PREVALENCE, RISK FACTORS, ANTIBIOGRAM AND *IN VITRO* ACTIVITY OF NANOENCAPSULATED BROMELAIN AGAINST BACTERIA ISOLATED FROM MILK OF DAIRY GOATS WITH SUB-CLINICAL MASTITIS IN THIKA EAST SUB-COUNTY, KENYA

PRECIOUS MAHLANGU

A thesis submitted to the Pan African University, Institute of Basic Sciences, Technology and Innovation in partial fulfillment of the requirements for the Degree of Master of Science in Molecular Biology and Biotechnology

2018

DECLARATION

I, the undersigned, declare that this is my original work and has not been submitted to any other college, institution or university for academic credit.

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LIST OF ABBREVIATIONS

CLSI: Clinical and Laboratory Standards Institute

CMT: California Mastitis Test

CN*S*: Coagulase Negative *Staphylococci*

D.V.O: District Veterinary Officer

E. coli: *Escherichia coli*

LPO: Livestock Production Officer

PCR: Polymerase Chain Reaction

S. aureus*:* *Staphylococcus aureus*

spp: species

ABSTRACT

Mastitis causes the greatest losses in dairy goat production. There is insufficient information on the prevalence and risk factors of sub-clinical mastitis and current antibiotic sensitivity in many counties in Kenya. The objectives of the study were to determine the prevalence and risk factors of subclinical mastitis in dairy goats, Thika East Sub-county, Kenya and thereafter, the associated bacterial pathogens, their antibiogram and sensitivity to nanoencapsulated bromelain. The *mec*A gene is responsible for resistance to beta-lactam antibiotics and its presence was determined in the *S. aureus* isolates. Goat level data was obtained for 110 lactating dairy goats of different breeds, parity and lactation stages. Farm level data on risk factors was obtained from 41 small-scale farmers using questionnaires. Milk was obtained from 110 lactating dairy goats and tested for mastitis using California Mastitis Test (CMT). The prevalence of subclinical mastitis at goat level was estimated to be at 50.9% using CMT out of which 86.5% yielded bacteria on culture. The significant risk factors associated with the occurrence of subclinical mastitis were cleaning schedule (p=0.022, OR=1.047) and parity of the goat (p=0.048, OR=1.37). A higher prevalence of sub clinical mastitis was observed for goats residing in houses cleaned at least once a fortnight. Does in the fourth and higher parity were most affected by subclinical mastitis. Milk samples from goats were cultured and bacteria identified using culture and standard identification methods. One hundred and sixty nine (169) bacterial isolates were obtained from culture of which 52 isolates from 7 major classes of isolated bacteria were tested for antibiotic sensitivity to six common antibiotics namely Penicillin G, Tetracycline, Norfloxacin, Chloramphenicol, Gentamycin and Streptomycin. Bromelain, chitosan and nanoencapsulated bromelain

were also tested for antibacterial activity against the major isolated bacterial groups. The Minimum inhibitory concentration was also determined. Fourteen different bacteria including; Coagulase Negative *Staphylococci* (20.7%), *Serratia* spp. (19.5%), *Citrobacter* spp. (16%), *Klebsiella* spp. (11%), *Staphylococcus aureus* (10.7%), *Enterobacter* spp. (6.5%), *Escherichia. coli* (5.9%), *Proteus* spp. (3%), *Corynebacterium* spp. (1.8%), *Morganella* spp. (1.8%), *Streptococcus* spp. (1.2%), *Providencia* spp. (0.6%), *Micrococcus* spp. (0.6%), *Staphylococcus intermidius* (0.6%) were isolated and identified from the milk samples. The identity of *Staphylococcus aureus* isolates was confirmed by the polymerase chain reaction using *S. aureus* specific 16S rRNA primers. All (100%) of isolates that had been identified as *S. aureus* using biochemical tests were confirmed by PCR. All (100%) the tested isolates were resistant to Penicillin G while 98% of the isolates were sensitive to Streptomycin. Bromelain and nanoencapsulated bromelain showed antibacterial activity against tested isolates. Nanoencapsulated bromelain was more effective against the tested isolates than bromelain. Due to lack of a positive control, the absence of the *mec*A gene in the *S. aureus* isolates was not definitive. In conclusion, the study showed that a large proportion (50.9%) of goats in the study area was affected by subclinical mastitis, the main bacteria being *Staphylococci* spp and coliforms. Most of the tested antibiotics can be used in the treatment of mastitis although resistance to Penicillin G and Tetracycline was noted in large number of isolates. *Citrobacter* spp was found to be the most resistant bacterial species. Bromelain, nanoencapsulated bromelain and chitosan were found to be effective broad spectrum antibacterial agents against bacteria causing sub-clinical mastitis in dairy goats. The low Minimum Inhibitory Concentration obtained for

nanoencapsulated bromelain warrants further testing *in vivo*. Farmers need to be trained on improved control of mastitis through adoption of good dairy husbandry and milking practices.

CHAPTER ONE

1.0 INTRODUCTION

Presently, there are over two billion people globally affected by the scourge of hunger and poverty, most of them being in sub-Saharan Africa(Ogola and Kosgey, 2012). The challenge faced by these countries in sub-Saharan Africa is to ensure food and nutrition security and one option is the promotion of goat farming. Goat production plays an important socio-economic role in many rural parts of the world (Boscos *et al.*, 1996; Castel *et al.*, 2010). The importance of goats as a source of meat and dairy products has been well documented (Haenlein, 2004).

Goat milk and its products have a major significance in human nutrition and have been used extensively in feeding starving and malnourished people (Haenlein, 2004). The milk is more nutritious than that from cows and can be consumed by people having cow milk allergies and gastro-intestinal disorders by having better digestibility (Haenlein, 2004).

Goat diseases and pests contribute significantly to low productivity of goats and impact negatively on both local and international livestock trade (GoK, 2009). Goat diseases can be divided into bacterial, viral, endoparasitic and ectoparasitic diseases (Radostits *et al.*, 2000). Bacterial diseases include pneumonia, brucellosis, mastitis and anthrax while Peste Dets Petits Ruminants (PPR), Foot and Mouth Disease and goat pox are viral diseases. Endoparasitic helminths and ectoparasites such as ticks are a major hindrance to goat production in tropics (Quinn *et al.*, 1994). Diseases such as Brucellosis, helminthiases and anthrax are zoonotic diseases that cause

substantial losses to farmers. Other diseases have an impact on the fertility, productivity and general herd health of the goats (Radostits *et al.*, 2000).

1.1 Caprine mastitis

Diseases such as mastitis have hindered the growth of dairy goat production systems (Shearer and Harris, 2003; Mbindyo, 2014). Mastitis in goats is an economically important disease associated with inflammation of the mammary gland and is characterized by changes in the physical characteristics of the udder or milk (Contreras and Rodríguez, 2011). Mastitis leads to a reduction in milk yield , lower the hygienic value of milk and unwelcome changes in the sensory quality and fatty acid profile of the by-products like cheese (Stuhr and Alruich, 2010; Arguello, 2017). The indirect losses from mastitis are the evident potential revenues not earned. Direct losses are seen in the incurred control costs which are actual expenditures related to treatments, preventive measures plus the additional labour used to administer the measures (Radostits *et al.*, 2000).

Intramammary infection of dairy goats are mainly of bacterial origin (Ndegwa *et al.*, 2000) and include several microbial agents such as *Mycoplasma, Coryneobacteria*, *Staphylococcus, Escherichia coli*, *Bacillus, Pasteurella*, yeast and other fungi (Abba *et al.*, 2014). The prevalence of subclinical dairy goat mastitis in Kenya was recorded as 28.7% using the California Mastitis Test (CMT) and 9.7% by bacterial isolation (Ndegwa *et al.*, 2000). In a study covering Meru, Nyeri, Embu counties, the overall prevalence of sub-clinical mastitis by CMT was 61% (Mbindyo, 2014). A similar study by Makau (2017) in Machakos County, Kenya reported a prevalence of 30.3% by CMT. These studies and others (Ameh and Tari, 1999; Mbilu, 2007; Bourabah *et*

al., 2013) revealed that in spite of widespread use of antibiotics to manage the disease, there is high prevalence of subclinical mastitis in dairy goats.

The economic implication of mastitis as a recurrent disease in dairy farming warrants further research into developing new technologies in antimicrobial therapy. Considering the extensive losses due to mastitis, research directed towards viable and safe alternatives should be considered. Mastitis in dairy goats is largely caused by bacteria, largely the genus *Staphylococci* (Boscos *et al.*, 1996). Although there are numerous studies on etiology of mastitis in cattle (Kateete *et al.*, 2013; Belayneh, 2013) only a few have been conducted in dairy goats (Bourabah *et al.*, 2013). Mastitis in dairy goat production is even more critical as the milk used for cheese is not pasteurised and poses risk of being transmitted to human beings (Merz *et al.*, 2016).

Several genera of bacteria have been identified to cause mastitis in dairy goats (Contrares *et al.*, 1997). Bacterial culture has been the gold standard for identification of the bacteria (Contrares *et al.*, 2007). However, molecular characterization using techniques like PCR is overtaking bacterial culture as it is more specific and takes a shorter time (Hoque *et al.*, 2018). One of the most important genus causing subclinical mastitis is the genus *Staphylococci*, with *Staphylococcus aureus* being the most important pathogen (Contrares *et al.*, 2003). Mastitis caused by *S. aureus* is contagious and produces thermostable enterotoxins which cause human food poisoning (Contatres *et al.*, 2007; Hoque *et al.*, 2018). There has been concern over some *S. aureus* species which are methicillin resistant

and are caused by the presence of the *mec*A gene which makes the bacteria resistant to beta- lactams, which is a public health concern (Ismail, 2017).

The predisposing factors for mastitis prevalence highly depend upon type of breed, stage of lactation, management practices and awareness of the dairy farmers (Lakshmi, 2016). Management strategies involve the extensive use of antibiotics to treat and prevent mastitis (Pieterse and Todorov, 2010). The efficacy of antibiotic treatment of mastitis depends on the pathogen virulence, clinical manifestation, antibiotic susceptibility of etiological agent and the efficiency of immunological system (Mbindyo, 2014). However, this has led to increased cases of antibiotics resistance (Oliver and Murinda, 2012; Idriss *et al.,* 2014; Preethirani *et al.*, 2015) and reduction in the number of antibiotics which can be used in the management of mastitis. Further consumers are averse to the presence of residues in milk (Mcewen *et al.*, 1991; Shearer and Harris, 2003; Pieterse and Todorov, 2010; Romero *et al.*, 2016; Berruga *et al.*, 2016). Further, the management of mastitis requires administration of the drugs through oral or parenteral means since their narrow teat canal cannot allow proper usage of intramammary tubes. Treatment through this route is often cumbersome and thus there is need to develop drugs which may be taken as in-feed and still confer a therapeutic effect (Davies and Davies, 2010). From the foregoing, it is clear that development of newer antibiotics especially from naturally occurring compounds for the treatment of mastitis is needed (Kumar *et al.*, 2016).

In recent years, acquired antimicrobial resistance in bacteria is an increasing threat in human as well as in veterinary medicine (Davies and Davies, 2010). Studies by Wakwoya *et al.*, (2006) in Ethiopia on lactating dairy goats reported on antimicrobial resistance of up to 83.7%. That study, among others (da Silva *et al.*, 2004; Ali *et al.*, 2010; Priya, 2016), showed that the misuse of antibiotics can lead to emergence and spread of antimicrobial resistance. However, similar studies in dairy goats are lacking in Kenya.

1.2 Antimicrobial potential of bromelain

There has been a growing research interest for the development of new antimicrobial agents to combat microbial resistance. The pineapple fruit, *Annanus comosus* has been widely used as a therapeutic plant in conventional and traditional medicine (Praveen *et al.*, 2014). These therapeutic qualities have been accredited to bromelain (Bhattacharyya, 2008). Bromelain is one of the enzymes found in the crude extract from the pineapple. This extract contains, among other components, a variety of proteinases, that have demonstrated (*in vitro* and *in vivo tests)* anti-edematous, anti-inflammatory, antithrombotic and fibrinolytic activities (Maurer, 2001). Indeed, bromelain is currently used as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases (Bromelain Monograph, 2010). The antiviral and antibacterial activity of bromelain has been exploited in treatment of bronchial and respiratory tract infections (Shweta, 2014). Bromelain also exhibits antihelmintic anti-candida and antifungal properties (Bromelain Monograph, 2010).

