.02	0.31
	No	16	42		
Selenium	Yes	32	52	0.01	0.90
	No	5	11		
Addition feed supplements	Feed all	21	47	0.70	0.42
	One group	16	16		
Money lost per annum	50000-150000	21	37	0.00	0.96
	>150000	16	26		
Sharing of paddock	Yes	16	21	0.20	0.70
	No	21	42		
Buying replacement heifer	Yes	11	15	0.02	0.90
	No	26	47		
Dry cow therapy	Yes	32	47	0.3	0.60
	No	5	16		
Frequency of checking SCC	Weekly	16	37	0.70	0.72
	More than	21	26		
Veterinary doctor visits	Regularly	26	52	0.40	0.54
	Seldom	11	10		

Dairy farmers who provided feed to animals during milking constituted 42% while those that did not were 58%; 68% of farmers fed all animals with additional feeds while 32% fed only one group of animal (either lactating or dry cows); neither of these variables was associated (P>0.05) with clinical cases of mastitis. Eighty four percent of farmers provided selenium while 16% did not without any association P>0.05) with MP.

About 58% of farmers used <150000 Rands on the treatment of mastitis while 42% used >150000 rands. Farmers who had a practice of having heifers and lactating cows to share the same paddock were 37% while 63% did not. Neither of these two

variables was associated (P>0.05) with MP. Dairy farmers with the policy of buying-in replacement heifers were 26% while 73% reared theirs without any association (P<0.05) with MP.

Seventy nine percent of dairy farmers practiced dry cow therapy while 21% did not. 53% of farmers checked somatic cell counts weekly, 47% more than one week; neither of these two variables being associated (P>0.05) with MP. Dairy farmers visited by veterinary doctors or practitioners were 78% while the rest (22%) were not, without any association (P>0.05) with MP. Results indicated that all farmers in KZN survey installed milking equipment properly, prevented liners from slipping, kept vacuum at claw, clean milking equipment adequately and kept liners and rubber parts free from cracks. However, none of them supplemented with sodium citrate.

Table 3. 3: Responses of dairy farmers on the mastitis challenge in KwaZulu-Natal province originally without changing their words

	Frequency
Should be taught	1
Advice people not to under milk	1
Good hygiene	2
Supplying drugs	1
Reducing source of infection	1
Environmental, stress management	1
Vacuum level control	1
Good management	1
Vaccinate cows	1
Cull more cows	1

Two farmers out of 11 suggested that good milking hygiene practices is important in the prevention and control of mastitis while others gave others suggestions

3.3.2 Entebbe, Uganda

Results indicate that male farmers had herd size of (2±0.33) and female (1±0.19) for cross breed with no association (P>0.05) between them. Male farmers had herd size

2cows and female farmers of 2 cows for Friesland breed with no association (P>0.05) between them. On table 3.4 the odds ratio indicated that male farmers are less likely to have mastitis than females. Mastitis is 3 times more likely to be prevalent in herds during the dry season than wet season.

Table 3. 4: **Odds ratios (OR) for gender of farmers, season of the year interacted with mastitis prevalence**

predictor	Odds	LCI	UCI
Gender (Male vs Female)	0.636	0.141	2.800
Season (Dry vs Wet)	3.143	0.709	15.830

Table 3. 5: association between breed, cow attribute and mastitis prevalence in Entebbe municipality

	Mastitis prevalence			
	Yes	No	χ^2	P-Value
Breed				
Cross	17	3	3.00	0.23
Friesland	43	33		
Local	-	3		
Period of lactation				
Early lactation	7	3	6.60	0.04
Mid lactation	23	0		
Late lactation	30	37		

The number of cross breed animals were 20%, 76% Friesland and 3% local with no association (P>0.05) between animal breed and MP (table 3.5). Ten percent of the cows were in early lactation, 25% in mid lactation and 67% in late lactation. There was a significant association (P<0.05) between stage of lactation and prevalence of mastitis.

Table 3. 6: Association between milking hygiene practices and mastitis prevalence in Entebbe municipality

		Mastitis prevalence			
		Yes	No	χ^2	P-Value
Milking hygiene practices:					
Washing teats	Yes	60	0	3.21	0.07
	No	33	7		
Pre- milking disinfection	Yes	50	10	0.43	0.51
	No	37	3		
Wearing gloves	Yes	3	0	0.70	0.40
	No	57	40		
Do you own boots?	Yes	37	17	1.09	0.30
	No	23	23		
Colour of boots	Black	57	29	0.21	0.65
	white	7	7		
Keeping bedding clean	Yes	30	17	0.20	0.70
	No	30	23		
Measures to minimise flies	Yes	60	37	1.60	0.22
	No	0	3		
Spraying and dip	Yes	57	37	0.09	0.80
	No	3	3		
Frequency of spraying	Regularly	48	17	3.20	0.08
	Seldom	14	21		
Role of milking hygiene	Agree	31	28	2.51	0.28
	Disagree	10	0		
	Neutral	21	10		

Sixty percent of farmers washed teats while 40% did not with a tendency of associating (χ^2=3.21; P<0.07) with prevalence of mastitis. Sixty percent of farmers carried out pre-milking teat disinfection while 20% did not without any association (P>0.05) with MP.

Three percent of farmers wore gloves while 97% did not. Farmers who put on boots were 54% while those that did not were 46%. Of those who wore boots, 86% put on black boots while 14% put on white boots. Forty seven percent of famers kept clean

bedding while 53% did not. Farmers who put measures to minimise flies were 97% while those who did not were 3%; none of the above variables was association (P>0.05) with MP.

Farmers that sprayed and dipped animals were 94% while those who did not were 6% with no association (P>0.05) with MP. Sixty five percent of farmers regularly sprayed while 35% did not with a tendency of associating (χ^2=3.21; P<0.08) with MP. Fifty eight percent agreed, 10% disagreed and 31% were neutral on whether milking hygiene practices affected mastitis occurrence; there was no association (P>0.05) with MP.

Table 3. 7: Association between nutrition and other management related factors on prevalence of mastitis in Entebbe municipality

		Mastitis prevalence			
		Yes	No	χ^2	P-Value
Selenium	Yes	40	27	0.00	1.00
	No	20	13		
Vitamin E	Yes	0	60	1.56	0.22
	No	3	37		
Additional supplements	Yes	30	30	1.90	0.17
	No	30	10		
Role of nutrition on mastitis	Agree	20	20	1.9	0.40
	Disagree	7	0		
	Don't know	33	20		
Ways to detect under milking	Yes	60	37	1.55	0.21
	No	.	3		
Records	Yes	50	30	0.31	0.60
	No	10	10		
Buying milk	Middle man	37	33	1.70	0.20
	Pass by customers	23	7		
Vet officer	monthly	3	0	1.6	0.22
	other times	60	37		
Association	Yes	17	13	0.11	0.75
	No	43	27		

Sixty seven percent of farmers supplemented dairy cow with selenium while 33% did not. Sixty percent of farmers supplemented cows with Vitamin E while 40% did not. Farmers who provided additional feeds constituted 60% while those that did not were 40%; neither of the above variables was associated (P>0.05) with MP. Forty percent of farmers agreed, 7% disagreed and 53% did not know whether nutrition has an effect on occurrence of mastitis without any association (P>0.05) with MP.

Farmers that practiced under milking were 97% while 3% did not with no association (P>0.05) with MP. Farmers that kept records were 80% while those that did not were 20%. Seventy percent of farmers sold milk to a middleman and 30% to walk-by customers. Only three percent of farmers were visited monthly by vet officer while 97% were visited less frequently. Thirty percent of farmers were attached to a dairy association while 70% were not; neither of the above variables was associated (P>0.05) with MP. Results indicated that all farmers in Entebbe municipality did not pre-strip, post milk and did not have knowledge on SCC but however knew that under milking could cause mastitis.

Table 3. 8: **Suggestions by farmers of Entebbe municipality on mastitis challenge originally without alteration of their words**

	Frequency
Hygiene	3
To educate us	2
To care for animals well	1
Supplying drugs	1
Need vet doctor to check on us	1
Help on feeds	1
Under milking shouldn't be done	1
Assist on inputs	1
Forming associations	1

Three farmers suggested that education was the way forward in prevention and treatment of mastitis (table 3.8) Two Farmers suggested milking hygiene practices while others gave other suggestions.

3.4 Discussion

Sixty percent of dairy farmers in Entebbe municipality had clinical mastitis in 2014 while 40% did not, indicating a great need for dairy farmers to improve their dairy management practices. Eighty six percent of the dairy farmers in KwaZulu-Natal province reported that 1-20 animals had clinical mastitis in the month the survey was done in 2014 and 14 % reported 21-70 animals. This is a high infection rate in just a month for dairy farmers who own 300-1200 animals. There is great need for dairy farmers in KwaZulu-Natal to also improve their dairy management practices. The fact that males farmers from Entebbe municipality are less likely to have mastitis than females farmer is contrasting to a study by Tesfaye *et al.* (2012) which showed that females were less likely to have mastitis than males. Tesfaye *et al*. (2012) explained that females were really involved in the practice of milking. Male farmers in Entebbe municipality were also directly involved in milking practice and this was the reason for less prevalence of mastitis.

The odds ratio on Entebbe survey suggested that mastitis was 3 times more likely to be prevalent in the dry season than wet season; this is attributed to the presence of horn-fly which are numerous in the dry than in the wet season acting as vector to transmit mastitis pathogens (David, 2015). In KwaZulu-Natal findings showed that mastitis was also more prevalent in summer and spring; this appears not to be in agreement with findings of Elbably *et al*. (2013) which showed dry season. However, in KwaZulu-Natal, warm to hot humid weather occurs in the wet season.

Some studies (Alemnew, 1999; Delelosse, 2010; Moges et al., 2011; Cai *et al*. 1994; Kherli et al., 1989) showed that cows in early lactation had high prevalence of mastitis due to low neutrophil functioning and greater negative energy balance. At Entebbe 14% of respondents had mastitis in early lactation, 23% in mid lactation and 67% in late lactation, contradicting the above reports. On the other hand 67% of KwaZulu-Natal farms had mastitis in early lactation, 5% in mid lactation and 26% in late lactation thus agreeing with the above reports, yet disagreeing with results at Entebbe.

Milking practices and milking machine malfunction contribute to mastitis (Damien, 2005). According to Jayaro *et al*. (2004) pre and post milking dipping were associated with lower risk of mastitis. These practices were applied by 88 and 95% of dairy farmers in KwaZulu-Natal, respectively. All dairy farmers in Entebbe municipality did not practice post milking dipping which is essential in providing protection to the teat end after milking. However, 60% practiced pre-milking dipping which had no relationship with prevalence of mastitis contradicting Jayaro *et al*. (2004). Other findings (Radostitis *et al*., 2000; Aminu *et al*., 2012) showed an association between MP (Mastitis prevalence) and washing of teats, washing hands and drying of teats. In all, 60% of dairy farmers washed teats which tended to be associated (χ^2=3.00; P<0.08) with MP, thus agreeing with Radostitis *et al*. (2000) and Aminu *et al*. (2012). Washing of hands and drying teats had no association with MP in KZN; 79% of KZN dairy farmers washed hands and even a lower proportion (27%) dried teats, despite that these practices are very essential in management and prevention of mastitis.

According to Kenji (2008) dirty bedding which refers to dampness and a lot of dung were the major causes of mastitis in dairy herds. However, finding from this study showed no association of dirty bedding with MP in Entebbe municipality perhaps because of low animal populations. In KZN, cleaning the parlour had no association with prevalence of mastitis because at least 95% of farmers kept their parlours clean. Damien et al. (2009) showed that dirty milking parlours caused mastitis in Irish dairy herds. According to Plozza *et al*. (2011) cows in herds without separate calving paddocks had higher SCC than those with separate calving. In KZN this practice tended to associate (χ^2=3.00; P<0.08) with MP, thus agreeing with Plozza *et al*. (2011). In addition providing separate calving paddocks for heifers and lactating cows prevented the spread of mastitis pathogens from unhealthy udders to udders of healthy animals. Results further indicated that the practice of providing shed helps to protect cows from heat stress and reduces movement of animals hence preventing the spread of mastitis pathogens by vectors and dirty farm areas; shed provision was not associated with MP and was practiced by only a few (27%) dairy farmers in KwaZulu-Natal.

In Entebbe the practice of wearing gloves during milking had no association with MP; this does not agree with the findings of Plozza *et al*. (2011) that wearing gloves would reduce mastitis. Dress code (putting on boots and the colour (white vs black) of boots) had no association with MP in Entebbe municipality. Aspects of dress code (including a white apron, head cover, white boots) are good for dairy industry as dirt is easily spotted and taken out, thus reducing milk contamination. Measures to minimise flies (that is dipping and spraying) were not associated with MP in Entebbe municipality probably because 97% farmers applied indigenous knowledge (use of

smoked cow dung) to chase flies. Secondly, 65%of farmers in Entebbe municipality sprayed regularly against ticks, which tended to reduce (χ^2=3.20; P<0.08) MP. In KwaZulu-Natal 64% sprayed to eliminate ticks and other flies besides horn flies which are vectors to mastitis. In this study the practice of replacing parts of milking machine had no association (P>0.05) with MP in KZN. However, Douglas (2011) reports that milking machine parts are supposed to be replaced regularly and should be functioning properly. Water at the mouth of teat caps cause liners to slip and damage teat ends increasing vulnerability to bacterial infection. However, the practice of preventing accumulation of water at mouth of teat cups was not associated with MP in KZN because 95% of dairy farmers practice it.

All farmers in Entebbe municipality did not pre-strip nor check for SCC. This is dangerous for milk consumers as there is no early detection of subclinical mastitis. Generally dairy farmers in KZN know how to maintain and keep milking machine in proper functioning order. Research done by Henriches (2009) showed that vitamin E and selenium supplementation enhanced the immunity of the mammary gland. Results from Entebbe showed that 67% of dairy farmer supplemented selenium and 60% supplemented with vitamin E which did not explain MP. Similarly, in KZN 84% of dairy farmers supplemented selenium without any association with MP. Dairy farmers in both places are quite serious in this area of nutrition but there is a great need for improvement and consistence in this practice. At KZN 42% of dairy farmers provided feed after milking which was unrelated to MP. This disagrees with findings of Tesfaye (2012) showing a strong association between provision of feed after milking and prevalence of mastitis. Providing feed after milking encourages cows to stand, helping in accelerating teat closure thus preventing entry of bacteria

as cows go to graze. According to Plozza *et al*. (2015) the practice of providing additional feeds had no association with MP, similar to results at Entebbe municipality.

That using dry cow therapy was unrelated to MP in KZN sharply contrasted the findings of Zeryhun *et al*. (2013) reporting an association between dry cow therapy and MP. This could be explained by the size of survey of which 77% were actively practicing dry cow therapy. Hiefers sharing the same paddock with lactating cows and buying-in replacement heifers are practices not encouraged in the dairy industry partly to avoid they spread mastitis. However, this practice was uncorrelated with MP. Also, frequent monitoring of somatic cell count and frequent regular visits by veterinary officers are encouraged for animal health reasons although this was not correlated with MP in KZN. Fifty eight percent of farmers in KZN lose 50000-150000 and 42% above 150000 rands on treatment of mastitis per annum and such losses could be attributed to numerous treatments due to high incidence rate of mastitis. Keeping records and monthly visits by vet officer are important in minimising mastitis but these practices were not associated with MP in Entebbe municipality Uganda.