In a study, Roselli *et al* (2007) indicated that bromelain can be an alternative to in-feed antibiotics which can be used to improve the health status of piglets by protecting against enterotoxigenic *E. coli*. Healthy cows and those with intramammary infections, when fed with bromelain, have been shown to have low levels of milk somatic cell counts, an indicator of absence of inflammation (Otani *et al.*, 1989). In dairy goats fed with bromelain at 7.4 grams/animal/day for 7 months, there was no case of sub-clinical mastitis while milk protein and fat was shown to have increased (Contreras *et al.,* 2009). However, the authors did not ascertain whether the lack of mastitis in the goats was due to the antimicrobial activity of bromelain. In mastitis, the mode of action of bromelain can occur in one of two ways, either through a reduction of the inflammation of the udder or by killing of mastitis causing organisms especially tissue invasive bacteria.

1.3 The statement of the problem

There has been an increase in adoption of dairy goats farming in developing countries such as Kenya over the years. However, despite the growing popularity of dairy goat production, farmers and extension workers have observed that mastitis is one of their main challenges (unpublished data, DVO, Thika East). There is therefore need to identify the risk factors, the bacterial species causing mastitis and drug resistance patterns of the bacteria associated with subclinical dairy goat mastitis the study area.

The pineapple enzyme, bromelain has been shown to reduce occurrence of sub-clinical mastitis in cows (Otani *et al*., 1989). Furthermore, dairy goats fed with

bromelain maintained a low prevalence rate of subclinical mastitis (Contreras *et al.*, 2009). In these studies, it was however not clear on the actual effect of bromelain to specific mastitis causing bacteria. However, antibacterial effects of bromelain have been reported on gastrointestinal bacteria (Eshamah *et al.*, 2013; Praveen *et al.*, 2014; Ali, 2015). However, there is dearth of information on effect of bromelain on bacteria causing diseases such as mastitis.

Oral administration of proteins like bromelain is a current challenge faced in the field of therapeutics. With high efficiency, low toxicity and good tolerance, peptides and proteins offer advantageous and biocompatible solutions to treat various diseases (Malhaire *et al.*, 2016). Proteins are fragile in biological environments and their hydrophilicity, plus their large molecular weight, leads to poor permeability which drastically limits their systemic use. To circumvent these difficulties, most protein therapies are administered by injection.

Nanoparticles, such as chitosan, might provide a solution for these treatments by firstly shielding them against harsh biologiacal environments and secondly serving as carriers that can cross the epithelia (Bilal *et al.*, 2017). Nanoparticles can also shield against chemical and enzymatic degradation and offer controlled release targeting, tolerability, improved uptake and translocation while overcoming the challenge of low availability (Bilal *et al.*, 2017).

The current research was geared towards determining the effect of nanoencapsulated bromelain on bacteria causing sub-clinical mastitis in dairy goats.

1.4 Justification of the study

Mastitis caused by bacteria is the greatest cause of losses in the dairy industry. Bacteria causing mastitis is diverse. Poor rural households dependent on protein from goat milk are prone to suffer from malnutrition and reduced income because of reduced milk yield due to mastitis. The Kenya Vision 2030 has earmarked increased productivity of livestock as one of the strategies to increase value in the agricultural sector. The national plan of promoting dairy goat production is aimed at addressing the number one Millennium Development Goal of alleviating extreme poverty and hunger. Diseases like mastitis hinder the realization of these goals.

Mastitis has a multi-factorial nature which is dependent on the host, environment and pathogen. This makes mastitis a complex disease needing proper management tools to reduce its occurrence. The prevalence of mastitis differs from area to area largely due to the differences in farm management. There is therefore a need to know the risk factors of mastitis to help new farmers in management and prevention of the disease.

Bacteria causing mastitis have become or are becoming resistant to a number of commonly used antibiotics. This antimicrobial resistance is a challenge, especially as some pathogens are zoonotic which may be transmitted to human populations. There is therefore a need to know the bacterial species associated with mastitis and their resistance profiles for treatment and surveillance of antimicrobial resistance patterns.

Kenya is leading producer of pineapples in the world. However, pineapples waste is an important cause of pollution in tropical countries, and its use as a source of bromelain for goats could be important not only for economical reasons but also for

environmental ones. Bromelain extracted from pineapple may be a drug in the treatment of mastitis. However, bromelain can be digested by rumen microbes. If used for treatment, higher concentrations of bromelain would be needed to reach the required therapeutic values.

Nanotechnology provides alternative approaches for development of novel antimicrobials that do not rely on the existing pathways of antibiotic action. Coating bromelain with nanomaterials like chitosan reduces the amount of bromelain needed to get a therapeutic effect. It is therefore important to investigate the effects of nanoencapsulated bromelain on bacteria associated with subclinical mastitis as a step in the possible development of a drug for mastitis.

1.5 Research Hypothesis

1. Nanoencapsulated bromelain has no antibacterial effect on bacteria

1.6 Objectives

1.6.1 General Objective

To determine the prevalence, risk factors, antibiogram and *in vitro* activity of nanoencapsulated bromelain on bacteria isolated from milk of dairy goats with sub-clinical mastitis.

1.6.2. Specific objectives

1. To determine the prevalence and risk factors associated with subclinical mastitis in lactating dairy goats in Thika East Sub County

2. To isolate, identify and determine the antibiotic sensitivity of bacteria causing sub-clinical mastitis in dairy goats

3. To determine *in vitro* activity of nanoencapsulated bromelain on bacteria isolated from milk of goats having sub-clinical mastitis

4. To identify *Staphyloccous aureus* using PCR and determine the presence of *mec*A gene in the isolates

1.7 Scope and limitations of the study

The study was limited to investigation of bacteria causing sub-clinical mastitis in lactating dairy goats of different breeds, parity and lactating stages from farms in Thika East Sub County. The study focused on the bacteria only and evaluation of the sensitivity to only six commonly used antibiotics as well as activity of nanoencapsulated bromelain against bacteria causing mastitis in goats. The positive controls for. *S aureus* and the *mec*A gene could not be accessed for use as positive controls in the study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Goat Production in Kenya

The Government of Kenya, in its report on the Kenya Livestock Policy of 2008 reported that research within the livestock sub-sector has in the past focused mainly on different types of cattle, especially dairy cattle and other livestock species which have significant impact (GoK, 2009). The government therefore committed itself to promote research and research work on other livestock species, including goats.

Goat production has been shown to play an important socio-economic role in many rural parts of the world in contributing to food and nutrition security (Ogola and Kogsey, 2012). Goats form an integral component of the livestock in Kenya and the goat population was estimated to be at 27, 740, 153 in 2009 (Wanjiru, 2011). The goat population in Kenya is predominantly indigenous Galla and East African goats (GoK, 2009) which are reared in arid and semi-arid areas. One million of these are dairy goats (Mbindyo, 2014). The majority of dairy goat breeds reared in Kenya are Toggenberg, Saneen and crossbreeds (Ndegwa *et al.,* 2001; Wanjiru, 2011; Mbindyo, 2014).

Goats are a source of income from sale of animals and their products, including skins, meat and milk for home consumption. They also provide manure, play important roles such as insurance against emergencies and as an investment in stock (Ogola and Kosgey, 2012). Goats are able to convert their feed into highly nutritious

milk and meat very efficiently (Castel *et al.*, 2010). They are able to adapt and utilize marginal forage, and survive under harsh conditions. This makes them a very valuable asset for subsistence farmers (Ogola and Kosgey, 2012).

2.1.1 Dairy Goats farming

Keeping dairy goats is sustainable as it requires small land size , less capital investment and improves the livelihood of the households through sale of milk and kids, which are usually in high demand (Wanjiru, 2011). Consequently, a number of projects have adopted the use of dairy goats as an intervention strategy in improving the livelihood of the disadvantaged in various communities in eastern Africa. (Ogola and Kosgey, 2012). The report by the Government of Kenya (2009) noted that dairy goat production has high potential for development given the high nutritional value of goat milk and their production efficiency with respect to their land utilisation.

Promotion of dairy goats in Kenya has been on the rise since Germany sponsored an integrated small livestock project that scaled the activities around Mt. Kenya in the 1980's (www. informationcradle.com). After the project ended, farmers came together through registered groups and formed the Dairy Goats Association of Kenya (DGAK) for sustainability of dairy goat projects. Community based goat improvement programmes rose in the 1990's. The most successful of these projects has been the one based in Meru District in Kenya (Ojango *et al.*, 2010).

The project sought to address the poor milk production and growth rate of the East African indigenous goat as a means of improving productivity and increasing

nutrition, income and overall livelihoods of the majority of the rural poor with limited livestock asset base (Ojango *et al.*, 2010). In the Mount Kenya region, where most of the dairy goats are reared, there are functional dairy goats associations, namely the Meru Goat Breeders Association (MGBA) in Meru County and Dairy Goat Association of Kenya (DGAK) in Nyeri and Embu Counties (Mbindyo, 2014). However, in Kiambu County where Thika East (area of study) is located there are no dairy goat associations which help farmers in breeding and advice in keeping of dairy goats.

The MGBA has been key in the promotion of dairy goat farming by ensuring the activities and coordination of dairy goat farmers, smooth execution of breeding programmes through efficient buck rotation in the county, helping in identification of goats, farmer trainings and record keeping for information (Mbindyo, 2014).

It was noted by Mbindyo (2014) that the MGBA were facing challenges of mismanagement, which has led to collapse of the milking plan, including closing down of the plant which has led to the lack of market for milk. Middlemen are now selling dairy product on behalf of the organization, leaving the farmers with less income than they should have earned. Buck rotation to farmers has also been affected and has resulted in inbreeding.

Goat milk differs from cow and human milk in having better digestibility, buffer capacity, alkalinity and therapeutic potential (Lad *et al.*, 2017). There are comparative differences between goat and cow milk in contents of enzymes,

minerals, vitamins, miscellaneous constituents and physical properties (Haenlein, 2004). Goat milk has been found to contain a better amount of essential amino acids than cow milk. Goat milk exceeds cow milk in monounsaturated fatty acids, polyunsaturated fatty acids and medium chain triglycerides, which all are known to be beneficial for human health, especially for cardiovascular conditions (Haenlein, 2004).

In many developing countries, goat milk has been used to feed starving and malnourished people as it is cheaper to produce and readily available (Haenlein, 2004). Therefore, any factor that adversely affects the quantity and quality of milk from the goat is of great impact to livelihoods of low resource people (Boscos *et al.*, 1996).

2.1.2 Diseases of goats

Goats suffer from many diseases common to ruminants such as pneumonia, anthrax, Foot and Mouth Disease (FMD), brucellosis, mastitis, Peste Dets Petits Ruminants (PPR) (Quinn *et al.*, 1994; Radostits *et al.*, 2000). Helminths are also a cause of decrease in productivity and some have a zoonotic potential (Stepek *et al.*, 2005). Overall, mastitis is the leading cause of losses in the dairy farming business (Bradley, 2002).

2.2 Mastitis in dairy goats

The GoK (2009) reported on the significance of the non-notifiable diseases like mastitis that affect large number of livestock in the country and which need sustained vigilance and surveillance in order to control.

Mastitis in goats is a global disease associated with inflammation of the mammary gland and is characterized by changes in the physical characteristics of the udder or milk (Priya, 2016). Mastitis is generally associated with poor hygienic practices and can be caused by the bruising of mammary tissue or teats from traumas, nursing, fly bites, or other wounds to the skin that provide an important barrier to infection (Radostits *et al.*, 2000).

Dairy goat farmers suffer severe economic losses due to intramammary infections (Merz *et al.*, 2016). Mastitis reduces dairy production and negatively modifies milk composition (Bourabah *et al.,* 2013). Indeed, mastitis is one of the important pathologies in goats with serious financial consequences (Bourabah *et al.*, 2013). The losses due to mastitis emanate from poor milk quality, reduced milk yield and increased expenditure on treatment and sometimes death due to the disease itself or through culling of affected livestock (Kateete *et al.*, 2013, Idriss *et al.*, 2014). Economic losses caused by mastitis include value of discarded milk, reduction in quality of milk and cost of treatment (Radostits *et al.*, 2000). It was noted by Haque *et al (*2015) that mastitis is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%).