In KZN 2 out of 11 dairy farmers said hygiene was the most important in minimising of mastitis, while others suggested different management factors. Out of 12 dairy farmers in Entebbe municipality, 3 suggested hygiene while 2 thought training by extension workers were key issues. This suggest education on mastitis by animal health workers and veterinary doctors would be of much importance to both areas**.**

3.5 Conclusion

In conclusion mastitis is a great challenge to small holder dairy farmers in Entebbe municipality and KZN. Dairy farmers did well on some milking hygiene practices like pre- milking teat disinfection and washing teats. They also practice management factors like good nutrition, record keeping, and checking for under milking. However they did not practice CMT test, fore milking and post milking disinfection. The percentage of those that put on gloves was low and dry cow therapy is not done at all in Entebbe municipality. Large scale dairy farmers in KwaZulu-Natal practiced proper maintenance and functioning of milking machine which is important in the prevention and control of mastitis. Few farmers provided feed to encourage animals to stand after milking. Some farmers practiced good nutrition management and hygiene practices. The study showed that mastitis is a challenge to large scale dairy farmers in KwaZulu-Natal and small scale dairy farmers in Entebbe municipality

therefore, there is great need to improve on the management practices as they are the major causes of mastitis. This also calls for alternative treatment therapies like trisodium citrate because mastitis pathogens have become resistant to antibiotics or this system of management meets the need of some consumers.

Chapter 4: The role of trisodium citrate in treatment of Sub-clinical mastitis

Abstract

Sub-clinical mastitis is the most dangerous form of mastitis and cause of loss in the dairy industry due to decreased milk production and milk that has to be discarded due to high somatic cell count. Mastitis pathogens have become resistant to antibiotics and dairy farmers incur more costs on buying more superior antibiotics. The objective of the study was to find out whether alternative treatment therapy trisodium citrate had an effect on SCC and growth rate of bacteria.

The treatment groups were as follows T1=control, T2=15gm, T3=30gm and T4=45gm. Trail 1 was done in sept 2014 n=18 and Trial 2 July 2015 n=18. The main variable in the study was somatic cells and covariates were breed, lactation number and milk yield. The trails lasted 10 days and data was recorded from day 1, 3rd 7th and 10th day. There was a gradual decrease of growth rate of bacteria for 1-3 under treatment.

A decrease was noted for colonies 4-10 with good results recorded against most of the treatments. Treatment did not react against bacteria growth of >10 colonies.

Milk yield had a significant ($P<0.05$) on SCC on day 1. Breed and lactation number had no significant ($P>0.05$) from 1, 3, 7 and 10th post treatment. Generally treatment had no significant ($P>0.05$).

Key words: Sub-clinical mastitis, growth rate, alternative therapy, SCC

4.1 Introduction

Mastitis is a major disease to the dairy industry leading to increasing use of antibiotics to counteract bacteria which is the major pathogen for this disease. There has been increased resistance of mastitis pathogens to antibiotics exemplified by increased resistance of staphylococcal isolates to different antimicrobial agents (Myllys *et al*., 1978; Pitkala, 2004). Research done by Ghaleb (2005) showed resistance of staphylococcus aureus mastitis and staphylococcus epidermis to ampicillin. Findings by Sadshiv and Kaliwal (2014) showed that Gram Positive mastitis pathogens were resistant to Methicillin, Penicillin, Oxacillin, Cefclor Norfloxacin and Tetracycline. Secondly, antibiotic residues have been found in milk building antibiotic resistance population of consumers. Some of these residues have also been identified as sources of cancer in human on long term basis and food poisoning for the short term (Hishem, 2013). Research done by Fathollah *et al*. (2013) showed that contamination of milk with antibiotics leads to allergic reactions and development of bacterial resistance and that tetracycline residues in milk consumed by infants less than 12 years caused permanent teeth discolouration.

Findings by Pritee *et al*. (2008) showed that a combination of Ashwaganda (50gm) vitamin E (5000IU), Zinc methionine (250mg), copper sulphate and selenium led to a recovery rate from mastitis of 80%. This pointed to good nutrition as an alternative treatment for antibiotics. Zinc is required in keratin formation and serves as part of the first line of defence of the udder against pathogenes. Thus inclusion of Zinc in diets reduced SCC of high producing cows (Barbano *et al*., 2008; Davidoc, 2013).

According to research done by Akbar *et al*. (2013), selenium + vitamin E+ Cu had a favourable effect on udder immune system and milk composition, without any change in milk production. In this same study the period of treatment was significant in the recovery of cows from mastitis. In this same study days or period was not significant on the recovery time from mastitis. Similarly, supplementation with selenium, vitamin E, Cu and other trace elements Co and Mn reduced somatic cell counts without any change milk production (Akbar *et al*., 2013; Chester *et al*., 2013). This makes use of other alternative therapies especially nutritional therapy to be an alternative to antibiotics.

Research done by Gaafar *et al*. (2010) showed that winter period had the most cases of elevated somatic cell counts (SCC) in animals followed by summer. Somatic cell counts tended to decrease with progress of lactation up to peak period but increased with the animals' number of lactations. In the same study Zinc methionine supplementation decreased somatic cell count, and changed the electrical conductivity and shortened the recovery time of affected animals (Gaafar *et al*.,2010). Spears and Weiss (2008) showed that Cr supplementation may affect cell and humoral immune responses. However, there was a difference between the treatment period and supplementation on SCC. Therefore, there is a great need to find out if trisodium citrate can affect mastitis treatment by reducing SCC and improving milk yield. Research done by Pablo (2013) showed that there was no association between parity and treatment groups on mastitis response to botanical therapy (PHYTOMAST). The objective of this study is to find out if additional trisodium citrate in cows' diet has an effect on SCC.

4.2 Materials and methods

The study was conducted in KwaZulu-Natal province at Fair field Dairy located at latitude 29° 28′ 0″ S, 30° 14′ 0″ E from August to September and in Denleigh farm located at latitude 29^{o} 19' 22.03", 30^{0} 15$^{"}$ 45.65"E from June to July 2015. Both places are found in the mist belt of KZN around PMB.

Experimental design: Lactating dairy animals were selected based on the level of sub-clinical mastitis (with 500000 or more cell/ml); these cows were greater than, 3 years of age and produced about 14 kg of milk. Dietary treatments comprised the control with no supplement (T1), supplementation with 15 g (T2), 30g (T3) and 45 g (T4) daily of trisodium citrate. These treatments were randomly assigned to 18 animals at Fairfield dairy (Trail 1: 11 Ayrshire and 7 Friesland) and to 18 Friesland cows at Denleigh farm (Trail 2).

4.2.1 Collection of milk samples

Each teat was scrubbed with pledged of cotton moistened with 80% alcohol separately. After discarding the first few drops of milk from each quarter, 10ml of milk was collected in sterilized bottles from the four quarters. Milk samples from each cow were collected in the following order: front right, front left, rear right and rear left to form a composite sample. Milk samples were collected during the 1st, 3rd, 7th and 10th day since the beginning sodium citrate treatment. These samples were stored overnight in freezer at 0^{0}C and transported in a cooler box with a cover to Allerton provisional vet laboratory the following morning for analysis of SCC, bacterial species and bacterial growth rate index.

4.2.2 Statistical analysis

Growth rate of bacteria was indexed 1-3, where 1 =1-3 colonies, 2: 4-10 colonies and 3 >10 colonies. Data on bacterial species number and the rate of growth in response to treatment was analysed using Proc Freq (SAS 9.8, 2011). The GLM of SAS 9.8 (2011) was used to determine the effect treatment on SCC.

4.3 Results

Gram negative bacteria and Gram positive bacteria. Gram negative bacteria included Escherichia.coli*,* Kleseilla pnuemoniae*,* Proteus mirabilis, pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermis and Enterobactor aerogenes. *Gram positive bacteria included* Staphylococcus aureus*,* Streptococcus agalactiae *and* Streptococcus uberis and Staphylococcus aerogenes.

Table 4. 1: **Interaction between treatment and species of bacteria**

	Species		χ^2	P-Value
	Mixed	Single		
T1	74	26	1.76	0.62
T2	81	19		
T3	68	32		
T4	73	28		

In the Table 4.1, 68 to 81% of species in each treatment were mixed and 19 to 32 were singles species. There was, however, no association (P>0.05) between treatment and species.

Table 4. 2: **Cross tabulation of rate of growth of bacteria in response to dietary treatment**

	T1	T2	T3	T4	χ^2	P-Value
Rate of growth						
1	25	19	23	20	1.99	0.92
2	28	38	33	30		
3	16	19	24	28		

In table 4.2, there was no association (P>0.05) of treatment with the rate of growth of bacterial species, although growth rate 2 was high in all treatments. The effect of supplementing cows with sodium tri-citrate are given on Table 4.3.The study demonstrated ridiculously high SCC from day 1-3, which reduced drastically by day 10. The SCC also increased (P<0.05) with initial SCC and decreased (P<0.001) with lactation number. Treatments had no effect (P>0.05) on SCC, neither did breed (P>0.05), lactation no (P>0.05) nor the covariate effect of milk yield (P>0.05). The mild yield was not affect by level of citrate supplementation or by any other variable.

Table 4. 3: Effect of various levels of sodium tri-citrate during various period on SCC in lactating cows and milk yield (kg/day)

	Days supplemented				
	1	3	7	10	Milk yield
LS					(kg/day)
T1	7.31	6.8	7.16	1.95	20.5
T2	7.01	6.6	7.15	1.92	18.7
T3	7.13	6.5	7.2	1.94	18.6
T4	7.32	8	7.32	1.99	21.2
RMSE	0.78	1.55	0.9	0.12	5.2
P Value					
Treatment	0.2	0.8	0.12	0.8	0.75
Breed	0.35	0.7	0.712	0.74	0.26
Lactation no	0.9	0.75	0.65	0.64	0.68
Milk yield	0.02	0.33	0.29	0.64	

4.4 Discussion

Somatic cell counts are a good predictor of udder health; the lesser they are the healthier the udder (Hillerton, 1999). Low citrate levels and free ca^{2+} are responsible for the initial lesion to the udder tissues. According to these studies (Dhillon et al., 1991; Dhillon *et al*., 1995: Singh *et al*., 1997), treating animals with trisodium citrate decreased the udder p^H creating an unsuitable environment for survival of bacteria. On the contrary in this study bacteria tended to grow slightly more under T4 than T3; in T4 more populations of greater than 10 colonies were prevalent.

In this study lactation number had no significant (P>0.05) effect on SCC from day 1, 3, 7 and 10th day thus agreeing with findings of Singh and Ludri (2001). Milk yield had a significant effect (P<0.05) on SCC on 1 day but not on other days (i.e. 3, 7 and 10th day); again agreeing with Singh and Ludri (2001). This high milk yield leads to flushing of bacteria hence reducing SCC. Breed had no significant (P> 0.05) on SCC which appears to be influenced by milking hygiene management. Treatment had no effect on SCC. However, SCC during various periods of sampling were significantly influenced by the initial SCC. Because somatic cell count would naturally count tissue cells, thus giving an index of white blood corpuscle in the mammary gland, it is possible that milking machines in these two farms could have inadvertently brought some injury to udder tissues.

4.5 Conclusion

This study showed that trisodium citrate lacks the potential to reduce the growth rate of bacteria. A radical reduction in SCC occurred on day 10. It could well be that higher levels of supplementation with tri-sodium citrate is required in KZN to demonstrate its therapeutic potential on sub clinical mastitis and presumably to clinical mastitis. Further studies should be done to clarify the therapeutic potential of tri-sodium citrate as an alternative to antibiotics and evaluation of herbs as an alternative for treatment of bovine mastitis.

Chapter 5:[1] Antibacterial activity and cytotoxicity of selected medicinal plants against bovine mastitis pathogens

Abstract

Bovine mastitis, a disease caused by inflammation of the udder, is one of the major causes of global dairy industry losses. Alternative therapies such as ethno veterinary medicine are worthy of further study since mastitis-causing bacterial pathogens are becoming increasingly resistant to conventional antibiotic therapy. Plant species were selected on the basis of their known antibacterial activity, use in ethno veterinary medicine and their ready availability for *in vitro* testing against a panel of bacterial species implicated in causing mastitis (both ATCC strains and clinical isolates). Water and acetone extracts were prepared from various plant parts of *Acacia nilotica*, *Tetradenia riparia, Aloe arborescens* and *Crassula multicava.* Antimicrobial activity was determined using a serial microdilution assay and cytotoxicity was evaluated against a mammalian kidney cell line using a tetrazolium-based colorimetric (MTT) assay.

Aloe arborescens and *Crassula multicava* were not generally active against tested bacteria. Acetone extracts of *A. nilotica* bark and *T. riparia* flower extracts were most active against Gram-positive bacteria. Activity against Gram-negative species, notably *Proteus vulgaris* and *Enterobacter aerogenes*, was also noted with MIC values as low as 0.0195 mg/ml. The best selectivity index (SI) value of 4.2205 was obtained by the *T. riparia* flower acetone extract against the field strain of *Streptococcus uberis.* Although toxicity of most of the extracts to mammalian cells

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was noted, good SI values indicate that activity was greater than toxicity for some extracts. These extracts, or purified active compounds derived from them, may prove useful in further investigations of alternative mastitis treatments.

Keywords*:* Bovine mastitis, ethnoveterinary medicine, antibacterial activity, cytotoxicity

5.1 Introduction

Mastitis is an inflammation of the mammary gland caused mainly by physical and microbial factors. Predominant mastitis-causing organisms are bacteria, fungi, yeasts and possibly viruses (Tiwari *et al*., 2013). Clinical mastitis is characterised by secretion of abnormal milk (i.e. watery milk or presence of flakes in milk) and inflammation leading to redness, swelling and hardness of the udder (Sharif and Muhammed, 2009; Chebel, 2007). It is generally accepted that the normal mammary gland of the dairy animal has a somatic cell count (SCC) of approximately 200 000 cells/ml with no bacterial infection. However, animals that have SCC greater than 200 000 cells/ml with the presence of bacteria in milk but no clinical signs of mastitis are considered to have subclinical mastitis (Chebel, 2007). This stage of mastitis is critical if left untracked and is of major importance in the dairy industry.

Mastitis is the most costly disease affecting dairy cattle, and is a major concern to the dairy industry throughout the world (Blosser, 1979; Mubarack *et al*., 2011; DeGraves and Fetrow, 1993). Worldwide losses due to mastitis range from €61 to €97 per cow on the farm per year (Hogeveen *et al*., 2011). However, if this disease is detected in the early stages, a greater portion of the loss can be reduced. According to Giesecke *et al*. (1971) the total value of annual milk losses due to mastitis in

South Africa is estimated at ZAR 29.68 million on farms. A deviation of SCC from normal (200 000 cells/ml) results in decreases in profit ranging from ZAR 491.48 to ZAR 1795.5 per cow per year, depending on the breed and production system (Banga *et al*., 2014).

The most common contagious bacteria causing mastitis among bovines are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, *Mycoplasma* species, *Staphylococcus chromogenes* (coagulase negative staphylococci), *Streptococcus dysgalactiae* and *Streptococcus uberis* (Erskine, 2001; Ahmad, 2001; Akram, 2002; Khan, 2002). The most prevalent mastitis pathogens occurring in animal surroundings such as bedding and manure have been found to be mainly environmental pathogens or coliforms. These include *Escherichia coli, Pseudomonas serratia, Enterobacter aerogenes, Klebsiella* species and *Proteus* species. However, in South Africa, the coagulase negative staphylococcus (*S. chromogenes*) was the most frequently isolated bacterial species in both lactating and dry cows from year 2000 to 2007, followed by *Staphylococcus aureus* and *Streptococcus agalactiae* (Petzer *et al*., 2009). It has been recorded that *S. aureus* is the most important mastitis pathogen in South Africa because of the economic losses it poses (Wilson et al., 1997).

The continuous use of antibiotics in the treatment of mastitis and other diseases in both humans and animals has led to multidrug resistant organisms, for example methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus* (VRE) and (extensively drug resistant) (XDR) tuberculosis (Alekshun and Levy, 2007). This results in the use

of high doses of antibiotics in animals such as dairy cows, leading to an increased amount of antibiotic residues in milk which is potentially hazardous to human health (Chockalingam *et al*., 2007; Dhanabalan *et al*., 2008). Futher danger of antibiotics is that they inhibit the normal defence mechanisms of the host by decreasing phagocyte function, rendering them inefficient (Aboul-Ela, 2002). Due to the increased antibiotic resistance of mastitis pathogens, high expenses incurred by farmers and risks posed to human health, there is a great need for an alternative natural treatment which is cheap and sustainable and more environmentally friendly (Azadi *et al*., 2011).

Alternative veterinary medicine can be defined as the utilisation of alternative therapies in the treatment of diseases (Loken, 2001). Therapies that fall into this category include homeopathy and phytotherapy. These alternative therapies have proven to be more frequently used on organic dairy farms than conventional farms worldwide (Henriksen, 2002). Phytotherapy in animal medicine can be simply described as ethno veterinary practices. The latter can be defined as knowledge, beliefs, experiences, skills, methods and practices regarding animal health care and management (Mathias, 2001; Akinpelus and Onakoya, 2006).

Medicinal plants have been used for a variety of purposes, including food preservation, pharmaceuticals and as natural therapies for many years. They have been used as natural antioxidants, antibacterial, cytotoxic, antiviral and fungicidal agents and nutrients (Gopinath *et al*., 2011). Antimicrobials obtained from plants have much therapeutic potential and are effective in treatment of infectious diseases in animals as well as humans. They may also simultaneously mitigate many of the

side effects that are associated with synthetic antimicrobials, for example drowsiness and death of red blood cells (Sharma *et al*., 2012). According to Kidd (1997) traditional veterinary medical practices constitute a rich heritage and have been used for centuries when there were no veterinarians and orthodox drugs. In South Africa only around 13% of plants used in ethno veterinary medicine have been evaluated for biological activity in targeted ethno veterinary studies (McGaw and Eloff, 2008). A large number of non-commercial, rural livestock owners in South Africa treat their own animals with traditional remedies (McGaw and Eloff, 2008). The rich cultural diversity in South Africa and many other developing countries is reflected in the use of plants as medicines and it is estimated that up to 60% of South Africans consult traditional healers, usually in addition to making use of orthodox medical services (Van Wyk *et al*., 1997).

Research done by Van der Merwe et al. (2001) in the North West province of South Africa recorded forty-five plant species from 24 families for 29 indications in animal medicine. The most important indications noted were retained placenta, diarrhoea, gall sickness, fractures, eye inflammation, general wellness, fertility problems, gastro-intestinal ailments, heart water, helminthiasis, coughing, red water and reduction of ticks. Masika *et al*. (2000) estimated that 70% of rural livestock owners in the Eastern Cape of South Africa use plants or plant based remedies to treat animal ailments. The study also showed that use of herbal remedies to treat animal diseases was widespread amongst small-scale farmers in the Eastern Cape, which was largely because of the low cost, convenience and ease of administration. Luseba and Van der Merwe (2006) made use of RRA (Rapid Rural Appraisal) methods to collect indigenous knowledge on animal disease treatment by Tsonga

people of South Africa. These remedies were thought to be more effective for treating some diseases and also as alternatives to expensive orthodox pharmaceuticals.

In this study, a number of plant species was selected for evaluation of antibacterial activity against a panel of microorganisms implicated in causing mastitis. These species were chosen on the basis of previously reported antibacterial activity as well as potential use in ethnoveterinary medicine for the treatment of various diseases. The list includes *Acacia nilotica* (bark and leaves)*, Aloe arborescens* (leaves) *Tetradenia riparia* (flowers and leaves), *Crassula multicava* (whole plant)

Acacia nilotica (L.) Delile (Fabaceae) is known as the Gum Arabic tree, Babul or Egyptian thorn. It is a multipurpose nitrogen fixing tree legume. It occurs from sea level to over 2000 m and withstands extreme temperatures greater than 50^{o}C but is sensitive to frost when young. It is widely spread in sub-tropical and tropical Africa from Egypt to Mauritania southwards to South Africa and in Asia eastwards to Pakistan and India. *Acacia nilotica* has antiseptic properties and is used in healing of wounds (Mattana *et al*., 2012).

Antibacterial activity of *A. nilotica* has been registered against *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella typhi* (Saini *et al*., 2008). The bark is used extensively for colds, bronchitis, diarrhoea, bleeding and leucoderma (Singh and Arora, 2009). There is no available information on the use of *A. nilotica* in ethnoveterinary medicine. However, some closely related species are being used for these practices, including *Acacia decurrens* where bark and leaves of this species

are used to treat uterus disorders in cattle (Masika *et al*., 2000). This species is used in the treatment of diarrhoea and intestinal parasites in poultry and also to treat fractures (Dold and Cocks, 2001; Van der Merwe *et al*., 2001). Minimum inhibitory concentration (MIC) values of 0.125 mg/ml for against *S. aureus* and 0.250 mg/ml against *E. coli* of *A. nilotica* alkaloids have been reported (Vijayasanthi *et al*., 2012). Antimicrobially active *A. nilotica* phytochemicals include saponins, anthraquinones, tannins, flavonoids, terpernoids, alkaloids and glycosides (Deshpande, 2013; Banso, 2009).

Aloe is an African genus occurring naturally in Africa and a few other parts of the world (Baker and Linley, 1983) with over 150 *Aloe* species found in southern Africa. Aloes are used to treat conditions such as diarrhoea, burns and septic wounds in southern Africa and Zimbabwe (Mbanga *et al*., 2010; Bisi-Johnson, 2010). The aloe plant is commonly used in rural poultry management in Zimbabwe (Mwale et al., 2005). Hutchings et al. (1996) recorded the use of *Aloe arborescens* Mill (Asphodelaceae) for the treatment of sick calves. According to work done by Pellizoni *et al*. (2012), active phytochemicals in *Aloe arborescens* mainly constitute phenols, anthraquinones and aloin.

Tetradenia riparia (Hochst.) Codd (Lamiaceae), also known as the ginger bush, is used for medicinal purposes by the Chagga, Pare, Meru and Maasai ethnic groups from North East regions of Tanzania (Njau, 2001). Ethnopharmacological information obtained from these ethnic groups through interviews indicate that the plant is used against dysentery, indigestion, constipation and malaria (Njau, 2001).*T. riparia* is one of the most important herbal species in South Africa (Shaik *et al*., 2014). It is also

used in the treatment of stomach aches, mouth ulcers, tooth aches, influenza and swollen legs (Njau *et al*., 2014). The Tswana people in South Africa use the leaves of this plant for gall sickness in cattle (Roberts, 1990; Hutchings *et al*., 1996).

Antibacterial and anthelmintic activity has been recorded for extracts of *Crassula multicava* Lem. (Crassulaceae) whole plant (Okem *et al*., 2012). Antibacterial MIC values against *E. coli* for the ethanol extract were 3.13 mg/ml, 1.56 mg/ml for the ethyl acetate extract and 6.25 mg/ml for the water extract (Okem *et al*., 2012). Against *S. aureus*, MIC values of 1.56 mg/ml, 1.56 mg/ml and 3.13 mg/ml were obtained for the ethanol, ethyl acetate and water extracts respectively (Okem *et al*., 2012).

The plant species listed above were selected because of their availability, sustainability and their usage in ethnoveterinary medicine, as well as their reported antimicrobial activity. The purpose of this *in vitro* study was to determine if extracts of these species can be exploited for treatment of bovine mastitis. Extracts of these plants were screened for activity against species of organisms implicated in causing mastitis, including clinical isolates and ATCC strains. Toxicity of each of these extracts was also tested against a mammalian cell line to ascertain whether activity noted was due to a general toxic effect.

5.2 Materials and Methods

5.2.1 Plant material collection and extract preparation

Tetradenia riparia (flowers and leaves)*, Aloe arborescens* (leaves)*, Crassula multicava* (whole plant) and *Acacia nilotica* (bark and leaves) plant material was

collected in August 2014 from the University of Kwazulu-Natal (UKZN) Botanical Gardens (first three species), and from the Sports Complex at UKZN, Pietermaritzburg campus. Plant parts were oven dried at 50^0C for 3-5 days. Voucher specimens PHILLIP 1, PHILLIP 2, PHILLIP 3 and PHILLIP 4 for each species respectively were identified and deposited in the Natal University (NU) Herbarium at the University of KwaZulu-Natal, Pietermaritzburg campus. The dried plant material was milled into powder through a 1 mm ring sieve using an Ultra Centrifugal mill (Zm 200 Restch, Germany) and stored at room temperature in air-tight containers.

Ground plant material (2 g) was extracted separately with 20 ml acetone, and distilled water in an ultrasonicator (Julabo GMBH Germany) for 15 min. In turn these extracts were filtered through Whatman No 1 filter paper into pre-weighed glass vials. The marc was re-extracted a further two times with 10 ml of extracting solvent and the filtrates were combined from each of the three extractions. These extracts were dried under a stream of air at room temperature. Water extracts were collected into pre-weighed glass jars and freeze dried using a Kieng freeze drier.

5.2.2 Bacterial cultures

Bacterial species used in the assay included (American Type Culture Collection) ATCC strains of Gram-positive species implicated in causing mastitis, namely *Staphylococcus aureus* (ATCC 25923), *Streptococcus uberis* (ATCC 700407), *Streptococcus agalactiae* (ATCC 13813) and the Gram-negative species *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus mirabilis* (ATCC 29245). Clinical field isolates of *Staphylococcus aureus*, *Staphylococcus chromogenes* and *Streptococcus uberis*

obtained from Allerton Provincial Veterinary Laboratory, Pietermaritzburg (South Africa) were included in the experiment. *P. vulgaris* and *E. aerogenes* clinical isolates were obtained from University of Zululand culture collection. Bacteria were cultured in sterile Mueller-Hinton (MH) broth (Oxoid) at 37^0 C and maintained at 4^0 C on MH agar.

5.2.3 Antibacterial microdilution assay

Minimum inhibitory concentration (MIC) values of extracts against different bacterial organisms were determined using a serial microdilution assay (Eloff, 1998). Plant extracts were re-suspended to a concentration of 10 mg/ml with acetone for organic extracts and sterile distilled water for aqueous extracts. A two fold serial dilution of 100 µl of the re-suspended extracts was prepared with sterile water in 96 well microtitre plates for each extract. Gentamicin (Sigma) was used as positive control and negative controls (acetone and bacteria free broth) were also included. The bacterial stock cultures cultured overnight in MH broth at 37°C were diluted with sterile MH broth to an approximate inoculum of 1 x 10^5 cfu/ml (Cos *et al*., 2006) and 100 µl was added in each of the wells. Microtitre plates were then covered and sealed with parafilm and incubated at 37^oC overnight. Bacterial growth was indicated by adding 40 µl of a 0.2 mg/ml solution of p-iodonitrotetrazolium chloride in sterile water (INT, Sigma) to each well and plates were incubated at 37°C for 30 min until development of colour caused by actively dividing cells. Bacterial growth in the wells was indicated by reddish pink colour, whereas clear wells, or those with much less intense colour, indicated inhibition by tested extracts. MIC values were recorded as the lowest concentration of extracts showing clear wells. The assay was repeated twice with replicates.

Pytochemicals only vary in quantities during different seasons but are the same with in species, this is the reason why pseudo replicates are used in this kind of studies (Dayana *et al*., 2012)

5.2.4 Cytotoxicity assay

Viable cell growth after incubation of African green monkey kidney (Vero) cells with test compound was determined using the tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983). Cells of a subconfluent culture were harvested and centrifuged at 200 x *g* for 5 min, and resuspended in growth medium to 5 x 10^4 cells/ml. The growth medium used was Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (FCS, Highveld Biological). A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtiter plate. MEM (200 µl) was added to wells of columns 1 and 12 to minimize the “edge effect” and maintain humidity. Plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator until the cells were in the exponential phase of growth. The MEM was aspirated from the cells, and was subsequently replaced with 200 µl of test compound at differing concentrations in quadruplicate. Serial dilutions of the test extracts and compounds were prepared in MEM. These cells were disturbed as little as possible during the aspiration of medium and addition of test substance. Microtitre plates were incubated at 37ºC in a 5% CO_2 incubator for 48 h with test compound or extract. Untreated cells and positive control (doxorubicin chloride, Pfizer Laboratories) were included.

After incubation, cells were washed with 150 µl phosphate buffered saline (PBS, Whitehead Scientific) and fresh MEM (200 µl) was added to each well. Following this, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and plates incubated for a further 4 h at 37ºC. After incubation with MTT, the medium in each well was carefully removed, without disturbing the MTT crystals in these wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well after which plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Chromate 4300) at a wavelength of 540 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

5.3 Results and Discussion

Results for antibacterial activity assays are presented in Tables 5.1 and 5.2 Plant extracts with MIC values less than 1.0 mg/ml were considered to have good antibacterial activity (Aligiannis *et al*., 2011) and those with MIC values less than 0.1 mg/ml were regarded as having significant activity (Kuete *et al*., 2010). Previous studies by Sharma *et al*. (2014) reported that acetone extracts of *Acacia nilotica* had antibacterial activity with MIC of 6.25 mg/ml and 12.5 mg/ml against *S. aureus* (HM 626197), for leaves and bark respectively. Table 5.1 shows that *Acacia nilotica* bark acetone extracts were more active the leaves against ATCC Gram-positive bacteria and the best MIC value was 0.039 mg/ml against *S. aureus* (American Type Culture

Collection) ATCC 25923 and *Streptococcus uberis* ATCC 700407, followed by *S. agalactiae* ATCC 13813 (MIC = 0.0703 mg/ml). *A. nilotica* bark water extracts were also active against these three strains with the highest MIC value of 0.1560 mg/ml for *S. aureus.* For leaf extracts the best MIC value was 0.1822 mg/ml against *S. uberis*. *A. nilotica* bark and leaves acetone extracts were active against all field isolates (Table 5.1) with MIC values less than 1 mg.ml and the best activity against *S. aureus* and *S. chromogenes* with MIC = 0.039 mg/ml. *A. nilotica* bark acetone extract was most active against *Staphylococcus epidermidis* with MIC = 0.0097 mg/ml, and good activity was also shown against *Enterobacter aerogenes* and *Proteus vulgaris*, as well as *Pseudomonas aeruginosa*.

Aloe arborescens and *Crassula multicava* water and acetone extracts were generally not very active against bacterial species tested, and neither were *Tetradenia riparia* leaf extracts, although the latter were more active than *A. arborescens* and *C. multicava*.