2.2.1 Prevalence of sub clinical mastitis in goats

Mastitis manifests either as subclinical, in which there's no visible symptom, or clinical, in which visible symptoms do occur, varying from mild to severe (Kateete *et al.*, 2013). As subclinical mastitis is less obvious and detectable only by measures of

milk's cellular content, it is 15-40 times more prevalent than the clinical form (Shearer and Harris, 2003). This is a concern among producers and veterinarians as there are no visible signs of the disease, which can eventually develop into the chronic clinical form of mastitis.

Subclinical mastitis represents a constant risk of infection for the whole stock (Stuhr and Aulrich, 2010). The prevalence of sub clinical mastitis in goats has been found to be different in many different regions and countries. In a review by Contrares *et al* (2007) it was reported that an annual prevalence of subclinical mastitis was 5-30% or higher in small ruminants. A prevalence of 29% was recorded by Boscos *et al* (1996) in Greece. In Nyeri, Kenya, Ndegwa *et al,* (2000) recorded a prevalence of 28.7%. In Nigeria, Abba *et al*, (2014) reported 10% prevalence in three states in Nigeria. In a study covering the Mount Kenya Region, Kenya, Mbindyo (2014) reported a prevalence of 61%. A prevalence of 53% was reported in Spain by Romero *et al*, (2016). This shows that sub clinical mastitis is a huge problem worldwide.

2.2.2 Causes of mastitis

The organisms responsible for mastitis can either be contagious or environmental (Radostits *et al.*, 2000). Mastitis is associated with viral, bacterial or fungi and their toxins (Bradley, 2002). Quite a large number of bacterial agents are present in environment, surrounding the sheds, beddings, contaminating the fodder and water where the animals are kept (Radostits *et al.*, 2000). Contagious pathogens, such as *Staphylococcus aureus,* are organisms adapted to survive within the host, especially the mammary gland and spread from goat to goat during milking (Bradley, 2002).

Environmental pathogens, such as coliforms on the other hand are not adapted to survive within the host but rather invade, multiply and cause an elevation of somatic cell counts (Bradley, 2002).

Bacteria such as *Staphylococcus aureus* and *Streptococcus agalactiae* are the important contagious pathogens in sub clinical mastitis (Preethirani *et al.*, 2015). *Escherichia coli, Klebsiella pneumoniae* and *Streptococcus uberis* are the predominant environmental pathogens (Radostits *et al.*, 2000), whereas *Streptococcus dysgalactiae* can be both an environmental and a contagious pathogen (Preethirani *et al.*, 2015).

The udder is the primary reservoir of contagious pathogens (Kateete *et al.,* 2013). Milk is a good medium for bacterial multiplication and udder infection is likely to occur in situations where health management is substandard (Bergonier *et al.,* 2003). Such infection, if not detected quickly and treated appropriately, may become chronic, causing morphological alterations in udder tissue (Alawa *et al.*, 2000). Under stressful conditions such as extreme temperatures, muddy and wet living conditions, or a sudden change in diet, a doe's immune system is compromised (Radostits *et al.*, 2000). It has been confirmed that the teat is an open gate for mastitis causal agents (Bourabah *et al.,* 2013).

The mode of spread of bacteria from the infected halves to the other half of udder is primarily at milking time (Bergonier *et al.,* 2003). Infection occurs when infectious agents reach the mammary gland and enters through the milk canal, interacts with the

mammary tissue cells and multiplies (Bergonier *et al.*, 2003). Bacteria like *S. aureus* produce a variety of extracellular protein toxins, including enterotoxins, toxic shock syndrome toxin, haemolysins and coagulase which destroy the mammary gland tissue (Leke *et al.*, 2017). The mammary gland tissue becomes inflamed and triggers an immune response which raises somatic cell counts. Intramammary infections caused by *S. aureus* are associated with peracute or acute cases of gangrenous mastitis (Contreras and Rodriguez, 2011).

Members of the genus *Staphylococcus* are the main etiological agents involved in all forms of mastitis in goats (da Silva *et al.*, 2004; Contrares *et al.*, 2007). *S. aureus* has been considered by researchers as the true mastitis pathogen with virulent factors (Hoque *et al.*, 2018). *Staphylococcus aureus* is the most important pathogen of caprine mastitis (Bergonier *et al.*, 2003; Contrares *et al.*, 2007). This is because it persists as the main pathogen causing mastitis (Vyletělová, *et al.*, 2011; Haran, 2012; Ismail, 2017). Intra-mammary infections caused by this pathogen necessitate special attention because *S. aureus* is the main cause of both clinical and subclinical mastitis (Aras *et al.*, 2012). In addition, *S. aureus* contamination of goat and sheep milk may cause S*taphylococcal* food poisoning, as many traditional caprine milk products are not subjected to pasteurization (Contrares *et al.*, 2007; Merz *et al.*, 2016).

2.2.3 Pathogenesis of mastitis

Mastitis destroys the milk-secreting cells and the scar or connective tissue replaces the milk secreting tissue, resulting in a permanent loss of productive ability of the affected animal. (Haque *et al.*, 2015). Mastitis manifests either as subclinical, in

which there’s no visible symptom, or clinical, in which visible symptoms do occur, varying from mild (flakes in milk, slight swelling of infected udder) to severe (abnormal milk secretions, hot swollen quarter/ udder, fever, rapid pulse, loss of appetite, depression and death) (Kateete *et al.*, 2013). Clinical mastitis is characterized by a varying degree of clinical abnormalities in the udder, milk and systemic effect. Sub-clinical mastitis is also significantly associated with a great increase of leucocytic cells in the milk, which are used as indicators of the condition (Shearer and Harris, 2003). Presence of the cells in the milk is also used as a measurement of milk quality and udder health (Mbindyo, 2014).

Subclinical mastitis is less obvious and may only be detectable by measures of the milk's cellular content (Shearer and Harris, 2003). Subclinical mastitis usually precedes the clinical form (Shearer and Harris, 2003). Subclinical mastitis is of long duration, is difficult to detect, reduces milk production, and adversely affects milk quality (Shearer and Harris, 2003). From the foregoing, it is clear that subclinical mastitis causes the most concern among producers and veterinarians because there are no visible signs of the disease. The subclinical form can eventually develop into the chronic clinical form of mastitis.

2.2.4 Risk factors of mastitis

Mastitis has a multi-factorial nature that predominates with a clear interaction between host, agent and environment (Thrusfield, 2007). Thus, as mastitis is a complex disease involving interactions of several factors of management, environment and factors relating to animal and causative organism, its prevalence varies from place to place (Ayano *et al.*, 2013). The prevalence of bacteria isolated

from milk of seemingly normal goats has also been attributed to the influence of factors such as differences in the time of sampling, the diagnostic criteria used; breed differences, different hygiene and management practices followed on each farm, age and parity of the animals and the milking method (Ndegwa *et al.*, 2001).

Poor management and sanitary conditions, lack of therapeutics and control measures like pre and post milking teat dipping are some major factors which play a vital role in the development of this disease in goats (Ali *et al.*, 2010). In a study by Paape *et al,* (2007), the effects of stage of lactation, parity, breed and state and area on prevalence of mastitis were studied. He reported that the prevalence of mastitis increased with parity and infection. Risk factors of goat mastitis have been studied (Boscos *et al.,* 1996; Zeng *et al.*, 1999; Sanchez *et al.,* 1999; Paape *et al.,* 2007). Parity has been identified as a risk factor (Boscos *et al.*, 1996; Sanchez *et al.,* 1999; Paape *et al.,* 2007). Age and lactation stage have also been reported as risk factors (Zeng *et al.*, 1999; Paape *et al.*, 2007).

2.2.5 Diagnosis of subclinical mastitis

Subclinical mastitis cannot be detected by clinical methods such as the inspection and palpation (Bradley, 2002). It can only be recognized indirectly by several screening methods including the California Mastitis Test (CMT), the modified white side test, somatic cell count, pH, milk electrical conductivity tests and catalase tests. The Somatic Cell Count (SCC) and the California Mastitis Test (CMT) are the most common tests used to screen mastitis in dairy goats. CMT has been recognized as a sensitive and rapid test to detect sub-clinical mastitis (Mbindyo, 2014).

The CMT test is based on the reaction between the CMT reagent and the DNA genetic material of the somatic cells. A higher concentration in somatic cells leads to a higher CMT score. CMT scores are directly related to average somatic cell counts (Bourabah *et al.*, 2013). However, other factors such as estrus may increase the somatic cell counts of milk and may be mistaken for sub clinical mastitis hence a more reliable test has to be used.

Bacteriological diagnosis is a more specific technique for detecting intramammary infection (Paape *et al.*, 2007). Research data suggest that microbiological culture of a single milk sample is reliable for detection of causal agent of the infection (Shearer and Harris, 2003). The determination of bacteriological status of milk samples was regarded as a "gold standard" for the determination of the udder health status (Stuhr and Aulrich, 2010). Recently, identification of mastitis causing organisms is being done using techniques such as polymerase chain reaction for identification of bacteria using 16s rRNA (Ismail, 2017).

2. 3 Identification of bacteria in milk

2.3.1 Use of culture for bacterial isolation and identification

The traditional method for bacterial isolation and identification is culture. The conventional method of cultural identification takes 5 to 6 days, even when rapid commercial systems are used for identification (El-Hadedy and El-Nour, 2012). The use of culture is time-consuming and classical techniques often lead to unreliable results due to doubtful responses of isolates in some tests (El-Hadedy and El-Nour, 2012). Fast and sensitive methods for identification are therefore needed**.**

2.3.2 Identification of bacteria using PCR

Fast and sensitive methods for identification of food-borne pathogens are important for microbiological safety throughout the food production chain (El-Hadedy and El-Nour, 2012). The huge demand for rapid microbiological methods has led to the development of multiple detection and identification systems that have considerably contributed to shorten the analysis time like the polymerase chain reaction (PCR) (Pinto *et al.*, 2005). PCR takes 48 hours and is now replacing the traditional culture method. Identification of *S. aureus* by PCR is now the gold standard (Ali, 2014). This is because PCR is rapid, easy to handle, sensitive and a specific valuable tool for routine testing (Pinto *et al.*, 2005).

The 16S rRNA gene sequence is the most common housekeeping genetic marker (El-Hadedy and El-Nour, 2012). It has been used extensively to study bacterial phylogeny and taxonomy (El-Hadedy and El-Nour, 2012). This is because of its

presence in almost all bacteria, it function has not changed over time and it is large enough for bioinformatics (El.-Hadedy and El-Nour, 2012).

2.4 Treatment of Mastitis

Antimicrobials are routinely used for therapeutic treatment of disease and at sub-therapeutic concentrations to prevent disease and for growth promotion (Pieterse and Todorov, 2010; Barlow, 2011; Kateete *et al.*, 2013). Antibacterial agents are generally defined as the agents which disinfect and eliminate adverse bacteria and work by either inhibiting the growth of bacteria or killing the bacteria (Zharfan *et al.*, 2017). With the global increase in antimicrobial resistance and zoonotic diseases, it has become important to periodically determine profiles and antimicrobial susceptibility patterns of pathogens associated with mastitis (Belayneh *et al.*, 2013). Indeed, the problem of antimicrobial resistance has been blamed in part on the heavy usage of antimicrobials in livestock production (Pieterse and Todorov 2010; Barlow, 2011).

Antibiotics are used extensively in the dairy industry to combat clinical and subclinical mastitis (Pieterse and Todorov, 2010; Barlow, 2011; Kumar *et al.*, 2016). The use of veterinary drugs to treat mastitis and other pathogens in dairy goats is a usual practice in current production systems (Berruga *et al.*, 2016). Strategies involving prudent use of antibiotics for treatment encompass identification of the pathogen causing the infection, determining the susceptibility/resistance of the pathogen to assess the most appropriate antibiotic to use for treatment, and sufficient treatment duration to ensure effective concentrations of the antibiotic to eliminate the

pathogen (Oliver and Murinda, 2012). The majority of antibiotics used are broad-spectrum antibiotics acting against gram-positive and gram-negative bacteria (Pieterse and Todorov, 2010).

Antibiotics like benzylpenicillin, cloxacillin, amoxicillin plus clavulanic acid, cephalonium and cefoperazone, erythromycin , tylmicosin, kanamycin, penicillin, ampicillin, erythromycin, or tetracycline have been recommended in the treatment of mastitis (Macdiarmid, 1978.). The efficacy of antibiotic treatment of mastitis in does depends on the cause, clinical manifestation, antibiotic susceptibility of etiological agent and the efficiency of immunological system (Mbindyo, 2014). To be effective, the drug has to exert specific antimicrobial activity at the site of infection and must have certain characteristics to be an effective agent in the mammary tissue (Pieterse and Todorov, 2010). The antimicrobial should have bactericidal rather than bacteriostatic action, because phagocytosis is impaired in the mammary gland (Pyörälä, 2009).