The acetone extract of *Tetradenia riparia* flowers showed strong antibacterial activity with MIC values below 0.1 mg/ml against several Gram-positive bacterial species (Table 5.1). Good MIC values were also achieved against *P. vulgaris, S. epidermidis* and *E. aerogenes.* Gram-positive bacteria are generally more responsive to antimicrobial substances because of permeability to foreign compounds, compared to Gram-negative bacteria, because they are multi-layered and bounded by an outer cell membrane (Sharma *et al*., 2014; Moyo *et al*., 2011; Aremu *et al*., 2010).

Regarding cytotoxicity results, *Acacia nilotica* bark acetone and water extracts were the most toxic of all extracts screened (Table 5.3), with LC_{50} values of 0.0332 and 0.0278 mg/ml respectively. This indicated that the selectivity index (SI) values (LC_{50} / MIC) were less than 1 against most bacterial species, so these plant extracts were more cytotoxic than they were antibacterial. Whereas MIC values were 0.0097 mg/ml and 0.0195 mg/ml against *S. epidermidis* and *E. aerogenes* respectively, SI values were 3.4227 and 1.7026, indicating much better activity than toxicity which encourages further studies of the antibacterial activity of this extract against these bacterial species.

Tetradenia riparia flower acetone extracts were also relatively antibacterial, but had an LC_{50} value of 0.0823 mg/ml against Vero cells. SI values ranged from 1.0551 to a promising 4.2205 where MIC values were reported between 0.078 and 0.0195, mg/ml respectively. The best SI value of 4.2205 was obtained against the field strain of *Streptococcus uberis.*

This study highlights and supports the value of parallel cytotoxicity testing to determine whether antibacterial activity is due to selective antibacterial activity or general toxic effects. The higher the SI value, the better the chances of a plant extract containing antibacterial substances that do not have general toxic effects to mammalian cells as well.

5.4 Conclusion

Mastitis is a disease causing significant economic impact worldwide, amongst commercial dairy farmers, it is also affecting the livelihood of rural small-holder farmers. The use of plant extracts to control bacterial infections is important in ethno veterinary medicine and it is interesting to study such plant species for potential efficacy against bacteria such as those implicated in causing mastitis.

In this study, *Aloe arborescens* and *Crassula multicava* acetone and water extracts did not show good antibacterial activity against the panel of bacterial species tested, but *Acacia nilotica* and *Tetradenia riparia* had promising activity. *A. nilotica* bark and *T. riparia* flower extracts prepared using acetone had the best activity against Gram-positive species, including those isolated from mastitis cases in the field. Activity against Gram-negative species, notably *Proteus vulgaris* and *Enterobacter aerogenes*, was detected with MIC values as low as 0.0195 mg/ml for the crude extract. Although toxicity of these extracts to mammalian cells was noted, good SI values indicate that activity is greater than toxicity for some extracts. Therefore, further research is warranted to isolate and characterize constituents of these extracts responsible for bioactivity.

Table 5. 1: Antibacterial activity of extracts of plant species used against mastitis (Staphylococcus and Streptococcus species*)*

Species	**Part**	**Extr[1]**	**MIC values (mg/ml) ± SD**						
			***S. aureus*[2] (ATCC 25923)**	***S. agalactiae* (ATCC 13813)**	***S. uberis* (ATCC 700407)**	***S. aureus* (field strain)**	***S. chromogenes* (field strain)**	***S. uberis* (field strain)**	***S. epidermidis* (clinical isolate)**
A. nilotica	BK	A	**0.0390**±0.00	0.0703±0.00	**0.0390**±0.00	**0.0390**±0.00	**0.0390**±0.00	**0.0780**±0.00	**0.0097**±0.00
		W	0.1560±0.00	0.3130±0.00	0.6250±0.00	0.3130±0.00	0.6250±0.00	0.3130±0.00	0.3130±0.00
A. nilotica	LF	A	0.6250±0.00	0.3130±0.00	0.1822±0.06	0.6250±0.00	0.1560±0.00	0.3130±0.00	0.1560±0.00
		W	0.6250±0.00	1.2500±0.00	1.2500±0.00	0.6250±0.00	1.2500±0.00	0.3130±0.00	0.6250±0.00
A. arborescens	LF	A	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	0.3130±0.00	2.5000±0.00
		W	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	2.5000±0.00
C. multicava	WP	A	1.8750±0.00	0.7292±0.26	0.3130±0.00	0.3130±0.00	0.9375±0.34	0.3130±0.00	0.6250±0.00
		W	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	>2.500±0.00	1.2500±0.00	2.5000±0.00
T. riparia	FL	A	**0.0780**±0.00	**0.0390**±0.00	**0.0780**±0.00	**0.0728**±0.01	**0.0780**±0.00	**0.0195**±0.00	**0.0390**±0.00
		W	1.2500±0.00	1.2500±0.00	1.2500±0.00	2.5000±0.00	1.2500±0.00	2.5000±0.00	2.5000±0.00
T. riparia	LF	A	0.2345±0.00	0.1170±0.043	0.3130±0.00	1.1560±0.00	0.1560±0.00	0.1170±0.04	0.1560±0.00
		W	2.5000±0.00	2.5000±0.00	2.500±0.00	2.5000±0.00	>2.500±0.00	2.5000±0.00	2.5000±0.00
Gentamicin (µg/ml)			0.0780±0.00	2.0833±0.63	2.500±0.00	0.2345±0.09	0.0254±0.01	1.2500±0.00	2.5000±0.00

[1]Extr. = extractant, A = acetone, W = water; [2]Bacteria: *S. aureus* = *Staphylococcus aureus*, *S. agalactiae* = *Streptococcus agalactiae*, *S. uberis* = *Streptococcus uberis*, *S. chromogenes* = *Staphylococcus chromogenes*) BK=Bark LF=Leaf WP=Whole plant FL=Flower

Table 5. 2: *Antibacterial activity of extracts of plant species used against mastitis (against Gram-negative bacterial species)*

Species	**Part**	**Extr[1]**	**MIC values (mg/ml) ± SD**					
			***E.coli*[1] (ATCC 25922)**	***K. pneumoniae* (ATCC 13882)**	***P. aeruginosa* (ATCC 27853)**	***P. mirabilis* (ATCC 29245)**	***P. vulgaris* (clinical isolate)**	**E. aerogenes (clinical isolate)**
A. nilotica	BK	A	0.1560±0.00	0.3130±0.00	**0.0780**±0.00	0.2738±0.08	**0.0390**±0.00	**0.0195**±0.00
		W	>2.500±0.00	1.2500±0.00	>2.500±0.00	0.3130±0.00	2.5000±0.00	0.3130±0.00
A. nilotica	LF	A	0.3130±0.00	0.3130±0.00	0.1560±0.00	0.6250±0.00	0.1560±0.00	0.1560±0.00
		W	>2.500±0.00	1.2500±0.00	>2.500±0.00	0.6250±0.00	2.5000±0.00	0.6250±0.00
A. arborescens	LF	A	0.6250±0.00	0.6250±0.00	>2.500±0.00	0.3130±0.00	>2.500±0.00	>2.500±0.00
		W	>2.500±0.00	>2.500±0.00	>2.500±0.00	2.500±0.00	2.5000±0.00	>2.500±0.00
C. multicava	WP	A	0.3130±0.00	0.1560±0.00	0.1560±0.00	0.6250±0.00	0.6250±0.00	0.6250±0.00
		W	>2.500±0.00	2.5000±0.00	2.5000±0.00	2.5000±0.00	2.5000±0.00	2.5000±0.00
T. riparia	FL	A	0.1560±0.00	0.3130±0.00	0.2607±0.09	0.2607±0.09	**0.0390**±0.00	**0.0195**±0.00
		W	>2.500±0.00	0.6250±0.00	1.2500±0.00	2.5000±0.00	2.5000±0.00	2.5000±0.00
T. riparia	LF	A	0.1560±0.00	0.3130±0.00	0.3130±0.00	0.3130±0.00	0.1560±0.00	0.1300±0.05
		W	>2.500±0.00	1.2500±0.00	1.2500±0.00	2.5000±0.00	2.5000±0.00	2.5000±0.00
Gentamicin (µg/ml)			1.2500±0.00	0.3130±0.00	0.3130±0.00	1.2500±0.00	2.5000±0.00	0.6250±0.00

[1]Bacteria: *E. coli = Escherichia coli*, *K. pneumoniae = Klebsiella pneumoniae*, *P. aeruginosa = Pseudomonas aeruginosa*, *P. mirabilis = Proteus mirabilis*, *P. vulgaris = Proteus vulgaris*, *S. epidermidis = Staphylococcus epidermidis*, *E. aerogenes = Enterobacter aerogenes*; clinical isolate = isolate obtained from culture collection of University of Zululand)

Table 5. 3: Cytotoxicity of selected plant extracts

Species	Part	Extractant	Average	SD
A. nilotica	BK	Acetone	0.0332	0.0078
		Water	0.0278	0.0009
A. nilotica	LF	Acetone	0.2187	0.0044
		Water	0.0688	0.0042
A. arborescens	LF	Acetone	0.4825	0.0237
		Water	>1	-
C. multifida	WP	Acetone	0.8160	0.1421
		Water	0.4879	0.0081
T. riparia	FL	Acetone	0.0823	0.0092
		Water	0.1784	0.0042
T. riparia	LF	Acetone	0.0513	0.0195
		Water	0.2738	0.0270
Doxorubicin				

Chapter 6: General discussion, conclusion and recommendation

6.1 General discussion

Sub-clinical mastitis is the most debilitating form of mastitis because there no visible sign like swelling, redness of udder but it causes hug losses to farmers because of decreased milk production and elevated SCC (Chebel, 2007). In chapter 3, farmers practiced pre-milking teat disinfection and washing teats in KZN and Entebbe. Farmers in Entebbe did not practice CMT test for milking and post milking disinfection probably because the test is expensive. Some of them practiced good nutrition, while some kept good records as a way of minimising mastitis.

In KwaZulu –Natal most of the farmers use machine milking and according to the study most of them practiced proper maintenance of the machine which minimises mastitis. Few farmers practiced provision of feed to animals to encourage standing which enables closure of teat sphincter hence avoiding invading bacteria.

In chapter 4, *Aloe arborescens* and *Crassula multicava* whole plant were not active against tested mastitis bacteria. However, acetone extracts of *Acacia nilotica* bark and *T. riparia* were active against Gram-positive bacteria. These extracts were active against Gram-negative bacteria, *proteus vulgaris* and *enterobactor aerogenes. Tetradenia riparia* flower extracts had good (selective index) SI value against field strain *streptococcus uberis,* other good SI values were noted against other mastitis causing bacteria indicating that *Acacia nilotica bark* and leaf extracts together with *Tetradenia flower* and leaf extracts could be potential treatment to animals with mastitis.

6.2 Conclusion

There is great need to improve on milking hygiene procedures and nutrition because mastitis is still a great challenge to farmers in Entebbe municipality and Kwazulu-Natal. The study indicated potential use of herbs in the treatment of bovine mastitis especially *Acacia nilotica* and *Tetradenia riparia* which were active against Gram-positive (American Type Culture Collection) ATCC strains, field isolates and Gram-negative clinical isolates. Trisodium citrate did not improve results within ten days of experimentation, but is deemed to have good therapeutic potential towards sub-clinical mastitis

6.3 Recommendations and further research

Extension workers in Uganda should educate farmers on the role of nutrition in udder health. They should also help farmers in sourcing quality mineral blocks and vitamin supplements from certified agro vet stores. Extension workers should remind farmers about hygiene in the dairy industry through seminars coordinated by the government. Farmers should be advised on acquiring the chemicals for CMT test which is not done by farmers in Entebbe municipality Uganda. They should be reminded of the need to have strip caps to check clinical mastitis.

KwaZulu-Natal province Department of Agriculture together with the Milk Producers Organisation of South Africa should organise training for farmers on good milk hygiene practices and educate them on the role of trace elements on udder health. Subsidies should give on the chemicals used on CMT tests. Governments vet laboratories like Allerton should make SCC test cheaper to farmers. The herbs used in this study have never been used to treat mastitis though they are well known

for their antibacterial activity. Therefore, further studies should be done to isolate the active compounds in this plants because they were very active against bacterial species that cause Mastitis. *Tetradenia riaparia* and *Acacia nilotica* were active *against Staphylococcus chromogenes* which is a commonly isolated bacteria in KwaZulu-Natal province based personal communication with Allerton vet laboratory. However, it was noted that herbs were not toxic to mammalian cells, thus necessitating further research and clinical trials on animals. It could well be that higher concentration and/or longer period of use of trisodium citrate is needed to demonstrate its conclusive role in managing sub-clinical and clinical mastitis.

List of references

Aboul-Ela, E., 2002. Cytogenetic studies on *Nigella sativa* seeds extracts and thymoquinone on mouse cell infected with schistomiasis using karyotyping. Mutation Research 516 (1-2), 11-17.

Agoston, G. Gram positive and Gram Negative anaerobic rods Gram positive spore forming rods.,2010. Http//: www.Mikrobiologica.sote.hu/eload/eng/2010-2011/ghidan.

Ahmad, R., 2001. Studies on mastitis among dairy buffaloes. Pakistan Veterinary Journal 21, 220-221.

Akabar, P., Mohammad, J.B., Sayed. M.H., Amirah, H.A., 2013. The effect of selenium, vitamin E and Copper injection on somatic cell count and milk composition. Journal of Farm animal Nutrition and physiology 9(1), 41-49.

Akinpelu, D.A., Onakaya, T.M., 2006. Antimicrobial activities of medicinal plants use in folklore remedies in south-western Nigeria. African Journal of Biotechnology 5(11), 1078-1081.

Akram, M.M.A., 2002. Studies on clinical mastitis in buffaloes with special reference to *S. aureus.* MSc Thesis. University of Agriculture. Faisalabad, Pakistan.

Albenzio, M., Taibi, I., Muscio, A and Sevi, A., 2002. Prevalence and etiology of subclinical Mastitis in intensively managed flocks and related changes in the yield of milk .Small Ruminant Research 43(3), 219-226.

Alekshun, M.N., Levy, S.B., 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell 128 (6), 1037-1050.

Alemnew, M ., 1999. Epidemiological and bacteriological investigation of bovine mastitis at modjo state owned dairy farm: DVM thesis Addis Ababa University. Ethiopia.

Aligiannis, N., Kalpoutzakis, E., Mitaku, S., Chinou, I.B., 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agricultural and Food Chemistry 49(9), 4168-4170.

Aminu, S., J.Abdullahi., J.Jibril, A ., Mohammed, A.A and Fasina, F.O. (2012). Sub-clinical mastitis and associated risk factors on lactating cows in savannah region of Nigeria BMC Veterinary Research 8,134.

Aremu, A.O., Ndhlala, A.R., Fawole, O.A., Light, M.E., Finnie J.F., van Staden, J. 2010. *In vitro* pharmacological evaluation and phenolic content of ten South African medicinal plants used as anthelmintics. South African Journal of Botany 76(3), 558-566.

Azadi, H.G., Farzaneh, N., Baghestani, Z., Mohamadi, A., Shahri, S.A.M.,2011. Effect of intramammary injection of *Nigella sativa* on somatic cell count and *Staphylococcus aureus* count in Holstein cows with *S. aureus* subclinical mastitis. American Journal of Animal and Veterinary Sciences 6(1), 31-34.