2.5 Antibiotic resistance and mastitis

2.5.1 Challenges in mastitis treatment

Treatment of sub clinical mastitis in dairy goats has faced challenges of administration of antibiotics and antibiotic drug resistance. The narrow teat canal of goats makes it difficult for intramammary infusion. Moreover, antibiotics which are routinely used for treatment are resisted by bacteria over time (Davies and Davies, 2010).

In recent years, acquired antimicrobial resistance in bacteria is an increasing threat in human and veterinary medicine (Davies and Davies, 2010). The indiscriminate and/or overuse of antibiotics can lead to the development of resistance among different bacterial strains (Kumar *et al.*, 2016). Antibiotic resistance has been attributed to overuse of antibiotics, inappropriate prescribing, extensive agricultural use, regulatory barriers and the lack of new antibiotics (Ventola, 2015).

The extensive use of antibiotics in the treatment and control of mastitis has possible implications for human health through increased risk of antibiotic resistant strains of bacteria emerging that may enter the food chain (Bradley, 2002). Increasing concerns for human health, primarily due to the emergence of antibiotic resistance in pathogenic bacteria, also necessitates the development of alternative anti-infective agents (Barlow, 2011).

The mechanisms by which bacteria acquires resistance to antibiotics include: production of enzymes which inactivate or modify antibiotics, changes in the bacterial cell membrane, prevention of the uptake of an antimicrobial, modification of the target so that it no longer interacts with the antimicrobial and development of metabolic pathways by bacteria which enable the site of antimicrobial action to be bypassed (Cheesebrough, 2006).

Staphylococcus aureus has been associated with high levels of antimicrobial resistance and the ability to cause chronic mastitis (Hoque *et al.*, 2018). The clinical form of mastitis responds poorly to antibiotics because of the development of a tissue

barrier that prevents penetration of antibiotics to the site of infection and it is estimated that only 70% of these *staphylococcal* infections responds to therapy (Quinn *et al.*, 1994).

Many *S. aureus* isolates produce beta-lactamase which render Penicillin G and similar antibiotics ineffective (Quinn *et al.*, 1994). Other antibiotics used against *S. aureus* may poorly penetrate into chronic lesions that can allow survival of bacteria (Quinn *et al.*, 1994). Thus, steadfast and speedy methods for detection of *S. aureus* in mastitic milk samples are crucial for the control of this disease, and economically sound udder health management (Hoque *et al.*, 2018).

2.5.2 Identification of *mec*A resistance in *S. aureus* isolates

S. aureus is frequently isolated from milk and poses problems as it expresses medically relevant virulence factors if encountered in dairy products such as the *mec*A gene (Sasidharan *et al.*, 2011). Methicillin resistant *Staphylococcus aureus* is mediated by penicillin binding protein PBP2a. The *mec*A gene is responsible for encoding this protein which shows low affinity for beta-lactam antibiotics (Sasidharan *et al.*, 2011). The modified PBP2a in MRSA isolates is therefore capable of replacing the biosynthetic functions of normal penicillin binding proteins even in the presence of β-lactam antibiotics, thereby preventing cell lysis (Akindolire *et al.*, 2015). It is because of this that *S. aureus* strains producing PBP2a are resistant to all beta-lactam antibiotics (Akindolire *et al.*, 2015).

Methicillin resistance is alarming as it has the potential for zoonotic transmission. The presence of virulence genes such as the *mec*A gene needs to be investigated for public health and safety. In Switzerland, Merz *et al* (2016) found genes encoding for

antibiotic resistance in 12 % of *S. aureus* isolated from some samples of goat milk. In another study in Pakistan, Ali *et al*, (2010) also recorded an antibiotic resistance of some bacterial isolates from goat milk to some antibiotics, with Penicillin G (42.13%) and ampicillin (46.15%) being the two top most resisted antibiotics. A high prevalence of antibiotic resistance of *S. aureus* isolated from goat milk to antibiotics was recorded in Brazil: amoxicillin (57.3%), streptomycin (46.8%), tetracycline (45.6%), lincomycin (45.6%) erythromycin (35.1%), rifampicin (25.7%), oxacillin (15.8%), norfloxacin (10.5%), doxycicline (10.5%) and enrofloxacin (2.9%) (Franca *et al*, 2012).

Similar work has not been done in the Thika region of Kenya where dairy goat keeping is becoming popular. Some authors have recorded the prevalence of *S. aureus* strains containing the *mec*A gene. In South Africa, Akindolire *et al* (2015) reported a 20.6% prevalence of the *mec*A gene in *S. aureus* isolated from milk of dairy cows. In Jordan, a prevalence of the *mec*A gene of 3.7% was reported (Ismail, 2017). In Uganda, 50% of the *S. aureus* isolates from bulk tank fresh milk contained the *mec*A gene (Aasimwe *et al*, 2017). Similar studies in detection of the *mec*A gene in *S. aureus* isolated from dairy goats' milk are lacking.

Reliable microbiological diagnosis of methicillin resistant *S. aureus* is essential for treatment, surveillance and control. The golden method for detection of methicillin resistant *S. aureus* is the Polymerase Chain Reaction (PCR) through detection of the *mec*A gene (Sasidharan *et al*, 2011).

2.5.3 Subclinical mastitis and food safety

Food borne diseases are an important public health problem because they affect human health and have an impact on economic and trade issues (Sasidharan *et al*, 2011). It has been reported that 2-6% of bacterial outbreaks are related to milk and milk products (Sasidharan *et al.,* 2011; Akindolire *et al*., 2015; Tessema *et al.*, 2016). *S. aureus* is one of the largest causes of food related, including milk, illnesses throughout the world (Ali *et al.*, 2016). It is also known as one of the most important agents of food poisoning globally (Sasidharan *et al.*, 2011).

2.5.4 Antibiotic resistance and antibiotic susceptibility testing

The treatment of mastitis is usually based on the clinical signs and where possible by utilizing results of the microbiologic culture obtained from milk samples (Barlow, 2011). Proper isolation and identification of the causative organism play significant role in prevention and control of the mastitis (Idriss *et al.*, 2014). With the global increase in antimicrobial resistance and zoonotic diseases, it has become important to periodically determine profiles and antimicrobial susceptibility patterns of pathogens associated with mastitis (Barlow, 2011). In developed countries, regular studies on antibiotic sensitivity of bacterial isolates are therefore mandatory for effective and economical treatment of the disease (Kumar *et al.*, 2016).

Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome (Franca *et al.*, 2012; Balouiri *et al.*, 2015). Antimicrobial disk diffusion tests are performed on the pathogens isolated from mastitis milk samples to determine the drug sensitivity profile of the pathogens

(Pieterse and Todorov, 2010). Such monitoring generates data of importance for therapeutic decisions and provides information on trends in resistance that might be cause for interventions regarding antimicrobial use (Mostafa *et al.*, 2017). However, this is rarely done in developing countries like Kenya.

2.6 Novel mastitis treatments

After treating goats with antibiotics, it is necessary to withdraw drug treatment to prevent antibiotics from building up in the milk and meat that can be hazardous to humans (Mcewen *et al.*, 1991). The main route of drug administration for treatment of mastitis in goats is parenteral and oral administration of the antibacterial in feed. This calls for observation of prolonged withdrawal periods which farmers do not adhere, the consequence being ingestion of milk containing antibiotic residues. A strong drive towards reducing antibiotic residues in animal food products has led to research in finding alternative antimicrobial agents (Pieterse and Todorov, 2010). There is research going on in identification of plants or their extracts as possible alternative drugs in the treatment of mastitis.

2.6.1 The therapeutic potential of Bromelain

Pineapples, *Ananas comosus* are native to Central and South America and are grown in several tropical and sub tropical countries including Kenya (Tochi *et al.*, 2008). Bromelain **(Figure 2.1)** is a general name for a family of sulfhydryl-containing, proteolytic enzymes obtained from *Ananas comosus* ("Bromelain monograph," 2010). The primary component of bromelain is a sulfhydryl proteolytic fraction. It also contains a peroxides, acid phosphatase, several protease inhibitors and organically-bound calcium (Bromelain monograph, 2010).

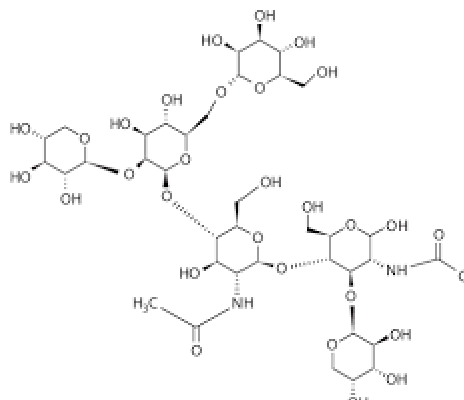


Figure 2.1: Structure of bromelain

The secondary structure of stem bromelain is relatively unchanged between pH 7-10, although this is irreversibly lost above pH 10 (Contreras *et al.*, 2009). Bromelain is made up of 212 amino acids and has a molecular weight is 33 kDa (Brattacharyya, 2008). It has catalytic activity at the active site, the sulfyhydryl group SH (Shiew *et al.*, 2010). Other components like phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates and several other protease inhibitors are also present in crude bromelain (Bhattacharyya, 2008).

Bromelain is considerably absorbable in the body without losing its proteolytic activity and without producing any major side effects (Shiew *et al.*, 2010; Pavan, 2012). Stem bromelain is rapidly absorbed across the gut epithelium without losing its biological activity and has been proposed as a therapeutic especially as an anti-inflammatory agent (Contreras *et al.*, 2008). Thus, bromelain has shown to exhibit various fibrinolytic, antiedematous, antithrombotic, and anti-inflammatory activities both *in vitro* and *in vivo* (Praveen *et al.*, 2014).

Bromelain has antibacterial activity and has been shown kill several bacteria (Shweta, 2014) Bromelain is a cysteine protease which cleaves glycyl, alanyl and leucyl bonds. Although its mode of action is not known, it is likely that it hydrolyzes some peptide bonds present in the bacterial cell wall (Ali, 2015). There is therefore minimum risk of bacteria acquiring resistance if the bromelain hydrolyses peptide bonds. Bromelain was found to be equally effective against both gram-positive and gram-negative organisms (Ashik *et al.*, 2016) .

In a study carried out by Praveen *et al* (2014), bromelain exhibited antibacterial efficacy on strains of both aerobic and anaerobic periodontal bacteria. The antibacterial effect of bromelain on fresh and overnight meat was assessed by Ali (2015) and concluded that crude bromelain was effective against *E.coli* and *Proteus* strains isolated from the meat. bromelain was recorded to be effective on *E.coli* and *L. monocytogens* by Eshamah *et al* (2013) Similar studies have not been done on bacteria causing mastitis.

The passage of drugs across the blood-milk barrier takes place by passive diffusion (Macdiarmid, 1978). For bromelain to be effective, it should be like the other antibiotics which have a low minimum inhibitory concentration (MIC), against the majority of udder pathogens, high bio-availability, low degree of serum protein binding, chemically be a weak base or otherwise highly non-ionized in serum, be sufficiently lipid-soluble to readily diffuse through membranes and have an extended half-life in the body (Macdiarmid, 1978). It should also be able to penetrate the mammary tissue and the drug dosages must be high enough to achieve and maintain therapeutic levels in milk. Nanotechnology can thus improve the drug delivery

system to deliver drugs in the correct amounts unchanged by the external environment.

2.6.2 Safety testing on the use of bromelain

Bromelain has been shown that it is well absorbed after oral application and it has no negative impact on health after prolonged use (Shiew *et al.*, 2010). Bromelain is safe and non toxic (Tochi *et al.*, 2008). It has a Lethal $Dose_{50}$ (LD_{50}) of greater than 10 g/kg in human beings (Bromelain Monograph, 2010). Toxicity tests on dogs, with increasing levels of bromelain up to 750 mg/kg administered daily, showed no toxic effects after six months (Shiew *et al.*, 2010). Dosages of 1.5 g/kg/day administered to rats showed no carcinogenic effects (Shiew *et al.*, 2010).