Baker, B. Linley, K., 1983. Flowers of the Veld. Longman, Zimbabwe. pp. 73-74.

Balasubaramanya, N.N., Lokesh,B.R., Ramesh,H.P., Krishnakantha,T.P ., 1995. Effect of Lactic microbes on superoxide anion generating ability of peritoneal macrophages and tissue. Indian Journal of Dairy Bioscience 6, 28–33.

Banga, C.B., Neser, F.W.C., Garrick, D.J., 2014. The economic value of somatic cell count in South African Holstein and Jersey cattle. South African Journal of Animal Science 44(2), 173-177.

Bannerman, D.D., Paape, M.J and Hare, W.R ., 2003. Increased levels of LPS- binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. Journal of Dairy Science 86(10), 3128-3137.

Banicka, C., Winnicka, A., Jozwik, A., Rzwuska, M., Strapkowska, N., Koscuczuk ,E., 2011. Relationship between somatic cell counts and bacterial pathogens in goats

Milk. Small ruminant research, 100(1), 72-77. http:dx.do org/10/016/J.small rum res 2011.04.014.

Barkema.H, W., Schukken, Y.H., Lam, T.J.G.M., Boeboet, M.L., Benedictus, G and Brand, A., 1999.Management practices associated with incidence rate of Clinical mastitis. Journal of Dairy Science 82(8), 1643-1654.

Baulez, M and Dussert,L., 2011. Organic Selenium in Ruminant diet can we expect extra-benefit beyond health. www.Hallem and Animal Nutrition.com

Beckly, M.S and T. Johnson,T., 1966. Five year study of a California mastitis test on a commercial dairy herd. Journal of Dairy Science 49, 746-746

Bernabucci, U., Ronchi, B ., Lacetera,W and Nardore, A., 2002. Markers of Oxidative status in plasma and Erthrocyytes of Transition Dairy Cows. During Hot season. Journal of Dairy Science. 85(9), 2173-2179.

Beppu.H., Shimpo, K., Chihara, T., Kaneko, T., Tamai, L., Yamaji, J., Ozaki, J., Kazuya, H., Sonada, J., 2006. Antidiabetic effect of dietary administration of Aloe arborescens miller components on multiple low disc streptozotocin induced diabetes in mice: Investigation on hypoglycemic action and systematic absorption dynamics of aloe components. Journal of Ethnopharmacology 103(3), 468-477.

Bhakadi, S., Konish,T ., Nuber, P and Fisher,W ., 1991. Stimulation of monokine Production by lipoteichoic acids .Infection and Immunity 59, 4614-4620.

Bisi-Johnson, M.A., Obi, C.L., Hattori, T., Oshima, Y., Li, S., Kambizi, L., Eloff, J.N., Vasaikar, S.D., 2011. Evaluation of antibacterial and anticancer activities of some South African medicinal plants. BMC Complementary and Alternative Medicine 11, 14.

Blackburn, P.S ., 1966. The variation of cell count of cow's milk through out lactation and from one lactation to next. Journal of Dairy Research 33, 193-198

Blosser, T.H., 1979. Economic losses from and the national research program on mastitis in the United States. Journal of Dairy Science 62, 119-127.

Bramley, A.J, Patel, H., Orielly, N.M., R.Foster,R and Foster, T.J., 1989. Role of Alpha toxins and Beta toxins in virulence of Stapylococcus aureus for mouse mammary gland. Infection and Immunity. 57(8), 2489-2494.

Broadely, C., Hoover, R.L ., 1989. Ceruloplasmin reduces adhesion and Scavanges superoxide during interaction of activated polymorphonuclear leukocytes with endothelial cells. American Journal of Pathology 135(4),647-655.

Brown, W.C., Rice-Ficht, A.C ., Estes,A.C and Estes, D.M ., 1998. Bovine type 1 and type 2 responses. Veterinary Immunology and Immunopathology 63(1-2), 45–55.

Bytyqui, H., Zaugg, U., Sherifi.K., Hamid, A.M., Muyi, G.S and Mehmeti, H., 2010. Influence of management and physiology Factors on somatic cell count in raw milk in Kosova . Veterinarski Arhiv 80(2), 173-183.

Cai, T.Q, Weston,P.G ., Lund, L.A., Brodie,B., Meckenna, D.J and Wagner, W.C ., 1994. Association between neutrophil functions and Periparturient disorders in cows. American Journal of Veterinary Research 55(7), 934-943.

Claxton, P.D and Ryan, D., 1980. Bovine mastitis Australian Bureau of Animal Health.

Chamberlain, A.T, and Wilkenson, J.M., 1996. Feeding the dairy cow. Chalcombe Publication 90-91.

Chebel, R.C., 2007. Mastitis effects in reproduction. NMC Regional meeting proceedings. University of California-Davis, Tulare, California, USA. http://www.nmconline.org

Cheruvanky, H. 2004. Method for treating hypercholesterolemia, hyperilipidemia and artherosclerosis united states. The Journal of Pathology 6(4), 733-799.

Chew, BP ., 1993. Role of Carotenoids in immune response. Journal of Dairy Science 76(9), 2804 -2811.

Chester, H.C., Vermiere , D., Brommelsiek, W ., Brokken,K ., Marx, G and Linn, J.G., 2013. The effect of trace mineral source on reproduction and milk production in Holstein cows. Professional Anima Scientist 29(3), 289-297.

Christina, S.,Wolfe, P Andrea, R.TCurrin, J., and Ken, E.L 2012. Practical methods for mastitis control. Communications and Marketing college of Agriculture and life sciences Virginia Poly Technique institute and state University. Http: www.ucds.ca

Christianen, M. and Neilsen, F., 1934. Infectious bovine mastitis. Proc 12th Intern Vet. Congr 2, 548-562.

Chockalingam, A., Zarlenga, D.S., Bannerman, D.D., 2007. Antimicrobial activity of bovine bactericidal permeability-increasing protein-derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis. American Journal of Veterinary Research 68(11), 1151-1159.

Cos, P., Vlietinck, A.J., Vanden Berghe, D., Maes, L., 2006. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology 106(3), 290-302.

Cotter, P.D., Hill, C and Ross, R.P., 2005. Bacteriocins developing innate immunity for food. Nature Reveviews Microbiology 3(10):777-788.

Criuckshank, R., Duiguid, J.P and Swain, R., 1970. A guide to the Laboratory Diagnosis and control of infection. The English language Book Society and E and S.Livingstone 39, 101.

David,J.B., 2015. The horn fly. Http:// entomology.unl.edu.

Davidoc, I ., Radinovic,M., Erdeljan,M., Cicovic, M.R, Stanic,I and Belic,B., 2013. Relationship between blood Zinc concentrations and udder health in dairy cows. Reveude Medicine Veterinaire 164(4), 183-190.

Damien. J,B., Micheal, L.D and Anne, M.H., 2005. A descriptive epidemiological study of mastitis in 12 Irish Dairy herd. Irish Veterinary Journal 58, 1.

Darsnaki, R.k., Rokhi, M.L., Alibadi, M.A and Issaza, K ., 2012. Antimicrobial activities of lactobacillus strains isolated from fresh vegetables Middle East Journal of scientific research 11(9), 1206-1219.

Dayana, R.G., Gobbo-Neto, L., Humberto, T.S., Norberto,P., Lopez,J.L., Callegari,L. 2012. Seasonal variation of major secondary metabolites present in Extract of Eremanthus mattogrossensis less (Asteracaea, Vernonieaes) leaves. Quim, Nova 35(11), 2139-2145.

Delelesse,G.D., 2010. Study on prevalence of bovine mastitis in cross breed dairy cow around Holeta areas, West shoa zone of Oromia, Ethiopia. Global Journal Veterinaria 5(6), 315-323.

Deshpande, S.N., 2013. Preliminary phytochemical analysis and *in vitro* investigation of antibacterial activity of *Acacia nilotica* against clinical isolates. Journal of Pharmacognosy and Phytochemistry 1(5), 23-27.

Dhanabalan, R., Doss, A., Jagadeeswari, M., Balachandar, S. Kezia, E., Parivuguna, V., Reena Josephine, C.M., Vaidheki, R., Kalamani, K., 2008. *In vitro* phytochemical screening and antibacterial activity of aqueous and methanolic leaf extracts of *Tridax procumbens* against bovine mastitis isolated *Staphylococcus aureus.* Ethnobotanical Leaflets 12, 1090-1095.

Dhillon, K.S. and Singh, J., 2009. Managing chronically mastitic cows. Veterinary Record, 8th ed. Dhillon, K.S., J. Singh and B.S. Gil. 1989. A new horizon in Mastitis treatment. J. Res. Punjab Agri. Univ. 26(3), 477-481.

Dhillon,K.S., Sandhu, H.S., Singh,T.J., Singh.J and Gill, B.S., 1991. Novel mastitis therapy in Buffaloes. Indian Journal of Animal Sciences 61, 1262-1264.

Dhillon, K.S., Singh, T.J., Sodhi,S.S., Sandhu, H.S., Dwivedi, P.N., Singh,J and Gill,B.S., 1995. Milk bacteriology: Pre and post trisodium citrate mastitis treatment in Buffaloes. Indian Journal of Animal Sciences 65, 9-11.

Dold, A.P., Cocks, M.L., 2001. Traditional veterinary medicine in the Alice district of the Eastern Cape Province, South Africa. South African Journal of Science 97, 375-379.

Douglas,J.R, Graeme, AM and Pamela, L.R., 2001. Evaluation of milking machine performance paper presented at VII international congress on bovine medicine Ovied Austrias Spain.http://www.uwex.edu/umril/pdf

Dziarski, R., 1991. Demostration of peptide glycan-binding sites on lymphocyte and Macrophages by Photo Affinity cross-linking. The Journal of Biological Chemistry 266(8), 4713-4718.

Elbably,M.A.,Emeash,H.H and Amaa, N.M., 2013. Risk factors associate with mastitis occurrence in Dairy Herds in Benisuef.Egypt. Worlds Veterinary Journal 3(1), 5-10.

Elbers.A.R, W., Miltenburg, J.D., Delange, D., Crauwels, A.P.P., Barkema, H.N and Schukken,Y.H., 1998.Risk factors for clinical mastitis in a random sample of dairy herds from Southern part of the Netherlands. Journal of Dairy Science 81(2), 420-426

Eloff, J.N., 1998. A sensitive and quick microplate to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64(8), 711-713.

Eamonn, M.M.Q ., 2010. Prebiotics and probiotics: Modifying and mining the microbiota. Journal of Pharmacological research 61(3), 213-218.

Emanuelson, U and Person, E., 1984. Studies on somatic cell counts in milk from Swedish dairy cows non-genetic causes of variation in monthly test-day results. Acta Agriculture scandinavica section A-Animal science 34(1), 33-34.

Erika, M, Casas, R.E and Morales, M., 2012. Bovine mastitis pathogens, prevalence and Effects on somatic cells. http://cdn.intechopen.com/pdfs-wm/39479.pdf

Erskine, R.S., Eberhert,R.S ., Grasso,P.S., Scholz,R.W .,1989. Induction of Eschericia coli Mastitis in cows fed selenium supplemented diets. American Journal of Veterinary Research 50(12), 2093-2100.

Erskine, R.S., Eberhart,R.S., Hutchison, L.S and Scholz, W., 1987. Blood selenium concentration and glutathione peroxidase activities in dairy herds with high somatic cell counts . Journal of the American Medical Association 190, 1417-1422.

Erskine, R.J., 2001. Mastitis control in dairy farms. In: Radostitis, O.M. (Ed) health, Food Animal Production Medicine 3rd edition, W.B Saudres Company Philadelphia Pennsylvania.

Fadlemoula, A.,Fahr,R.D.,Anacker,G and Swalve, H.H., 2007.The management practices associated with Prevalence and Risk factors of mastitis in Large scale Dairy animals in Thurungia Germany 1: Enviromental factors associated with prevalence of Mastitis. Australian Journal of Basic and Applied science 1(4), 619-624.

Fathollah,A., Maryam, M., Mohammad,J and Leila,A., 2015. Dietry exposure to tetracycline residues through milk consumption in Iran. Journal of Enviromental Health Science and Engineering 13, 80.

Fernadez, M.F., J.Beris, J and Barbes, C., 2003. Probiotic properties of human Lactobacillus strains to be used in gastric intestinal tracts. Journal of Applied Microbiology 94(3), 449-458.

Fitzpatrick, J.L, Cripps, P.J ., Hill, A.W., Bland, D.W and Stokes, C.R ., 1992. MHC class II expression in bovine mammary gland. Veterinary Immunology and Immunopathology 32(1-2), 13-23.

Fox, L.K .(2009). Prevalence and risk factors of heifer mastitis. Veterinary Microbiology 134 (1-2), 82- 88.

Gaafar, H.M.A., Basuioni, M.I., Ali, M.F.E., Shitu, A.A., Shama, A.S.E., 2010. Effect of Zinc methionine Supplementation on Somatic cell count in milk and mastitis in Freshian cows. Archiva Zootechnica 13(2), 36-46.

Ghaleb, M.B., 2006, Antibiotic resistance against staphylococcal isolates recovered from Sub-clinical mastitis in North Palestine.The Islamic University Journal 14(1), 1-9.

Giesecke, W.H., van den Heever, L.W., Du Toit, I.J., 1971. Bovine mastitis in Republic of South Africa. Bulletin de l'Office International des Epizooties. 76, 621.

Gitto, E.R. J., Reiter, M.K.,Karbownik, MTan, D.X ., Gitto, P.B Barberi, I Barberi, s., 2002. Oxidative stress in pre and perinatal period. Biology of Neonate Journal 81(3) , 146-157

Goff, J.P., 2006. Major Advances in our understanding of Nutritional Influences on Bovine health. Journal of Dairy Science, 89(4), 1292-1301.

Gonsalez, R.N and D.J, Wilson, D.J., 2003. Mycoplasma mastitis in dairy herd. Veterinary clinics of North America: Food Animal Practice 19(1), 199-221.

Gopinath, S.M., Suneetha, T.B., Mruganka, V.D., Ananda, S., 2011. Evaluation of antibacterial activity of *Tabernaemontana divaricata* (L.) leaves against the causative

organisms of bovine mastitis. International Journal of Research in Phytochemistry and Pharmacology 1, 211-213.

Guidry, A. and Miller, R.H.,1986. Immunoglobulin isotype concentrations in milk as affected by stage of lactation and parity. Journal of Dairy Science 69(7), 1799–1805.

Halliwell, P. J., Gutteridge, M.C., 1999. In: Free radicles in Biology and medicine tenth (ed) Oxford University press New York USA.

Hamann, J., Ostreas, O, Magntz, M and Woyke, W., 1994. Functional parameters of milking units with regard to teat tissue treatment. International Dairy Federation Bulletin No.297.

Harmon, R.J., 1994. Physiology of mastitis and factors affecting somatic cell counts. Journal of Dairy Science 77(7), 2103-2112.

Harmon, R.S and Heald, C.W. 1982. Migration of polymornuclear leukocytes into bovine mammary gland during experimentally induced staphylococcus aureus mastitis. American.Journal of Veterinary Research 43(6), 992.

Hashemi, S.R and Davoodi, H., 2012. Herbal plants as new immune-stimulator in poultry industry: A review. Asian Journal of Animal and Veterinary Advances 7(2), 105-116.

Heinrichs. A,J.,Costello., S.S.,Jones, C.M ., 2009. Control of heifer's mastitis by nutrition. Veterinary Microbiology, 134 (1-2), 172-176.

Henriksen, B., 2002. Stor interesse for homeopati og urtemedisin hos norskokobonder Praksisnytt 7, 55-58.

Heumann, D., Barraj, C., Severin, A., Clausen, M.P., Tomasz, A ., 1994. Gram positive cell walls stimulate synthesis of tumour necrosis factor alpha and Interleukin. 6 by human monocytes. Infection and Immunity 62 (7), 2715-2721.

Hill, A.W, Heieghan, J.J ., William, M.R., 1983.The opsonic activity of bovine milk whey for Phagocytosis and non-encapsulated E.coli. Veterianry Microbiology (8)(3), 293-3000.

Hillerton, J.E. (1999). Balancing mastitis and quality Proc. British mastitis Conference. Stonelergh UK pp: 31-36.

Hillerton J, E., 1999. Redifining mastitis based on somatic cell count. IDF Bulletin 345, pp.4, 6.

Hisham,I.,2013. Introduction to Veterinary drug residues:Hazards and risks. Http:www.sustech.edu.com

Hogan, J.J., Pankey,J.W and Smith,K.L.,1987. Stapylococcus species other than staphylococcus aureus. In: Proceeding of the national mastitis council 26 th Ann meeting, orlando Florida pp-21-32.

Hogan, J.S., 1997. Occurrence of clinical and subclinical Environmental Streptococcal, 59-75Mastitis In: Proceedings of symposium on udder Health Management for Enviromental Streptococci 1997 National Council inc. Anington Tey.

Hogan, J.S., Weiss, P Todhunter, D.A., Smith, K.L., Schoenberger, P.J., 1992. Efficacy of an Escherichia coli J5 vaccine in an experimental challenge Trial. Journal of Dairy Science 75(2), 415-422.

Hogan, J and Larry, K.J ., (2003). Coliform mastitis. Veterinary Research, 34.507-519.

Hogeveen, H., Huijps, K., Lam, T.J.G.M., 2011. Economic aspects of mastitis: new developments. New Zealand Veterinary Journal 59(9), 16-23.

Hutching, A., Scott, A.H., Lewis, G .,Cunningham, A.B., 1996. Zulu medicinal plants an inventory, Natal University press, Pietermaritzburg

Hyvonen, P.T., Haarachiltanen, T., Lehtolainen,T., Heikinen, J., Isomaki,R and

Pyrola, S., 2010. Concentration of bovine lactoferrin and citrate in milk during experimental endotoxin mastitis in early verses late lactating dairy cows. Journal of Dairy Research 77(4),474-480.

Irene, K., Modestas,R., Vytautas,S., Raimundas,M., Asta,P., Ceslova.B.A., 2011.Prevalence of gram Positive Bacteria in cow's mastitis and their susceptibility of Beta-Lactam Antibiotics . Veterinary Medicine Zootechnics 56(78), 65-75.

Isolauri, E., Musema, H., Arvola,T., Rantela, I and Arvilommi, H ., 1993.Lactobacillus strain GG Reverse increases intestinal Permeability induced by cow milk in suckling rats. Gastroenterlogy 105(6),1643-1650.

Jayarao, B.M., Pillai, S.R., Swant, A.A., Wolfgang, D.R and Hedge, N.V., 2004. Guide lines for monitoring bulk tank somatic cell bacteria counts. Journal of Dairy Science 87(10), 3561- 3573.

Jaarsveld, E ., 2002. Veld and Flora. Botanical Society of South Africa pp 63-65

Jamuna, M and Jeevaratrum, K ., 2004. Isolation and partial characterisation of bacteriocins from pediococcus species. Applied Microbiology and Biotechnology 65(4), 433-439.

Jingar, S.C., Mehla, R.K., and Singh, M. (2014) climatic effects on occurrence of clinical mastitis in different breeds of Cows and Buffloes. Archivos de.Zootecnia 63 (243),473-482

Joe, H and Harry, S.K., 2002. Coliform mastitis Department of Animal sciences, Ohio Agriculture Research and Development Centre. The Ohio State University.

Jones, GM., Pearson, R.E., Labbaugh, G.A and Heald, C.W., 1984. Relationship between somatic cell count and Milk production. Journal of Dairy Science 67(8), 1823-1833.

Jones.G,M., 2009. Http:// pubs ext .vt .edu

Kaila, M, Isolauri,E., Soppi, E., Virlanen, E., Laine,S ., Anvilommi, H., 1992. Enhancements of the circulating antibody secreting cell response in Human Diarrhoea by a Human lactobacillus strain. Pediatric Research 32(2), 141-144.

Kellog, D.W., Harrison,J.H ., Hancook,D.D., Todhunter,D.A ., Conrad, H.R ., 1990. Effect of Vitamin E and Selenium Supplementation on incidence of Clinical Mastitis and duration of Clinical symptoms. Journal of Dairy Science 67(6), 1293-1300.

Kelmanson, J.E., Jagar,A.K and Vanstaden, J. (2000). Medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69(3), 241-246.

Kehrli, M. E and Shuster, D.E ., 1994. Factors affecting somatic cells and their role in the health of Bovine mammary gland. Journal of Dairy Science 77(2), 619-627.

Kehrli, M.E, Button, J.L., Nonneke, B.J and Lee, E.K., 1999. Effect of stress on leukocyte trafficking and immune responses. Implications for Vaccination. Advances in Veterinary Medicine 41, 61-68.

Kehrli, M.E, J.L Burton and B.J Nonnecke., 1999. Effects of stress on Luekocyte trafficking and immune response: Implications for vaccination. In: Schultz editor. Advances in Veterinary medicine Vol 41. Veterinary vaccine and diagnosis. New York Academic press p61-81.

Kehrli,M.E., Nonnecke, B.J., Roth, J.A., 1989. Alterations in bovine neutrophil function during the peripatum period. American Journal of Veterinary Research 50, 207-2014.

Kevin, M., Rigby, F., Daleo, F., 2012.Neutrophilis in innate host defense against staphylococcus aureus infections. Seminars in immunopathology 34, 237-259

Khan, M and Khan, A ., 2006. Basic facts in dairy animals: a review Pakistan Veterinary Journal 26 (4) :20 4-208.

Khan, A.Z., 2002. Comparative aspects of prevalence of mastitis in buffaloes and crossbred cows and antibiotic susceptibility profiles of isolates. MSc Thesis. University of Agriculture, Faisalabad, Pakistan.

Khate, K and Yadav,B.R., 2010. Incidence of Mastitis in Sahiwal cattle and Murrah buffaloes of closed organised herd. Indian Journal of Animal Sciences 80(5), 467-469.

Khuzwayo, J.K.2011. MSC Thesis ESSENTIAL OIL COMPOSITION AND SOME BIOLOGICAL ACTIVITIES OF *TETRADENIA RIPARIA.*

Kidd, R., 1997. Herbfest focuses on natural healing. Veterinary Medicine 92(9), 768 769.

Kimura, K, Goff,J.P ., Reinhardt,T.A ., 2001. Parturition blunts calcium signals in immune cells. Abstract presented at Great lakes Immune Fluoresence conference, October 2001, Milwaukee, WI.

Kiremidjian, J., Roy, M., Wisle,H.L ., Cohen, M.W and Stotzky,G., 1990. Selenium and immune cell functions, Effect of lymphocyte proliferation and production of interleukin 2. Proceedings of the society for Experimental Biology and Medicine 193, 136-142.

Kiremidjian and Stotzky, G., 1987. Selenium and immune response. A review. Enviromental Research 42(2), 277-303.

Kuete, V., 2010. Potential of Cameroonian plants and derived products again microbial infections: a review. Planta Medica 76, 1479–1491.

Lacy, H.J., 1998. Physical characteristics of the teat canal and the relationship with infection proc 37th Annual meeting of National Mastitis Council pp 54-61.

Lalman, D and C.Murphy., 2004. Vitamin and mineral nutrition of grazing cattle Department of Animal science, Okhalhoma Co-operative Extension service, Division of Agriculture science and Natural resources Okhalhoma state university.

Lee, E.K and Kherili, M.E., 1998. Expression of adhesion molecules on neutrophil of periparturient cows and neonatal calves. American Journal of Veterinary Research 59(1), 37-43.

Le francois.L., 1994. Basic aspects of Intra Epithelial lymphocyte immunobilogy In: Ogra,P.L, Mestecky, J ., Lamn, M.E ., Scrober, W., Mcghez,J.R ., Biennstock, J (eds) Hard book on mucosa immunology Academic press San Deigo 287-290.

Loken, T., 2001). Alternative therapy of animals - homeopathy and other alternative methods of therapy. Acta Veterinaria Scandinavica .Supplementum. 95, 47-50.

Lui, P.T, Stenger, J ., Wenzel, L.L., Tan, B.H et al., 2006. Toll like receptor triggering of a vitamin D-mediated Antimicrobial response. Science 311(1), 5768 -1770-3

Luseba, D., van der Merwe, D., 2006. Ethnoveterinary medicine practices among Tsonga speaking people of South Africa. Onderstepoort. Journal of Veterinary Research 73, 115-122

Martin, G and Andrew, B., 2004. Clinical forum staphylococcus aureus mastitis in cattale Uk Vet 9:4.2

Mackay, C.R and Hein,W.R., 1991). Marked Variations in γδ T cell numbers and distribution through out the life of sheep. Current Topics in Microbiology Immunology 173, 107-111.

Mallard, B.A, J.C Dekker,J.C., Ireland, M.J., Leslie, K.E Sharit, C ., Vankampen, C.L Wayter, L and Wilkie,B.N., 1998. Alteration in immune responsiveness during peripartum period and Its Vanofication of dairy cow and calf health. Journal of Dairy Science 81(2) 585-595.

Martin, G and Andrew, B., 2004. Clinical forum staphylococcus aureus mastitis in cattale Uk Vet 9:4.2

Maria, V.B.M., Bevilaqua,C.M.L., Palha,M.D.C., Braga,R.R., Schawanke, K ., Rodrigues, S.T., Lameira.O.A ., 2011. Ethno Veterinary Knowledge of the inhabitants of Marajó Island, Eastern Amazonia. Brazil. Acta Amazonica 41(2), 233-242.

Masika, P.J., van Averbeke, W., Sonandi, A., 2000. Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. Journal of the South African Veterinary Association 71(2), 87-91.

Mathias, E., 2001. Introducing ethnoveterinary medicine. www.ethnovetweb.com/what is evm.pdf. Accessed 4 October 2015. Mattana, C.M., Satorres, S.E., Escobar, F.,

Mattana, M,C ., Satorres, E.S.,Sabini, C., Sabini, L., Fusco, M., Alcaraz, L.E., 2012. Antibacterial and cytotoxic activities of *Acacia aroma* extracts. Emirates Journal of Food and Agriculture 24(4), 308-313.

Mbanga, J., Mangoma, N., Saidi, B., 2010. An evaluation of the antimicrobial activities of *Aloe barbadensis*, *A.chabaudii* and *A. arborescens* leaf extracts used in folklore veterinary medicine in Zimbabwe. Journal of Animal and Veterinary Advances 9(23), 2918-2923.

McGaw, L.J., Eloff, J.N., 2008. Ethnoveterinary use of Southern African plants and scientific evaluation of their medicinal properties. Journal of Ethnopharmacology 119(3), 559-574.

Mein, G.A., Williams,D.M ., Reinemann, D.J ., 2003. Mechanical forces applied by the teat cap liner and responses of the teat proc 42nd Annaul meeting of National Mastitis Council.

Miller, R.H and Paape, M.J ., 1985. Relationship between milk somatic cell count and milk yield In: proc Am. Mtg. Natl. Mastitic counc.p.60.

Moges, N.Y., Faw, A.J and Belihu, K., 2011. A cross sectional study on prevalence of subclinical mastitis Associated Risk Factors in and around Gondar, Northern Ethiopia. Internation Journal of Animal and Veterinary Advances 3 (6), 455-459.

Monreu, M.C., Ducluzeau, R ., Guy, G.D., Muller,M.C., 1987. Increase in population of duodenal immunoglobulin A plasmolysis in exertive mice associated with different living or Dead bacteria strain of intestinalorigin. Infection and Immunity 21, 532-539.

Morse,D., Delorenzo,M.A., Wilcox ,C.Jet al .,1993. Climatic effects on occurrence of clinical mastitis in dairy cattle. Journal of Dairy Science 76(9), 2561-2578.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.

Moxely.J.E., Kennedy, B.W ., Downey, B.R and J Bowman, J.S.T.,1978. Survey of milking hygiene practices and their relationships to somatic cell counts and milk production. Journal of Dairy Science 61(11), 1637-1644.

Moyo, M., Finnie, J.F., van Staden, J. (2011). Antimicrobial and cyclooxygenase enzyme inhibitory activities of *Sclerocarya birrea* and *Harpepyllum caffrum* (Anarcadiaeceae) South African Journal of Botany 77(3), 592-597.

Mubarack, H.M., Doss, A., Dhanabalan, R., Venkataswamy, R., 2011. In vitro antimicrobial effects of some selected plants against bovine mastitis pathogens. Hygeia Journal for Drugs and Medicines 3(1), 71-75.

Mustacich, D. and Powis, G 2000. Thioredoxin reductase. Biochemical Journal 346 (1), 1-8.

Mungabe, E,O., Tenhagen, B.A., Kassa , Tet al., 2004. Risk factors for dairy cow mastitis in central highlands of Ethiopia. Tropical Animal Health and Production 36 (5), 463-472.

Mwale, M., Bhebhe, E., Chimonyo, M., Halimani, T.E., 2005. Use of herbal plants in poultry health management in the Mushagashe small scale commercial farming area in Zimbabwe. International Journal of Applied Research in Veterinary Medicine 3(2), 163-170.

Myllys, V., Asplund, K., Brofeldt, E., Hirvela-koski, V., Honkanen-Buzalski, T.,Juntulas.J and Kulkas,T., 1998. Bovine mastitis in finland 1998 and 1995. Changes in prevalence and antimicrobial resistance. Acta Veterinaria. Scandinavica 39(1), 119-126

Nazke, R.P.1981. Element of mastitis control. Journal of Dairy Science 64(6), 1431-1442.

Najera, O., Gonsalez, C ., Toledo, G Lopez, L and Ortiz.R., 2004. Flow cytometry of study of lymphocyte Subjects in Malnurished and well-nourished children with bacterial infections. Clinical and Diagnostic Laboratory Immunology 11(3), 577-580

Neijenhuis,F, Mein , C.A ., Brit ,S.S., Reinemann, D.J., Hillerton, J.E., Farndworth, R., Baines , J.R Hemling, T., Ohnstad,I ., Cook, N., Morgan,W.F., Timms, L., 2001. Evaluation of Bovine teat condition on commercial dairy herds. A relationship between teat-end callosity or Hyper keratosis and Mastitis. Proc.AABP-NMC. International symposium of Mastitis and Milk quality, Vancouver BC, Canada, PP 362-366.

Nickerson, S.C ., 1987. Resistance mechanisms of the bovine udder: New Implications for mastitis control at teat end. Journal of American Veterinary Medical Association 191(11), 1484-1488.

Nooruddin,M., .Ali, M.I., and Debnath, N.C.,1997. Retrospective epidemiologic study of periparturient diseases in dairy cows. Clinical mastitis. The Bangladesh-Veterinarian, 14: 43-47.