2.6.3 Nanoparticles as drug delivery systems

Challenges in chemotherapy include use of large size materials in drug delivery, some of which include poor bioavailability, in *vivo* stability, solubility, intestinal absorption, sustained and targeted delivery to site of action, therapeutic effectiveness, generalized side effects, and plasma fluctuations of drugs (Emeje *et al.*, 2012). Nanotechnology offers unconventional approaches for fighting microbes that do not rely on the existing pathways of antibiotic action (Halbus *et al.*, 2017). This makes it possible to address the challenge of antimicrobial resistance by using nanoparticles with engineered antimicrobial action designed to target specific pathogens (Halbus *et al.*, 2017). Discoveries of new antimicrobial agents are being sought to improve antimicrobial actions without negative side effects or stimulating natural antimicrobial resistance (Tachaboonyakiat, 2017).

Nanoparticles have the ability to protect drugs from the degradation in the gastrointestinal tract, and can allow target delivery of drugs to various areas of the body (Emeje *et al.*, 2012). Nanoparticles are able to penetrate tissues and are easily taken up by cells, allowing for efficient delivery of drugs to target sites of action (Halbus *et al.*, 2017). Nanocarriers can reduce the toxicity and other adverse side effects in healthy cells by accumulating the drugs in target diseased tissues and also reduce the required dose of drug (Ma *et al.*, 2017).

Several materials are used for formation of nanoparticles (Andronescu and Grumezescu, 2017). Nanoparticles are broadly divided into organic and inorganic/ synthetic and natural (Halbus *et al.*, 2017). Inorganic nanoparticles are synthesised from various metals such as copper, gold and silver (Emeji *et al.*, 2012). Other elements such as carbon are also used (Halbus *et al.*, 2017). Organic nanoparticles include polysaccharides like cellulose, starch and chitosan and lipid based nanoparticles (Halbus *et al.*, 2017). The drawback in using inorganic nanoparticles is the potential toxicity and side effects feared as a result of the accumulation of the metals in the body as they are not degradable and flushed out of the body like organic nanoparticles (Ma *et al.*, 2017).

The selection of nanoparticles coating material depends on the type of active constituents and its characteristics and the site of application of the encapsulated active agents (Pandit *et al.*, 2016). The advantage of using polysaccharides as drug carriers is due to the fact that they are: available as natural resources, low cost, biodegradable, nontoxic, stable, and hydrophilic (Liu *et al.*, 2008). These polysaccharides offer a wide structural diversity and properties due to their chemical

composition and wide range of molecular weights. The presence of different reactive groups in the structure of polysaccharides makes them amenable for easy chemical and biochemical modification (Emeji *et al.*, 2012). An example of such a polysaccharide is chitosan.

2.6.4 Nanoencapsulation of bromelain

Bromelain works well over a wide pH range in gastrointestinal tract. The limitation of bromelain in ruminants has been their rapid movement in the gastrointestinal tract, it takes about 20 minutes to pass through the small intestines (Stepek, *et al.,* 2005). There is also the possibility of degradation by rumen microbes. These factors have led to recommendation of multiple dosages. Some studies have shown that bromelain can be inactivated by low pH found in the abomasum and this shows the need for encapsulation of the bromelain so that sufficient therapeutic levels reach the site of action (Shiew *et al.*, 2010).

In a study that was carried out by Bernela *et al*, (2016), bromelain was encapsulated in Katira gum nanoparticles and it was concluded that the anti-inflammatory potential of bromelain was enhanced by the encapsulation. This was attributed to enhanced absorption due to reduction in particle size or protection of bromelain from acid proteases.

The most common method of preparing chitosan nanoparticles is by the ionic gelation method of chitosan with tripolyphosphate (Fan *et al.*, 2012; Sawtarie *et al.*, 2017). Mixing of dilute aqueous chitosan and sodium tripolyphosphate (TPP) solutions generates nanoparticles ranging between tens and hundreds of nanometers

in diameter (Sawtarie *et al.*, 2017). These nanoparticles have been studied extensively for drug and gene delivery (Sawtarie *et al.*, 2017).

2.6.5 Chitosan

Chitosan (**Figure 2.2**) and its derivatives are natural polysaccharides derived from chitin (Yang *et al.*, 2014). It is the second most abundant polysaccharide in nature after cellulose (Issa *et al.*, 2005). It is approved as a food additive in some countries. Chitosan has reactive amino and hydroxyl groups and chelates many transitional metal ions (Al-Remawi, 2012). It has active amino groups which grant it its biological properties (Ma *et al.*, 2017). The presence of primary amine groups in repeating units of chitosan grants it several properties (Divya *et al.*, 2017).

Figure 2.2: Chemical Structure of chitosan

Chitosan has been used to coat nanoparticles made of other materials, in order to reduce their impact on the body and increase their bioavailability (Ma *et al.*, 2017). The advantages of using this polymer include availability, low cost, high biocompatibility, biodegradability and ease of chemical modification (Yang *et al.*, 2014). The activity of chitosan is limited by factors like low solubility (Sawtarie *et al.*, 2017). Chitosan is only soluble under acidic conditions of <6 (Ma *et al.*, 2017).

Chitosan can be used to nanoencapsulate proteases including bromelain (Fan *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of study area

The study was carried out in Thika East Sub-county of Kiambu County (**Figure 3.1**). The sub-county is located in the central part of the country about forty kilometres from Nairobi. It lies between latitudes 1°S and 1' south of the Equator and longitudes 37°' and 5' east. Rainfall is bimodal and ranges from 500mm - 1,300mm while average temperature is 18.7°C. According to Livestock Production Officers (LPOs) in the study area, a moderate number of farmers have taken up the dairy goat farming although no proper census has been undertaken (personal communication, Thika East Sub-county Veterinary Officer).

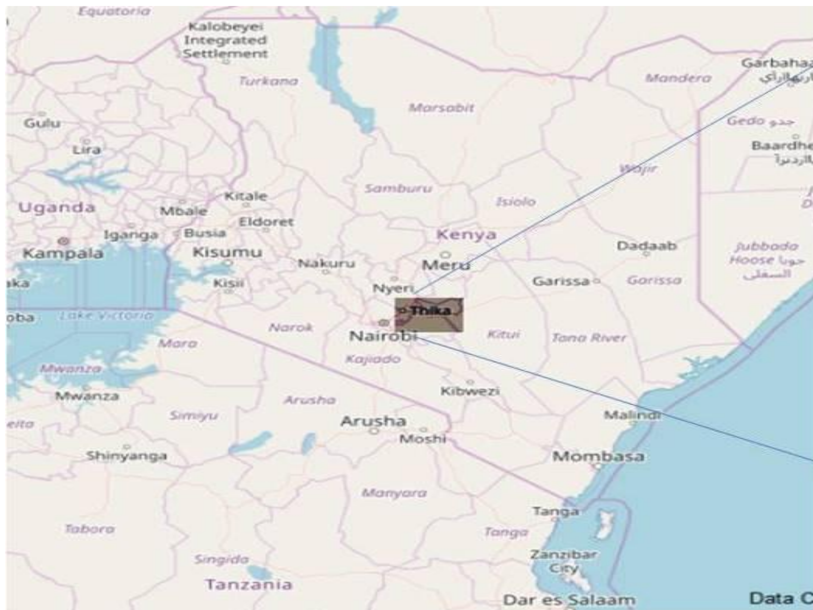


Figure 3: Map showing location of Thika (Google Maps)

3.2 Study design, sample size determination and administration of questionnaires

A descriptive, cross sectional study design was used. The sample size of 110 lactating dairy goats was calculated using an adjusted formula for small populations by Thrusfield (2007).

$$n = \frac{1.96^2 P_{exp}(1 - P_{exp})}{d^2}$$

Where:

n = required sample size;

P_{exp} = expected prevalence;

d^2 = desired absolute precision.

Ndegwa *et al* (2000) found the prevalence of subclinical mastitis of goats in Nyeri part of Kenya to be 28.7%. Thus, in the current study a prevalence of 28.7% was used to calculate the sample size. Using the formula above, 315 goats were supposed to be sampled.

In relatively small populations like the one of dairy goats in Thika East Sub-county, it is possible to select a smaller sample size than one from a theoretically infinite population to achieve the same degree of precision using the adjusted formula by Thrusfield (2007).

$$N_{adj} = N \times n / N + n$$

Where n is the sample size calculated from above and N is the Size of the study population.

Given that the size of the study population (lactating dairy goats) was approximately 150 lactating goats (Personal communication, DVO, Thika East Sub-county), a sample size of 101 does was calculated. A total of 110 does were sampled.

The sampling unit of interest was individual smallholder dairy goat farms whose goat flocks size ranged between 1 and 10. Only farms with lactating goats were visited. Since there were no formal list of dairy goat farmers in the study area, the snowball technique and sampling to redundancy method (Goodman, 1961) was used as a sampling strategy to locate the farmers. The initial farmers were identified with the help of the local extension officers. Thereafter, these farmers helped in further identification of other farmers with lactating goats until all the farmers in sub-county were covered. Using this strategy, a total of 41 farmers was identified from which the goats were sampled from. At all the farms, details of the lactating goats including age, breed, parity and lactation stage and were obtained from the farmer through administration of a questionnaire (Appendix 1 and 2).

3.3 Sampling of milk and California Mastitis Test

The milk samples were collected aseptically using the method by Quinn *et al* (1994). Briefly, the does were restrained and thereafter the teats were scrubbed with cotton wool saturated with 70% ethanol. The teats were dried using a disposable towel. The first three streams of milk were discarded.

The California Mastitis Test (CMT) was carried out according to manufacturer's instructions. Three (3) ml of milk from separate teats was milked into a CMT paddle

and an equal amount of a commercial CMT reagent (Immucell RP, USA) was added to the paddle. The CMT paddle was rotated in a circular motion to thoroughly mix the contents. Gel formation was observed within 20 seconds. The results were read on a score of 0-3. A score of 0, trace and 1 was considered negative while a score of 2 and 3 were considered positive (Appendix 3). Following the CMT, 5ml of milk from CMT positive udders was collected into sterile universal bottles.

The milk samples of the CMT positive samples were placed in cool boxes with ice packs and transported to Jomo Kenyatta University of Agriculture and Technology (JKUAT) laboratory for bacteriological culture and isolation within 12 hours.

3.4 Culture and identification of bacteria

The identification was carried out using the identification protocols described by Quinn *et al*, (1994) and Cheesbrough, (2006). Sheep blood agar (Himedia, India) and MacConkey agar (Oxoid, UK) was prepared according to manufacturer's instructions. The agar was left to set and stored in a refrigerator until use. A 100µl milk sample was inoculated onto both sheep blood agar and MacConkey agar. The milk was allowed to dry and streaking was done using a sterile loop. The plates were incubated at 37ºC for 24-48 hours.

The morphology of the bacterial colonies obtained was checked for the colony size, shape, texture and colour. Haemolysis of the red blood cells in the sheep blood agar was also checked for by observing any colour change in the media around and under the colonies. After 48hours, plates with no growth were recorded as no growth. Plates with mixed growth were subcultured to obtain pure colonies. Pure cultures

were then examined for morphology and gram staining characteristics. Morphological properties were also noted (Quinn *et al.,* 1994). .

Biochemical tests were used to further identify bacteria according to methods described by Quinn *et al.,* (1994) and Cheesebrough (2006). The tests included the catalase test for *Staphylococcal* and *Streptococci* identification, oxidase test, coagulase test using rabbit plasma for identifying *S. aureus* producing coagulase enzyme, fermentation tests to differentiate *Enterobacteriaceae*, indole test to differentiate *E. coli* from other *Enterobacteriaceae,* urease test to identify organisms such as *Proteus*. Mannitol test, motility test, citrate test and MR-VP test were also used for identification.

Results of haemolysis, colony morphology, dichotomous keys of gram staining and biochemical tests were used for identification (Quinn *et al.*, 1994 and Bergey *et al.*, 1984).

3.5 Antibiotic Susceptibility Tests

Bacteria of the same genus of species were recorded as present or absent in a doe. Both udders were considered. Antimicrobial susceptibility test was conducted on randomly selected bacteria from 7 major classes (n = 52) isolated during the study period. Susceptibility was tested against 6 commonly used and readily available antibiotics using the Kirby- Bauer disc diffusion method (CLSI, 2015). Six antibiotic disks of different concentrations namely Penicillin G (10 IU), Gentamycin (10µg), Streptomycin (10µg), Tetracycline (30µg), Chloramphenicol (30µg) and Norfloxacin (10µg) were used. The discs were sourced from Oxoid (UK). Pure colonies of the

isolate to be tested were diluted in sterile saline solution to standardize to a 0.5 McFarland standard.