Okem, A ., 2011. Thesis: Pharmacological activities of selected South African medicinal plants.

Okitoi, L.O., Ondwasy, H.O ., Siamba, D.N and Nkurumah, D ., 2007. Traditional herbal preparations for indigenous poultry health management in Western Kenya. Livestock Research for Rural Development 19, 5

Olatoye, I.O and Ehinmowo, A.A., 2010. Oxytetracycline residues in edible tissues of Cattle slaughted in Akure Nigeria. Nigerian Veterinary Journal 31(2), 93-103.

Oliver, S.P and sordillo,L.M., 1988. Udder health in periparturient period. Journal of Dairy Science 71(9), 2584-2606.

Olde, R.R.G.M., Barkema, H.B., Stryhn, H., 2007. The effect of season on somatic cell count and incidence of clinical mastitis. Journal of Dairy Science 90(4), 1704-1715.

Oshima, M and Fuse, H ., 1981. Citric acid Concentration in Sub-clinical mastitis Milk. Journal of Dairy Research 48(3), 387-392

Osteras,O Solverod, L., 2009. Norwegian mastitis control programme. Irish Veterinary Journal 62: 26-33.

Overton, T.R., Piepenbrink, M.S and Waldron, M.R., 2000.Interaction of liver Metabolism and Health in transitional Dairy cows. Page 251 in proc.cornell, Univ, N.Y. Ithaca.

Overton, T.R and Walden,M.R ., 2004. Nutritional management of transition dairy cows: Strategies to optimise metabolic health. Journal of Dairy Science 87:105-119.

Owens, W.E., Ray, C.H., Watts, J.L., and Yancey, R.J. (2001). Comparison of success of antibiotic therapy during animal production. International Journal of Antimicrobial agents 17, 431-437.

Pablo, P., Hubert,K ., Han, B., Jaun, V and Carlos.R., 2013. Efficacy of botanical preparation for the intramamary treatment of clinical mastitis on organic dairy farm. The Canadian Veterinary Journaal 54 (5), 479-484.

Pankey, J.W., Barker, R.M ., Twoney, A and Duirs, G., 1982. Comparative efficacy of dry cow treatment against Stapylococcus aureus. New Zealand Veterinary Journal 30(1-2), 13-5.

Park, Y.H., Fox, L.K., Hamilton, M.J and Davis, W.C ., 1992. Bovine Mono nuclear leukocyte. Sub population in peripheral blood and mammary gland secretions during lactation. Journal of Dairy Science 75(4), 998-1002.

Peeler.E,J., Green, MJ., Fitzpatrick, J.L., Morgan, K.L and Green, L.E., 2000. Risk factors associated with clinical mastitis in low somatic cell count British dairy herds. Journal of Dairy Science 83(11), 2464-2472.

Pendigon, G., Vintini, E ., Alverez, S., Medina, M ., Medizi, M ., 1999. Study of possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. Journal of Dairy Science 82(6), 1108-1114.

Petzer, I.-M., Karzis, J., Watermeyer, J.C.,van der Schans, T.J., van Reenen, R., 2009. Present trends in udder health and emerging mastitogenic pathogens in South African dairy herds. Journal of the South African Veterinary Association 80(1), 17-22.

Philpot, W.N and Nickerson,S.C ., 1999. Mastitis counter Attack west falia surge LLC: Illinois.

Pitkala, A., Havari, M ., Pyonala,S., Myllys, V., Honkanen, T.B., 2004. Bovine mastitis in Finland 2001. Prevalence, distribution of bacteria and antimicrobial resistance. Journal of Dairy Science 87(24), 833-2441.

Plozza, K ., Lievaart, J.J Potts ,G and Barkena, H.W.., 2011. Subclinical mastitis and associated risk factors on dairy farmers in New South Wales .Australian Veterianry Journal 89 (1-2), 41-46

Prasad, A.S., Bao, B ., Back, Jr., Kucuk, O ., Sarkar, F.H., 2004. Antioxidant effect of zinc in humans. Free radicle Biology and medicine 37(8), 1182-1190.

Pritee, G., Upadhyay, A.K., Gangwar.N.K and Rasput, M.K.S., 2008. Relationship of mineral and Vitamin Supplementation with mastitis. Veterinary world 1(4),103-104.

Quadri, L.E.N., Sailer,M ., Roy, K.L., Vederas, J.C and Stiles, M.E ., 1994. Chemical and genetic characterization of bacteriocins produced by Carnobacterium piscicola LV17B. Journal of Biological Chemistry 22 *(*269), 12204–12211.

Quinn, P. J., Carter, M.E.,Markely, B., Carter, C.R., 1999. Clinical Veterinary Microbiology Harcourt Publishers ltd: London.

Radostitis, O.M., Gay, C.C ., Hinchchaliff, K.W ., Constable, P.D. (2007). Veterinary medicine 10th ed saunders, Elsevier, Philadelphia pp, 673-748.

Rahal, A and Kumar, A . (2009). Tulsi: A miracle herb in the hands of traditional house lady. Proceedings of the 1O th Annual Conventional of Indian Association of Lady Veterinarians and National Symposium on Contribution of Women Veterinarians in Upliftment of LivestockProduction Through New Technologies,(CIALV'09) , college of Veterinary Sciences and Animal Husbandry, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur.

Rahman, M.A., Bhuiyan, M.M.U., Kamal,M.M and Suddin, M.S., 2009. Prevalence and risk factors of mastitis in dairy cows. The Bangladesh Veterinarian 26(2), 54-60

Rai, K.W., Kumari,S ., 2006. Pytochemical Investigation of the stem of cassia Linn. Asian journal of Chemistry 18(1), 763-765.

Rainard, .P and Poutrel, B ., 1995. Deposition of complement components on Streptococcus agalactiae in bovine milk in the absence of inflammation. Infection and Immunity 63(9), 3422–3427.

Reneé, P, Todorov, S.D., 2010. Bacteriocins exploring alternatives Antibiotics in mastitis treatment. Brazilian Journal of microbiology 41(3), 542-562.

Reugg, P.L., 2009. Management of mastitis on organic and conventional dairy farms. J.Animal Sci 87(13) 43-55.

Riberio, M.G., Motta, R.G ., Paes,A.C ., Allendorf, S.D., Siqueira, A.K ., Fernades, M.C and Hara, G.H., 2008. Peracute bovine mastitis caused by Klebseilla pnuemonia Arquivo Brasileiro de Medicina Veterinária e Zootecnia 60(2), 485-488.

Richie, E. R., Bass, R., Meistrich, M.L and Demmison, D.K ., 1982. Distribution of T lymphocyte subsets in human colostrum. The Journal of Immunology 129(3), 1116–1119

Riollet, C, Rainard, P and Poutrel,B., 2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with Escherichia coli and Staphylococcus aureus. Clinical and Diagnostic Laboratory Immunology 7(2**)** 161–167.

Roberts. M., 1990. Indigenous healing plants. Southern Book Publishers. Halfway House

Rumke, P, Visser.D. Kwa, H.G., Harf, A.A., 1971. Radio- Immuno assay of lactoferrin in blood plasma of breast cancer patients , lactating and normal women; prevention of

false high levels caused by leakage from neutrophil leucocyte in Vitro. Folio Medica Neerlandica 14,156-16

Saba, L, Mohammud, F ., Sahida , H and Naveeda, A.K., 2011. Antibacterial and cytotoxic Activities of Acacia Nilotica Linn (Mimosceae). Methanol extracts against spectrum Beta-Lactamase Producing Escerichia coli and Klebseilla. Tropical Journal of Pharmaceutical Research 10(6) 687-697

Sadashiv, S.O and Kaliwal, B.B., 2014 Isolation,characterisation and antibiotic resistance of Bacillus spp from Bovine mastitis in region of north Karnataka, india. International Journal of Current Microbiology and Applied Science 3(4), 360-373.

Samad.M,A ., 2008. Animal Husbandry and Veterinary Science, Volume 11 Lep Pub no.11. Bangladesh Agriculture University campus mymesingh Pp 1184-1189.

Sampimon, O., Bakema,H.W ., Brends,L J., Sol,J and Lam,T., 2009. Prevalence of intrammary infection in Dutch dairy herds .Journal of Dairy Research 76(2),129-136.

Sandholm, M., Kaartinen, L and Pyorala, S ., 1990. Bovine mastitis why does antibiotic always work? An over view .Journal of Veterinary Pharmacology Therapeutics 13(3), 248- 260.

Santos, M.V., Fonseca, L.F.L., 2007. Estrat ́egrias para controle de mastite e meshoria da qualidade do leite. Sao Paulo: Marole 314 p

Sapna, M, Swati,R ., Anil ,K and Mnene, V., 2011. Medicinal attributes of acacia nilotica linn. A comprehensive review on ethno.pharmacological claims. International Journal of Pharmaceutical & Life Sciences (ISPL) 2 (6), 830-837.

Scalleti, R.W., Trammel, D.S ., Smith, B.A ., 2003. Role of dietary copper in enhancing resistance to *Escherichia coli* Mastitis. Journal of Dairy Science 86 (4), 1240-1249.

Schalm, O.W and Mead, J.W., 1943. The Effect of incomplete milking on chronic mastitis caused by *S.agalactiae*. Journal of Dairy Science 26(9),823-832.

Schanbacher, F.l, Goodman, R.E and Talhaik, R.J., 1993. Bovine mammary lactoferrin. Implications from messenger ribonucleic acid mRNA sequence and Regulation contrary to other milk proteins. Journal of Dairy Science, 76(12), 3812-3831.

Schonewille, J.T., Vant, W.A.T., T.Walterse,T and A.C Beyman, A.C., 1999. Hypocalcaemia industry intravenous administration of ethylenedian tetraacetate and its effects on excretion calcium in urine of cows fed high chloride diet. Journal of Dairy Science 82(6), 1317-1329.

Schroeder.J,W., 2012. Milk quality evaluation tools for farmers. North Dakota state University .https://www.ag.ndsu.edu/pubs/ansci/dairy/as1131.pdf

Seegers,H., Fourichon, C., Beaudeau, F., 2003. Production effects rated to mastitis economics in dairy cattle herds. Veterinary Research 34(5), 475-491.

Seigler,D.S., 2003 Photochemistry of Acacia-sersu lato.Biochemical systematic and Ecology 31(8), 873

Selsted, M.E., Tang,Y.Q ., Morris, W.L., Mcquire, P.A., Nonotry, M.J., Smith, W., Henschen, A.H and Cullor, J.S., 1993. Purification, primary structures and antibacterial activities of Beta-defensins, a new Family of antimicrobial peptides from Bovine neutrophils. The Journal of Biological Chemistry 268(9), 6641-6644.

Shaik, S., Mkize, L., Khumalo, M. Singh, N., 2014. *Tetradenia riparia*-mediated synthesis of nano-gold particles. Digest Journal of Nanomaterials and Biostructures 9 (2), 567-573.

Shafter-Weaver.K.A., Pighetti, G.M and Sordillo, L.M., 1996. Diminished mammary gland lymphocyte functions pararell shifts in trafficking patterns during postpartum period. Proceedings of The Society for Experimental Biology and Medicine 212(3), 271-280.

Sharif,A and Muhammed,G ., 2009. Mastitis control in dairy animals. Pakistan Veterinary Journal 29(3), 145-148

Sharma, N., Singh,W.K., singh,O.P., Pendly,V ., Verma, P.K ., 2011. Oxidative stress and oxidant status during transition period in dairy cows. Asian-Australasian Journal of Animal Science 24(4), 479-484.

Sharma, M., Mandloi, A.K., Pandey, G., Sahni, Y.P., 2012. Antimicrobial activity of some medicinal plants against fish pathogens. International Research Journal of Pharmacy 3, 4.

Sharma, C., Aneja, K.R., Surain, P., Dhiman, R., Jiloha, P., Measi, V., Kaur, M., 2014. *In vitro* evaluation of antimicrobial spectrum of *Acacia nilotica* leaves and bark extracts against pathogens causing otitis infection. Journal of Innovative Biology 1 (1) 51-56.

Shennan, D.B and Peaker, M ., 2000. Transport of milk constituents by the mammary gland physiology. Reviews, 80 (3), 925-951.

Shyamapada, M., D.M Manisa, D.M., Krishnedu, S., Nishite, K ., 2011. In-Vitro Antibacterial activity of three Indian species Against Methicillin Resistant. *Stapylococcus aureus*. Oman medical Journal 26 (5), 319-323.

Shook, G.E .(1993). Genetic Improvement of Mastitis through selection on somatic cell count. Veterinary clinics of North America: Food Animal Practice 9(3), 563-577

Shukken.Y,H., Grommers, F.J., Vandegeer, D., Erb, H.N and Brand,A.(1990a). Risk factors for clinical mastitis in herds with low bulk somatic cell count. 1. Data and risk factors for all cases. Journal of Dairy Science , 73(12), 3463-3471.

Shukken.Y,H.,Grommers, F.J., Vandegeer, D.,Erb, H.N and Brand, A (1991b). Risk factors for clinical mastitis in herds with low bulk somatic cell count.2.Risk factors for Escherichia coli and Staphylococcus aureus. Journal of Dairy Science, 74 (3), 826-832.

Silvia,F., Depetrino, S.E., Deforrat, M.E.E.B.B., Meson,O ., Perdigon, G .,1995. Protective ability of certain lactic acid bacteria against an infection with candida albicans in a mouse immunosupression model by corticoid. Food and Agriculture Immunology 7(4), 365-373

Singh, R., Arora, S., 2009. In vitro evaluation of peroxyl radical scavenging capacity of water extract/fractions of *Acacia nilotica* (L.) Willd. ex Del. African Journal of Biotechnology 8(7), 1270-1272.

Singh,T, Varindra, J, Singh, J and Dhillon, K.J., 1997. Effect of trisodium citrate mastitis treatment on some milk constitutes. Indian Journal of Animal Sciences 67, 1055-1056.

Singh,M and ludri, R.S., 2001. Influence of stages of lactation, parity and season on somatic cell count. Asian-Australasian Journal of Animal sciences 14(12), 1775-1780

Smith, C.F., Klopper, R.R., Crouch, N.R., 2008. Aloe aborescens Asphodelacaea Aloaceae and Cities. Haseltonia 14, 189-198.

Smith, K.L ., Todhunter, D.A and Schoenberge,P.S ., 1985. Environmental mastitis cause, prevalence. Journal of Dairy Science 68 (6), 1531-1553.

Smith, K.L and Hogan, J.J ., 2003. Epidemiology of Mastitis proc 3rd Int. Mastitis Tel Aviv, Israel, 56, 3-12.

Sordillo, L.M and Babuik, L.A ., 1991. Controlling acute Escherichia coli .Mastitis in periparturient period with recombinant bovine interferon-gamma .Veterinary Microbiology 28(2), 189-198.

Sordillo, L. M., Shafer-Weaver, K and DeRosa, D ., 1997. Immunobiology of the mammary gland. Journal of Dairy Science 80(81), 851–1865.

Souto, Li.,Minagwa, C.Y., Telles, E.Q ., Garbuglio, M.A., Amako, M ., Melville, P.A ., Dais, R.A .,Sakata,S.T and Benitez,N.R ., 2010. Correlation between mastitis occurrence and the count of microorganisms in bulk raw milk of bovine dairy herds in four selective culture media. Journal of Dairy Research 77(1), 63-70.