Mueller Hinton Agar (Himedia, India) plates were inoculated with standardized inoculums of the test organism using sterile cotton swabs. The respective plate was seeded uniformly by rubbing the swab against the entire agar surface. Each antimicrobial impregnated disk was applied onto the surface of the inoculated plate by using sterile forceps. The plates were incubated at 35°C for 16 to 18 hours. The interpretation of the growth inhibition zones and classification of isolates as susceptible, intermediate, and resistant were done following the guidelines of the Clinical and Laboratory Standards Institute (2015).

3.6. In vitro activity testing of bromelain on isolated bacteria

3.6.1 Bromelain extraction

Bromelain was extracted from fresh pineapple stem using the ammonium sulphate precipitation method (Kahiro *et al.*, 2017). Fresh pineapples were washed with 0.1% hydrogen peroxide solution. Stems were peeled off and cut into small pieces. The pineapple peels were weighed and disrupted by grinding in a blender in the presence of sodium acetate buffer and filtered. The juice obtained was labeled as stem crude extract.

The crude extract was centrifuged for 50 minutes at 4 000 g. The supernatant was collected and stored at 4°C. Ammonium sulphate salt (6.6g) was added pinch by pinch to 15ml of the supernatant under ice conditions. The mixture was stirred continuously on a magnetic stirrer for 45 minutes. The solution was then incubated

overnight at 4°C. Afterwards, the suspension was centrifuged at 10,000 g for 10 minutes at 4°C. The pellet was collected and dissolved in 10 ml of 10mM Tris HCl buffer.

Purification was done using dialysis. The dissolved pellet was placed into a 10kDa dialysis membrane and secured. The membrane was placed in a beaker containing 100mM Tris HCL buffer and allowed to stand overnight. The liquid which remained in the membrane was collected and labeled as purified bromelain and stored at -20°C.

3.6.2. Nanoencapsulation of bromelain

Blank chitosan nanoparticles were prepared by ionic gelation method (Fan *et al*, 2012).Low molecular weight chitosan (Sigma, U.S.A) was dissolved in 1.5 %w/v acetic acid solution. Sodium tripolyphosphate (1% w/v) was made by dissolving 1g of sodium tripolyphosphate in 100ml of distilled water. A 4mg/ml solution of bromelain was made using distilled water.10ml of bromelain solution was mixed with 10ml of sodium tripolyphosphate and rotary mixed for 1 minute. Twelve milliliters of the bromelain–tripolyphosphate mixture was drawn into a syringe and added to 20ml 1%chitosan solution drop-wise with vigorous and continuous stirring. The resultant nanoparticles suspension was centrifuged at 4000 g for 50minutes. The suspension was washed with distilled water and air-dried. Storage was done at a temperature of -20 ^{o}C.

3.6.3 In vitro antibacterial activity of nanoencapsulated bromelain

Antibiotic sensitivity was tested using the agar well diffusion method. Different concentrations of encapsulated bromelain, bromelain and 1% chitosan were used to determine susceptibility of the isolated bacteria. Six different concentrations, in triplicate were used. Streptomycin was used as the reference drug.

Mueller Hinton Agar plates were inoculated by spreading 100 µl of the bacterial inoculum. Four holes, each 8mm in diameter, were aseptically made in the media using a tip. Fifty microlitres of the six different concentrations of commercial and extracted bromelain, encapsulated bromelain and 1% chitosan was introduced into each of the wells, in triplicate. The plates were left to stand for two hours to allow the extracts to sink into the media before incubation at 35ºC for 16 hours. The zones of inhibition were measured and the mean diameter determined. The zones of inhibition were compared to the Streptomycin values (CSLI, 2015) to determine susceptibility or resistance. Concentrations were calculated to determine the concentration delivered by 50µl.

3.6.4 Minimum Inhibitory Concentration Determination

The broth microdilution method was used to determine the MIC (CLSI Standard, 2015). Stock solutions of nanoencapsulated bromelain (4mg/ml), extracted and commercial bromelain (100mg/ml) and chitosan (10mg/ml) were made. One milliliter (1ml) of nanoencapsulated bromelain, commercial and extracted bromelain and chitosan were added to 9ml of Mueller Hinton Broth. 50 µl of broth was placed in the wells 2-12 of the 96 well microtitre plates.

One hundred microlitres (100µl) of the test reagent was added into well 1 in triplicate. Streptomycin was used as a positive control standard. One gram of Streptomycin was dissolved in 2.2ml of distilled water. The test reagent (50µl) was transferred from well 1-11 and well 12 were left free of the test drug. Bacterial suspensions were standardized to 0.5 McFarland's and diluted 1:150 in Mueller Hinton Broth. Fifty microlitres (50µl) of inoculum was transferred into each well and incubated at 35˚C for 16-18 hours. The MIC was the determined as the last well were there was no visible bacterial growth in natural light. Concentrations were calculated to determine the concentration delivered by 50µl.

3.7 Identification of *S. aureus* using the polymerase chain reaction

The samples which had been identified as *S. aureus* using gram stain and biochemical methods (catalase, coagulase and mannitol salt test) were further confirmed using the polymerase chain reaction as described by Ismail (2017). DNA extraction was done using the Zymo *Quick*-DNA Miniprep Plus Kit (Zymo, U.S.A). The concentration of DNA was checked using a Nanodrop spectrophotometer (PCR^{max} Lambda, Vacutec, South Africa). The isolates were confirmed by *S. aureus* specific 16s rRNA using the primer sequence F-5'GTAGGTGGCCAAGCGTTATCC 3' and R-5' CGCACATCAGCGTCAG 3' (Ismail, 2017).

A 20 µl reaction mixture containing 4µl Master mix (One Taq 2X Master Mix with standard buffer, New England Biolabs) , 0.5µl each of 16S rRNA *S. aureus* specific primers, 1µl genomic DNA and 14µl of nuclease free water was prepared for each

PCR amplification. A 100bp ladder was used. The master mix was used as a negative control.

PCR amplification was carried using a thermocycler (ProFlex PCR System, Applied Biosystems, U.S.A). The PCR cycling conditions were: Initial denaturation at 94˚C for 5 minutes, 35 cycles of denaturation at 94˚C for 60 seconds, annealing at 50˚C for 60 seconds and extension at 72˚C for 60 seconds. Final extension was done at 72˚C for 10 minutes and cooling to 4˚C. 5µl of products of PCR amplification were analysed using 2% agarose gel electrophoresis (Sigma Aldrich, USA) on tris-acetate-ethylene-diamine-tetra acetic acid (TAE) buffer containing fluorescent red dye. Visualisation of the gel was done using UVITECH Cambridge gel documentation system to view bands of 230bp amplicon size.

3.8 Determination of presence of *mec*A gene using polymerase chain reaction

The identified *S. aureus* isolates were also tested for the presence of the *mec*A gene. The primer sequences Forward: 5'AAAATCGATGGTAAAGGTTGGC 3' and Reverse: 5'AGTTCTGCAGTACCGGATTTTGC 3' (Murakami *et al.*, 1991; Ali *et al.*, 2017) were used. A 20 µl reaction mixture contained 4 µl Master mix, 0.5 µl each of *S. aureus* primers, 1 µl genomic DNA and 14 µl of nuclease free water. The PCR cycling conditions were as in 3.7 above except the annealing temperature was 56˚C.

Visualisation was done using UVITECH Cambridge gel documentation system to view bands of amplicon size of 533bp.

3.9 Data and statistical analysis

The coded data was entered into MS Excel (Microsoft, USA) spread sheet and exported to SPSS (Microsoft, USA) and R for data analysis. Descriptive statistics were presented as Tables. A *chi* square test was used to evaluate associations between risk factors and mastitis infection ($p<0.05$). Logistic regression was used to test individual risk factors and their strength of association in mastitis infection. The odds ratio was used to determine the strength of associations identified in the logistic regression procedure. The mean of the three plates/ isolate for the zones of inhibition was calculated and used to compare with the CLSI values.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of farms and sampled goats

A total of 41 farms were sampled from the study area. Most of the farms were a quarter acre (80.5%) and the largest acreage was one and a half acres. The majority of goat houses (75.6%) were raised timbers, while others (22%) were earthen. Zero grazing (85.4%) was mostly practiced though some farmers practiced open grazing (12.2%) and tethering (2.4%). Most of the farmers (63.4%) were not aware of occurrence of mastitis in their flocks. The frequency of milking the does ranged from once (80.5%) to twice (19.5%) a day. All the farmers indicated that they did pre- and post-milking teat cleaning procedures. Most of the farmers (95.1%) consumed the milk at home **[Table 4.1]**. The average number of goats kept by farmers was five.

Table 4.1: Characteristics of dairy goat farms in Thika East Sub- County, Kenya

Farm Details	Characteristic	Frequency	%
Farm Size	Quarter acre	33/41	80.5
	Other	8/41	19.5
Housing	Raised Timbers	31/41	75.6
	Earthen	9/41	22
	Open	1/41	2.4
Grazing system	Zero	35/41	85.4
	Open	5/41	12.2
	Tethering	1/41	2.4
Other Livestock	Yes	40/41	97.6
	No	1/41	2.4
Frequency of milking	Once	33/41	80.5
	Twice	8/41	19.5
Selling of milk	Yes	2/41	4.9
	No	39/41	95.1
Awareness of mastitis	Yes	26/51	63.4
	No	15/41	36.6

4.2 Prevalence of sub-clinical mastitis and identification of the bacteria

Using the California Mastitis Test (**Plate 4.1**), the prevalence of subclinical mastitis at doe and udder level was found to be 50.9% (56/110) and 40.5% (89/220), respectively. Out of the samples which were positive for subclinical mastitis by CMT, 86.5% (77/89) gave a positive bacterial culture. A total of 169 isolates of bacteria were obtained from the cultures (**Appendix 4**).

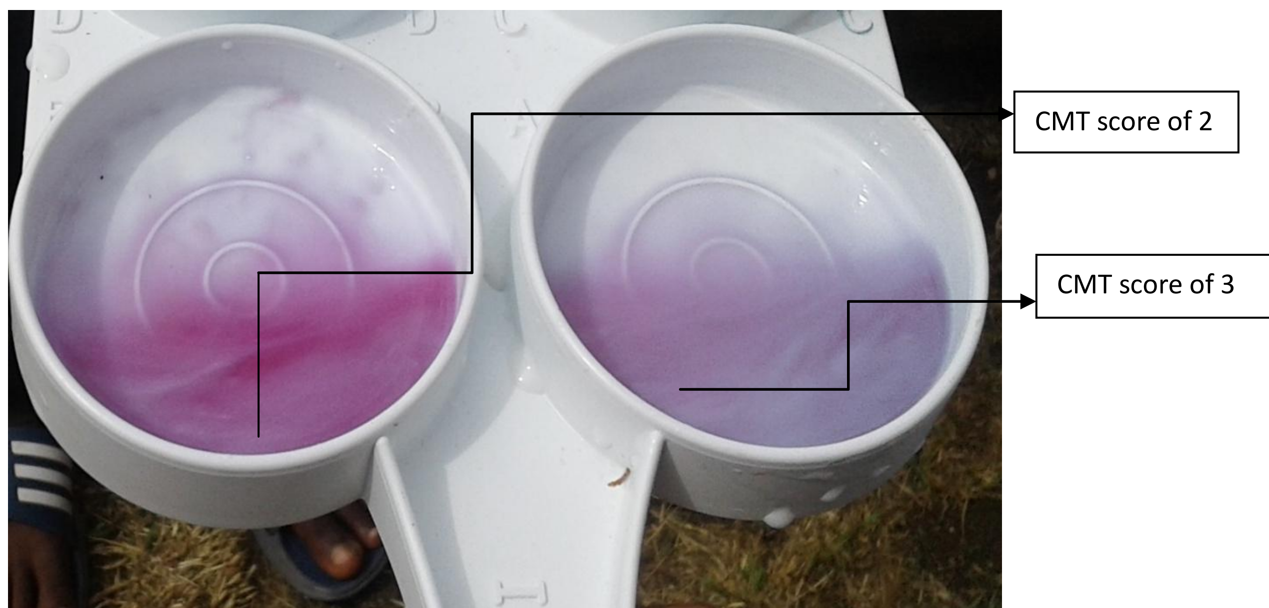


Plate 4.1: Positive California Mastitis Test milk samples

The results of the bacteria isolated and their frequency is given in Table 4. 2 below. In descending order the isolated bacteria were Coagulase Negative *Staphylococci, Serratia* spp, *Citrobacter* spp, *Klebsiella* spp, *Staphylococcus aureus, Enterobacter* spp, *E. coli, Proteus* spp, *Corynebacteria* spp, *Morganella* spp, *Streptococcus* spp, *Providencia* spp, *Micrococcus* spp. and *Staphylococcus intermedius*.

Table 4.2: Laboratory bacterial culture results for goats sampled in Thika East Sub-County, Kenya

Bacterial Species	Number of isolates	Proportion (%)
Coagulase Negative *Staphylococci*	35	20.70
Serratia spp	33	19.53
Citrobacter spp	27	15.98
Klebsiella spp	19	11.24
Staphylococcus aureus	18	10.65
Enterobacter spp	11	6.51
E. coli	10	5.92
Proteus spp	5	2.96
Corynebacterium spp	3	1.78
Morganella spp	3	1.78
Streptococcus spp	2	1.18
Providencia spp	1	0.59
Micrococcus spp	1	0.59
Staphylococcus intermedius	1	0.59
Total	169	100

4.3 Relationship between prevalence and risk factors

The results of the CMT were used to evaluate the relationship between prevalence and risk factors. In terms of doe breeds, the highest prevalence of subclinical mastitis was German alpine (66.7%), followed by crosses and the least affected were Kenyan alpine. However, there was no significant (p=0.3934, OD=1.059) differences in the prevalence of sub-clinical mastitis in the different breeds **[Table 4. 3].**

Table 4. 3: Effect of breed on prevalence of subclinical mastitis in dairy goats, Thika East Sub-County as identified by CMT

Breed	Frequency (positive does)	Proportion positive (%)	p-value
German alpine	6/9	66.7	0.3934
Crosses	20/46	56.5	
Toggenburg	10/20	50	
Others	5/10	50	
Kenyan alpine	9/25	36	

The highest prevalence of subclinical mastitis was found in the early lactation stage while lowest prevalence was found in the mid lactation stage at 41% and the late lactation stage had a prevalence of 49%. There was no significant difference (p=0.4251, OD=0.803) between the prevalence of mastitis in the different lactation stages **[Table 4.4**].

Table 4.4: Effect of lactation stage on prevalence of subclinical mastitis in dairy goats, Thika East Sub-County, as identified by CMT

Lactation stage	Frequency (positive does)	Proportion (%)	p-value
Early	14/18	78	0.4251
Mid	16/39	41	
Late	26/53	49	

Key: Early: 1 day-3 months, Mid: 3-6 months, Late : >6months

The lowest prevalence of sub-clinical mastitis was found in goats in the first parity **[Table 4.5**] and a significant (p=0.0477, OR=1.370) increase of prevalence was noted as the parity increased.

Table 4.5: Effect of parity on prevalence of subclinical mastitis in dairy goats, Thika East Sub-County, as identified by CMT

Parity	Frequency (positive does)	Proportion (%)	p-value
1	15/35	43	0.0477
2	22/46	48	
3	7/12	58	
4 and above	12/17	71	

The highest prevalence of subclinical mastitis was found in lactating does whose houses were cleaned fortnightly and there was a significant [p=0.022, OD=1.047] increase in prevalence in goats where there was less cleaning **[Table 4.6].**

Table 4.6: Effect of cleaning schedule on prevalence of subclinical mastitis in dairy goats, Thika East Sub-County, as identified by CMT

Cleaning schedule	Frequency (positive does)	Proportion (%)	p value
Weekly	30/65	46	0.022
Fortnightly	19/26	73	
Daily	4/12	33	
Irregular	3/7	42	

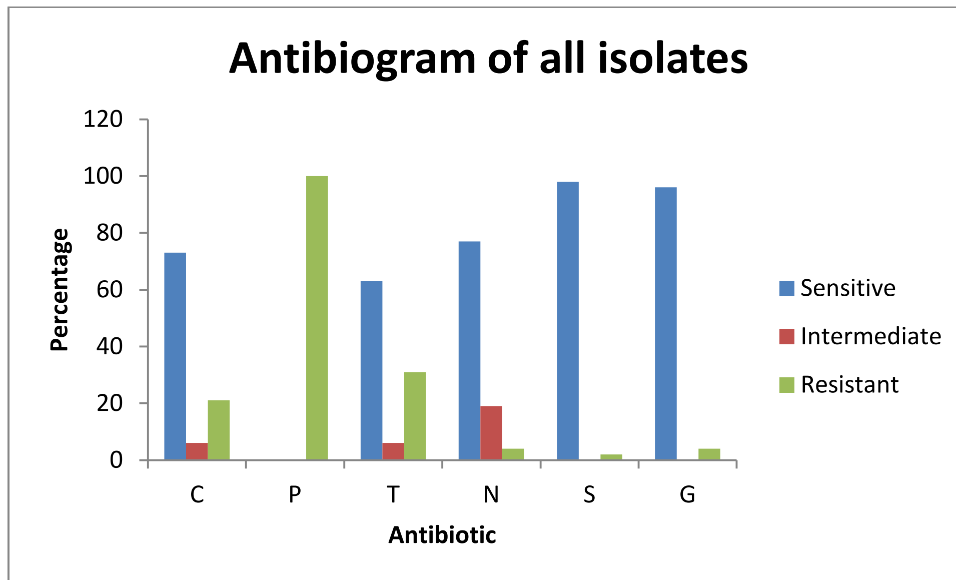
4.4Antibiotic susceptibility testing results

In overall, the bacteria isolates were most sensitive to Streptomycin (98%) and least sensitive to Tetracycline (63%) (**Table 7, Figure 3**). Of the isolates, 31% were resistant to Tetracycline, followed by Chloramphenicol (21%). All (100%) the isolates tested were resistant to Penicillin G **[Table 4. 8**].

Table 4.7: Antibiogram of bacterial isolates obtained from milk of dairy goats with subclinical mastitis to antibiotics, Thika East Sub-County, Kenya

(n=52)

Antibiotic	Sensitivity	Intermediate	Resistance
Streptomycin	51/52 (98%)	0 /52 (0%)	1/52 (2%)
Gentamycin	50/52 (96%)	0/52 (0%)	2/52 (%)
Norfloxacin	40/52 (77%)	10/52 (19%)	2/52 (4%)
Chloramphenicol	38/52 (73%)	3/52 (6%)	11/52 (21%)
Tetracycline	33/52 (63%)	3/52 (6%)	16/52 (31%)
Penicillin G	0/52 (0%)	0 /52 (0%)	52/52 (100%)



Key: C-Chloramphenicol, P: Penicillin G, T: Tetracycline; N: Norfloxacin, S: Streptomycin; G: Gentamycin

Figure 4.1: Graphical presentation of antibiotic profile of all isolates

Table 4. 8: Resistance patterns of bacterial isolates obtained from milk of dairy goats with subclinical mastitis to six antibiotics, Thika East Sub-County, Kenya

	% Resistance					
Bacteria	**C**	**P**	**T**	**N**	**S**	**G**
S. aureus (n=8)	0	100	25	25	0	0
CN*S* *(n=10)*	0	100	20	0	10	0
Serratia (n=9)	33	100	22	0	0	0
Klebsiella (n=8)	25	100	37	0	0	25
Citrobacter (n=7)	57	100	57	0	0	0
Enterobacter (n=6)	0	100	17	0	0	0
E.coli (n=4)	50	100	0	0	0	0

Key: C-Chloramphenicol, P: Penicillin G, T: Tetracycline; N: Norfloxacin, S: Streptomycin; G: Gentamycin

The *S. aureus* isolates tested were most sensitive to Gentamycin and Streptomycin (100%). The isolates were also sensitive to Chloramphenicol (88%), Tetracycline and Norfloxacin (75%) **[Table 4.9].** The *S. aureus* isolates were resistant to Penicillin G (100%) and to Tetracycline and Norfloxacin (25%).

Table 4.9: Antibiogram of *S. aureus* isolates obtained from milk of dairy goats with subclinical mastitis, Thika East Sub-County, Kenya

(n=8)

Antibiotic	**Sensitivity**	**Intermediate**	**Resistance**
Streptomycin	8/8 (100%)	0 /8 (0%)	0 /8 (0%)
Gentamycin	100	0 (0%)	0 (0%)
Chloramphenicol	7/8 (88%)	1/8 (12%)	0 (0%)
Tetracycline	6/8 (75%)	0 (0%)	2/8 (25%)
Norfloxacin	3/8 (37.5%)	3/8 (37.5%)	2/8 (25%)
Penicillin G	0 (0%)	0 (0%)	8/8 (100%)

Coagulase Negative *Staphylococci* isolates were sensitive to Gentamycin (100% of the isolates), Chloramphenicol (90%), Streptomycin (90%), Norfloxacin (80%). Reduced sensitivity was noted to Tetracycline (70%) **[Table 4.10].**

Table 4.10: Antibiogram of Coagulase Negative *Staphylococci* isolated from milk of dairy goats with subclinical mastitis, Thika East Sub-County, Kenya

(n=10)

Antibiotic	**Sensitivity (%)**	**Intermediate (%)**	**Resistance (%)**
Gentamycin	10/10 (100%)	0/10 (0%)	0/10 (0%)
Chloramphenicol	9/10 (90%)	1/10 (10%)	0/10 (0%)
Streptomycin	9/10 (90%)	0/10 (0%)	1/10 (10%)
Norfloxacin	8/10 (80%)	2/10 (20%)	0/10 (0%)
Tetracycline	7/10 (70%)	1/10 (10%)10	2/10 (20%)
Penicillin G	0/10 (0%)	0/10 (0%)	10/10 (100%)

Multidrug resistance refers to bacteria resistant to at least three drugs. In this study, multidrug resistance was found in 10 bacterial isolates (19.2%) namely 2 *S. aureus* isolates (Penicillin G, Tetracycline and Norfloxacin), 3 *Citrobacter* isolates (Penicillin G, Chloramphenicol and Tetracycline), 1 *Serratia* isolate (Penicillin G,Chloramphenicol and Tetracycline) and 2 *E.coli* isolates (Penicillin G, Tetracycline and Chloramphenicol) and 2 Klebsiella isolates (Penicillin G, Tetracycline and Chloramphenicol) **[Table 4.11].**

Table 4.11: Multidrug resistance among bacteria isolated from milk of dairy goats with subclinical mastitis, Thika East Sub County, Kenya

Bacterial Isolates	Number of resistant drugs		
	1	**2**	**3**
CN*S* (n=10)	7	3	0
Serratia (n=9)	5	3	1
S. aureus (n=8)	6	0	2
Klebsiella (n=8)	3	3	2
Citrobacter (n=7)	2	2	3
Enterobacter (n=6)	5	1	0
E. coli (n=4)	2	0	2

4.5 Antibacterial activity of bromelain to bacterial isolated from milk of dairy goats with subclinical mastitis using the Agar Well Diffusion Method

4.5.1 Antibacterial activity of extracted bromelain

The antibacterial activity of extracted bromelain against bacterial isolates was dose dependent. Antimicrobial activity was noted at 5000µg/ml for 13 out of 14 (92.9%) isolates. Only one (7.1%) *Citrobacter* isolate was not sensitive to extracted bromelain at 5000µg/ml. Out of 14 isolates, only 6 (42.9%) isolates were susceptible to extracted bromelain at 2, 500 µg/ml. A total of 8 out of 14 (57.1%) were not susceptible to extracted bromelain at 2, 500 µg/ml. Except for one isolate (Klebsiella isolate number 23) that were sensitive at 1 250µg/ml, all the isolates were sensitive. No antibacterial activity was noted for all isolates below 1, 250 µg/ml **[Table 4.12].**

Table 4. 12: Sensitivity of selected bacterial isolates obtained from milk of dairy goats with subclinical mastitis to extracted bromelain