Spain, J.(1993). Tissue integrity: a key defence against mastitis infection the role of

Zinc proteinates and a theory for mode of action. In: Lyons, T.P (Ed) Biotechnology in feed industry, proceedings of the 9th Annaul symposium, Alltech Technical publication, Nicholasville KY USA pp 53-58.

Spears, J.W ., 2000. Micro nutrients and Immune function in cattle. proceedings of Nutrition society 59(4), 587-594.

Spears, J.W and Weiss, W.P. (2008). Role of antioxidant and trace elements in health and immunity of transitional dairy cows. Veterinary Journal 176(1), 70-76.

Stephen, C.N., Kautz, M.F and Elizabeth, L.N ., 2013. Managing mastitis in dairy heifers Animal and Dairy science Department University of Georgia, Athens.http:// extension.uga.edu/publications/files/pdf

Suriyasathatporn ,W., Schukken ,Y.H., Nelsene, M et al., 2000. Low Somatic cell count : A risk factor for subsequent clinical mastitis in dairy herd. Journal of Dairy Science 83 (6),1248-1255.

Suskind, D., Murthy, K.K and Suskind, R.M.,1980. The malnourished child: An overview Nestle Nutr. Workshop Ser 19, 1-17

Taguchi, T, , Acher,W.K Fusihashi,K., Yamamoto, M., McGhee,J.R Bluestone,J.A Kigomo,H (1991). Noval function for intestinal intra epithelial lymphocytes: Marine D+ γ/δ TCR^{+} T cells produces IFN-y and IL5 Journal of Immunology 147, 3736-3744.

Tamilselvam, B., Almeida, R.A., Dunlap, J.R., and Oliver, S.P 2006. *Streptococcus uberis* cells internalizes and persists in bovine mammary epithelial cells. Microbial Pathogenesis 40,279-285.

Tesfaye,A., H,Mekonnen., S,Rahwa and D,Dendin., 2012 The effect of management practices on small holder Dairy farms in Nazereth Ethiopia. World journal of Agriculture Sciences 8(2), 218-222

Tichaczek, P.S., Nissen-Meyer, J., Nes, IF.,Vogel, R.F and Hammes,W.P .,1992. Characterization of the bacteriocins curvacin A from Lactobacillus curvatus LTH1174 and sakacin P from Lactobacillus sake LTH673. Systematic and Applied Microbiology 15,460–468

Tiwari, J.G., Babra, C., Tiwari, H.K., Williams, V., De Wet, S., Gibson, J., Paxman, A., Morgan, E., Costantino, P., Sunagar, R., Isloor, S., Mukkur, T., 2013. Trends in therapeutic and prevention strategies for management of bovine mastitis: an overview. Journal of Vaccines and Vaccinations 4(2),1000176

Toyang, N.J., Wanyama, J ., Nuwanyakpa, M and Django, S ., 2007. Ethno Veterinary medicine: A Practical approach for Treatment of cattale diseases in Sub-Saharan Africa 2nd Edn, Agronisa. The Netherlands, ISBN-13 978929013668, pg 87.

Treece, J.M., Morese, G.E and Lievy, C ., 1996. Lipid analyses of bovine teat canal Keratin. Journal of Dairy Science 49(10),1240-1244

Tufano, M.A., Cipollaro, D.G., R.Innelo , Galdinero, M ., Galdiero. F ., 1991. Protein A and other surface components of staphylococcus aureus stimulate production of IL-1α, IL-6 TNF AND IFN-Y. European Cytokine Network 2(5), 361-360.

Vacek ,M., Stadnik, L., Stipkova, M., 2007. Relationship between the incidence of health disorders and reproductive traits of Holstein cows in Czech Republic. Journal of Animal Science 52 (8), 227-235.

Vankampen, C and Mallard,B.A. (1977). Effects of peripartum stress and Health on circulating bovine lymphocyte subsets. Veterinary Immunology and Immunopathology 59(1-2), 79-91.

Van, Wyk. B-E., Smith, G. (2004). Guide to aloes of South Africa, second Ed. Briza Publications, Pretoria.

Verpoorte, R .(1998). Antimicrobial activity in Alkaloids biochemistry, ecology and medicinal applications eds Margret F.Robert and micheal Wink , springer publication 397-426.

Wallenberg.G,J., Vanderpoel,H.M.,Van Oirschot, J.T., 2002. Viral infection and bovine mastitis:a review . Veterinary Microbiology 88(1), 27-45

Weiss, W.P., Hogan,J.J ., Smith, K.L and Holbert, K.H., 1990. Relationships among Selenium, Vitamin E and mammary gland Health in commercial dairy Herd . Journal of Dairy Science 73(2), 381-390.

Weiss, W.P and Spears, W.P ., 2006. Vitamin and trace minerals affects an immune function of ruminants In: K .Jersen, T.Huelplund, Weilsen M.O (Eds) Ruminant physiology wageningen Academic Publisher .Utretdet, the Netherlands pp 473-496.

Weiss, W.P., Hogan,J.S., Todhunter, D.A and Smith, K.L ., 1997. Effect of vitamin E. Supplementation in diets with cow concentration of selenium on mammary gland health of dairy cows. Journal of Dairy Science 80(8),1728-1773.

Williams, P., Laubert, P.A .,Brown, M.R.W., 1988. Penetration of immunoglobulin through the Kebs*eilla* capsule and their effect on cell surface hydrophobicity. Journal of Medical Microbiology 26(1), 2935.

Whitaker, D.A., Earynes, H.F Aitchison, K and Kelly, S.M ., 1997. No effect of dietary Zinc Proteinate on clinical mastitis infection rate recovery and somatic cells in dairy cows. Veterinary Journal 153(2) 197-203.

White, F and Rattry, E.A ., 1965. Diurnal variations in the cell content of cow's milk. Journal of Comparative Pathology 75(3),253-261.

Worobo, R.W., van Belkum,M.J ., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, ME ., 1995. A signal peptide secretion-dependent bacteriocin from Carnobacterium divergens. Journal of Bacteriology 177 (11) 3143–3149.

Woolford, M.W ., 1997. Prospectives of Mastitis from "Dolonunder"proc 36 Th Annaul meeting of National mastitis council pp 56-64.

Zeryhun,T., Aya,T and Bayecha,R., 2013.Study on prevalence of bacteria pathogens and associated risk factors of bovine mastitis in smallholder dairy farms in and around Addis Ababa, Ethiopia. Journal of Animal and Plant Sciences 23(1),50-55

Zhao, X and Laccase,P., 2007. Mammary tissue damage during bovine mastitis causes and control. Journal of Animal Science 86(13),57-65

Appendix

Entebbe questionnaire

The purpose of this questionnaire is to evaluate management associated risk factors on prevalence of mastitis in Entebbe municipality Uganda

Therefore, your help in answering the questions below will highly be appreciated.

In case of any inquiries regarding the study contact +27 781 157 3530

Name of the farmer…….

Municipality…….

Contact of the farmer……..

NO	Questions	Response

1 Herd size ……

2 Indicate the name of breed if mixed give the name of others and their number……..

(I) Factors related cow

3 How many cows are in lactation?.....

4 Which breed record most cases if mixed?…….

5 How many animals in lactation have mastitis?.....

6 Which stage of lactation do you record most cases of Mastitis? Early ☐ Mid ☐ Late ☐

7 Which period of the year do you record most cases of mastitis? Dry season ☐ Wet season ☐

8 What measures do you set up to counter this?.....

(II)Milking hygiene

9 Do you wash teats with minimum water? Yes ☐ No ☐

10 Did you have mastitis last year? Yes ☐ NO☐

11 How many times? Once ☐ Twice☐ Thrice ☐

12 If no choose from the following ☐ Water is expensive

☐ I get bothered to fetch

☐Other

specify……..

13 Do you dry the teats thoroughly after Pre-milking teat dis infection with individual paper towel or clean individual cloth? Yes ☐ NO☐

14 Do you wear gloves during milking? Yes ☐ NO ☐

15 If no choose from the following reasons ☐ They are expensive

☐ Do not know where to get them

☐ Never thought about it

☐ Not necessary

16 Do you put on apron while milking? Yes ☐ No ☐

17 If yes indicate the colour?.....

18 If no Choose from the following reasons ☐ it's expensive

☐ Don't where to get them

☐ Never thought about

☐ It's not necessary

19 If it's expensive you agree that putting on an apron is good which colour should it have?

☐White

☐Black

☐Blue

☐ Colour doesn't matter

20 Do you put on boots while milking? Yes ☐ No ☐

21 If yes which colour……

22 If no Choose from the following reasons? ☐It's expensive ☐ Not necessary other specify……

23 Do you use pre-strip as part of your preparation with strip-cup before cows are milked? Yes ☐ No ☐

24 Do you apply post milking disinfectant after milking? Yes ☐ NO☐

25 Do you know that undermilking and overmilking are causes of mastitis? Yes ☐ NO ☐

26 Do you have any ways to detect this? Yes ☐ NO ☐

27 Do you keep bedding free from Manure? Yes ☐ NO ☐

28 Do you know it's important to know Somatic Cell Count of each individual cow? Yes ☐ No ☐

27 If yes why……

29 Do you use CMT test to check Somatic cell count? Yes ☐ NO ☐

30 If no choose the following ☐ Its expensive ☐We don't know this ☐ It's not necessary

31 How oftenly do you check Somatic cell count? Weekly ☐ 2 weeks ☐ Monthly ☐ other specify……..

32 Do you have measures of minimising flies at your farm? Yes ☐ No ☐

33 If yes which measures? ……

……

34 Do you spray your animals? Yes ☐ No ☐

35 How often? Weekly ☐ 2 weeks ☐ Monthly ☐

36 Milking hygiene practices plays a big role to the onset mastitis? Strongly agree ☐ Agree☐ Strongly Disagree ☐ Disagree ☐

(III)Nutrition Factors

37 Do you supplement your animals with Sodium Citrate? Yes ☐ NO☐

38 If no choose ☐ It's expensive ☐ Do not know that it's important ☐ Do not where to get it

39 Do you supplement your animals with selenium? Yes ☐ No ☐

40 Do you supplement your animals with and Vitamin E? Yes ☐ No☐

41 Do you feed additional supplements to your cows choose one of the following options? To all lactating cows and dry cows ☐ only lactating ☐ Only dry cows☐

42 Does Nutrition play a role in the onset of mastitis? Strongly agree ☐ Agree ☐ strongly disagree ☐ Disagree

(IV)Other Factors

43 Do you buy replacement heifers? Yes ☐ No☐

44 Do you keep record of your animal's health? Yes ☐ No☐

45 If yes give reasons……
……..

46 Do you use dry cow therapy as a treatment programme for mastitis? Yes ☐ No☐

47How much money do you loose in treatment of animals with mastitis per annum?.....

48 How many cases of mastitis do you have on average per year?

49 Who buys your milk? Processing company☐ Middle man ☐ Walk by customers☐ other specify……..

50 Does a veterinary officer visit your farm? Monthly ☐ 3 months☐ other Specify……..

51 Do you have an association of small scale holder dairy farmers? Yes☐ No ☐

52 Give recommendations on the challenge of mastitis?

THE END

KwaZulu-Natal questionnaire

The purpose of this questionnaire is to Evaluate the Effect management Aspects on the prevalence of mastitis in Kwazulu Natal.

Therefore, your help will highly be appreciated in Answering the questions below.

In case of any inquiries regarding this study contact 078 157 3530

Name of the Farm:

……..

Name of the Municipality……

…

Contacts of the Farmer……..

NO Questions

Response

1 Herd identification

Numeric ☐

2 Herd size

Numeric ☐

3 Indicate name of Breed and Number of animals if mixed indicate Name of other Breed and Number animals ……

4. How many animals are in lactation? 100-300 ☐ 301-700 ☐ 701 Above ☐

4.1 How many animals currently in lactation have mastitis? 1-20 ☐ 21-70 ☐ 71 Above ☐

4.2 Which periods of the year are many cases of mastitis recorded in your herd? Winter☐ spring☐ summer ☐ autumn☐

4.3 Which stage of lactation do you record most cases of mastitis? Early ☐ Mid ☐ late ☐

4.4 Which cows record the most cases of mastitis in your herd? Older ☐ younger ☐ Both ☐

5 Do you use pre-strip as part of your preparation before cows are milked? YES☐ NO☐5.1 Do you wash hands / wear gloves during milking? YES ☐ NO☐

5.2 Do you wash teats as part of your preparation with warm solutions? YES ☐ NO☐

5.3 Do you clean the milking parlour before and after milking? YES☐ NO☐

5.4 Do you have separate calving paddock for your cows? YES☐ NO☐

5.5 Do you apply post milking disinfectant after machine removal? YES ☐ NO☐

5.6 Do you dry the teats thoroughly after Pre-milking teat dis infection with individual paper towel or clean individual cloth? YES ☐ NO☐

5.6.1 If yes how long? 30-45s ☐ 10-15s ☐ 15-29s ☐ don't know ☐

5.6.2 Do you have sheds for your animals on your farm? YES ☐ NO ☐

5.6.3 How often do you dip or spray your animals? Weekly ☐ 2 week interval ☐ other☐

6 How regularly are parts of your milking equipment replaced? Monthly☐ Annually☐ ☐other

6.1 Has the milking equipment been installed properly? YES☐ NO ☐

6.2 Do you avoid slipping of teat liners during milking? YES ☐ I don't know ☐

6.3 Are liners and other rubber parts free from cracks or holes? YES☐ NO ☐

6.4 Do you prevent water from accumulating at the mouth of the teat cup during milking? YES ☐ NO ☐

6.5 Do you keep vacuum at claw during peak milk? YES ☐ NO☐

6.6 Is the vacuum pump, vacuum distribution tank, and pipelines of adequate size for the number of milking units? YES☐ NO ☐

6.7 Is the milking equipment cleaned adequately? YES☐ NO ☐

6.8 When do you shut off vacuum for the milking machine? Before removing machine ☐ After removing the machine☐

7 Is feed provided after milking to encourage cow to stand for at least an hour? YES ☐ NO ☐

7.1 Do you supplement your animals with sodium citrate? YES☐ NO☐

7.2 Do you supplement your animals with selenium and vitamin E? YES ☐ NO☐

7.3 Do you feed additional supplements to your lactating cows, choose one of the following options. To all lactating and Dry cows ☐ only lactating cows ☐ only dry cows ☐ other options☐

8 How much money are you loosing from animals with mastitis per Annum?

8.1 How many cases of clinical mastitis do you have on average per year? .50-80 ☐ 81-100 ☐ 101 Above

9 Do heifers and lactating cows share the same paddock? YES ☐ NO☐

9.1 Do you buy replacement heifers? YES ☐ NO☐

9.2 Do you use dry cow therapy as treatment programme for mastitis? YES ☐ NO☐

9.3 How frequently do you check somatic cell count (SCC) Monthly ☐ Weekly ☐ other?

9.4 What kind of treatment do you give animals infected with Mastitis? Antibiotics ☐ Herbal extracts ☐

9.5 What is the intensity of mastitis on your farm? Low ☐ Medium ☐ High ☐

9.6 How often does a veterinary officer visit your farm?

Monthly ☐ 3months ☐ other☐

9.7 Do you keep records of your Animals Health? YES☐ NO☐

9.8 Give suggestion on how you think you the challenge of mastitis can be solved?