Bacteria	Isolate	Concentration of extracted bromelain (µg/ml)					
	ID	5000	2500	1250	625	312.5	156.25
Enterobacter	53	S	S	NS	NS	NS	NS
	65	S	NS	NS	NS	NS	NS
Citrobacter	51	S	NS	NS	NS	NS	NS
	121	NS	NS	NS	NS	NS	NS
Klebsiella	44	S	S	NS	NS	NS	NS
	23	S	S	S	NS	NS	NS
E. coli	41	S	NS	NS	NS	NS	NS
	11	S	NS	NS	NS	NS	NS
Serratia	30	S	S	NS	NS	NS	NS
	1	S	NS	NS	NS	NS	NS
CNS	12	S	NS	NS	NS	NS	NS
	50	S	S	NS	NS	NS	NS
S. aureus	8	S	NS	NS	NS	NS	NS
	37	S	S	NS	NS	NS	NS

Key S- Sensitive NS-Not Sensitive

4.5.2 Antibacterial activity of commercial bromelain to bacterial obtained from milk of goats with sub clinical mastitis to commercial bromelain

The activity of commercial bromelain at 5, 000µg/ml was dose dependent and also lower than that of extracted bromelain with 10 out of 14 (71.4%) isolates susceptible. The remaining 4 (28.6%) isolates were not susceptible at the same concentration. At the concentration of 2, 500 µg/ml, 6 (42.9%) of the isolates were susceptible to

commercial bromelain while 8 (57.1%) were not susceptible. At the concentration of 1, 250 µg/ml only one (7.1%) of the isolates was susceptible to commercial bromelain while the rest (92.9%) was not susceptible at these concentrations. All the tested bacterial isolates were not susceptible to commercial bromelain at 625 µg/ml and 156.25 µg/ml **[Table 4.13].**

Table 4.13: Sensitivity of selected bacterial isolates obtained from milk of dairy goats with subclinical mastitis to commercial bromelain

Bacteria isolate	**Isolate ID**	**Concentration of commercial bromelain (µg/ml)**					
		5000	**2500**	**1250**	**625**	**312.5**	**156.25**
Enterobacter	53	S	S	NS	NS	NS	NS
	65	S	NS	NS	NS	NS	NS
Citrobacter	51	NS	NS	NS	NS	NS	NS
	121	NS	NS	NS	NS	NS	NS
Klebsiella	44	S	S	NS	NS	NS	NS
	23	S	S	S	NS	NS	NS
E. coli	41	NS	NS	NS	NS	NS	NS
	11	S	NS	NS	NS	NS	NS
Serratia	30	S	S	NS	NS	NS	NS
	1	S	S	NS	NS	NS	NS
CNS	12	NS	NS	NS	NS	NS	NS
	50	S	S	NS	NS	NS	NS
S. aureus	8	S	NS	NS	NS	NS	NS
	37	S	NS	NS	NS	NS	NS

Key S- Sensitive; NS- Not Sensitive

4.5.3 Antibacterial activity of nanoencapsulated bromelain to bacteria isolated from milk of goats with sub clinical mastitis

The antibacterial activity of nanoencapsulated bromelain was dose dependant with all isolates sensitive at 200µg/ml and 100µg/ml. All the 14 bacterial isolates were sensitive to nanoencapsulated bromelain at 200µg/ml and 100µg/ml. All isolates except one (7.1%) *Citrobacter* isolate were sensitive to nanoencapsulated bromelain at 50µg/ml. A total of ten (71.4%) isolates, namely two (100%) *S. aureus* isolates, two (100%) CN*S* isolates, two (100%) *E.coli, two (100%) Klebsiella*, one (50%) *Enterobacter* isolate and one (50%), *Citrobacter* isolates were sensitive to nanoencapsulated bromelain at 25µg/ml.

The remaining four (28.6%) isolates, namely two (100%) *Serratia*, one (50%) *Citrobacter* and one (50%) *Enterobacter* not susceptible to nanoencapsulated bromelain at 25µg/ml. All (100%) bacterial isolates tested were not susceptible to nanoencapsulated bromelain at 12.5µg/ml and 6.25 µg/ml **[Table 4.14]**.

Table 4.14: Sensitivity of bacterial isolates obtained from milk of dairy goats with subclinical mastitis to nanoencapsulated bromelain

	Isolate	Concentration of nanoencapsulated bromelain (µg/ml)					
Bacteria	**ID**	**200**	**100**	**50**	**25**	**12.5**	**6.25**
Enterobacter	53	S	S	S	NS	NS	NS
	65	S	S	S	S	NS	NS
Citrobacter	51	S	S	S	S	NS	NS
	121	S	S	NS	NS	NS	NS
Klebsiella	44	S	S	S	S	NS	NS
	23	S	S	S	S	NS	NS
E.coli	41	S	S	S	S	NS	NS
	11	S	S	S	S	NS	NS
Serratia	30	S	S	S	NS	NS	NS
	1	S	S	S	NS	NS	NS
CNS	12	S	S	S	S	NS	NS
	50	S	S	S	S	NS	NS
S. aureus	8	S	S	S	S	NS	NS
	37	S	S	S	S	NS	NS

Key S- Sensitive; NS-Not Sensitive

4.5.4 Sensitivity of isolates isolated form milk of goats with sub clinical mastitis to chitosan using agar well diffusion method

All bacterial isolates were sensitive to chitosan at 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml,15.625µg/ml and 8µg/ml.

4.5.5 MIC Determination

The MIC of Streptomycin was 22.2µg/ml for 12 (85.7%) isolates and 44.4µg/ml for 2 (14.3%) isolates (one *Citrobacter* and one CN*S* isolate). Extracted bromelain and commercial bromelain did not show any inhibition in all the isolates tested **[Table 4.15].**

The MIC of the nanoencapsulated bromelain to bacterial isolates differed in all the isolates tested. Of the isolates, 9 out of 14 (64.3%) had an MIC of 25µg/ml. An *E. coli* isolate (7.1%) had an MIC of 50µg/ml. The MICs of 2 (14.3%) isolates of *Klebsiella* and *S. aureus* isolates was 100µg/ml and 2 (14.3%) other isolates of *Klebsiella* and *S. aureus* isolates was 200µg/ml **[Table 4.15].**

The MIC of bacterial isolates tested to chitosan differed among isolates. A majority of the samples, 8 (57.1%) of the isolates had an MIC of 15.625µg/ml. The highest MIC recorded was 31.25µg/ml for 3 (21.4%) of the samples. The lowest MIC was 4µg/ml for 2(14.3%) isolates. Only one (7.1%) of the isolates had an MIC of 8µg/ml **[Table 4.15].**

Table 4.15: MIC of selected bacterial isolates to different antibacterial agents

Isolate	MIC (µg/ml)				
	Extracted Bromelain	**Commercial Bromelain**	**Nanoencapsulated Bromelain**	**Chitosan**	**Streptomycin**
Enterobacter (53)	>5000	>5000	25	4	22.2
(65)	>5000	>5000	25	15.625	22.2
Citrobacter					
(51)	>5000	>5000	25	31.25	22.2
(121)	>5000	>5000	25	15.625	44.4
Klebsiella					
(44)	>5000	>5000	100	15.625	22.2
(23)	>5000	>5000	200	15.625	22.2
E.coli (41)	>5000	>5000	50	8	22.2
(11)	>5000	>5000	25	15.625	22.2
Serratia					
(30)	>5000	>5000	25	15.625	22.2
(1)	>5000	>5000	25	31.25	22.2
CN*S* (12)	>5000	>5000	25	31.25	44.4
(50)	>5000	>5000	25	4	22.2
S.aureus (8)	>5000	>5000	200	15.625	22.2
(37)	>5000	>5000	100	15.625	22.2

4.6 Identification of *S.aureus* using 16s rRNA

A total of 18 samples which had been positively identified as *S. aureus* using results of morphology, haemolysis and biochemical tests were confirmed using PCR. The 16s rRNA fragments with expected amplicon size of 230bp were obtained as shown in the agarose gel picture **[Plate 4.2]**. All the 18 isolates were positive for the *S. aureus* specific 16S rRNA.

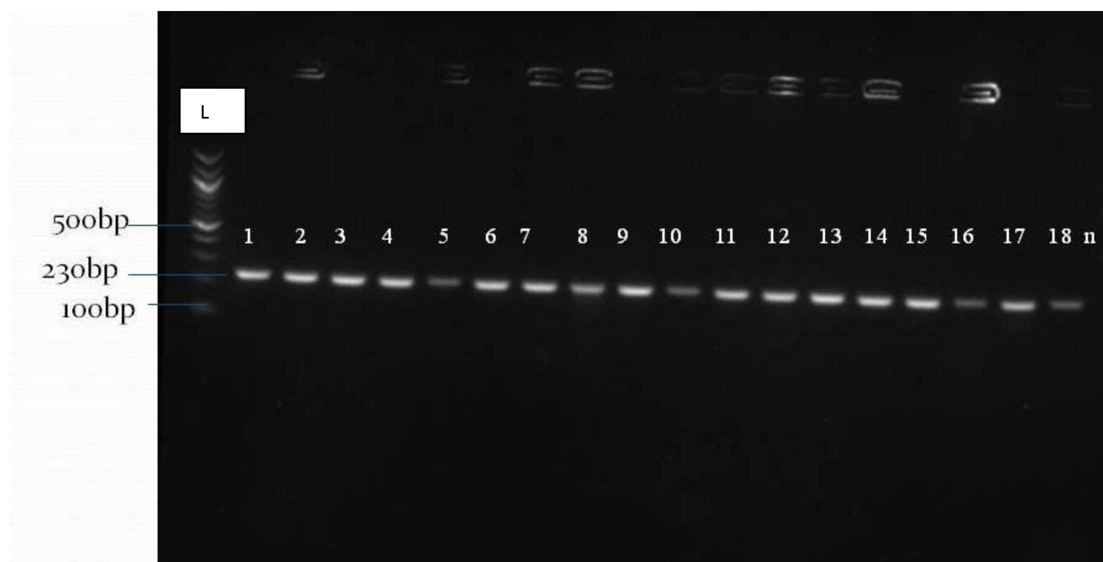


Plate 2: Gel picture of *S. aureus* specific PCR, L: 100bp ladder, Samples 1-18, negative control

4.7 Detection of *mec*A gene

The *mec*A gene was not amplified in all the 18 isolates tested for its detection. However, there was no positive control, it could not be concluded that the isolates were negative for the *mec*A gene.

CHAPTER FIVE

5.0 DISCUSSION

The findings of this work on the prevalence of subclinical mastitis (50.9%) based on CMT was higher than reported in Greece (29%) (Boscos *et al.*, 1996), Spain 30% (Contrares *et al.*, 1995), Pakistan (38%) (Pirzada *et al.*, 2016), India (19.89%) (Mishra *et al.,* 2018) and Nyeri, Kenya (28.7%) (Ndegwa *et al.*, 2001). The results were comparable to the ones found in Bulgaria (Hristov *et al*, 2016) which found a prevalence of 44.2%. The results were lower than those reported in Mount Kenya region (Mbindyo, 2014) which found a prevalence of 61% and in Tanzania (Mbilu, 2007) which found a prevalence of 76.7%. The differences in prevalence of mastitis have been attributed to the differences in host and management factors that influence intramammary infection of goats (Islam *et al.*, 2012).

Mastitis has a multi-factorial nature with a clear interaction between host, agent and environment (Thrusfield, 2007) therefore all the three factors have to be considered in management of the disease. The differences in prevalence in Kenya of 61% and 30.3% respectively (Mbindyo, 2014; Makau, 2017) might be due to differences in farm management and the size of the herds encountered. Mbindyo (2014) carried out her work in the Mount Kenya region where rearing of dairy goats is done on a large scale while Makau (2017) carried out his work in Machakos where the highest number of dairy goats kept per farm were six.

It is also possible that the herds with high prevalence of subclinical mastitis may have been herds which have been shown to be positively associated with not only

mastitis, but other infectious diseases (Makau, 2017). The high prevalence of subclinical mastitis encountered shows a high prevalence of sub clinical mastitis in the study area, which may be attributed to lack of knowledge on dairy goat farming, poor hygiene, lack of standard milking procedures, lack of proper pre and post udder washing and none usage of teat dips (Ndegwa *et al.*, 2001). In addition to this, the sampling method that was used in this study and the differences in sample size may also explain the differences in prevalence.

In this study, there was a significantly higher (p=0.02) prevalence of mastitis in does whose houses were cleaned every two weeks compared to those which were cleaned more frequently. These results are in agreement with those by Ali *et al.,* (2010), Bergonier *et al.,* (2003), Mbindyo (2014), Bourabah *et al*., (2013) and Mbilu *et al.,* (2007). Environmental mastitis caused by organisms such as coliforms tend to thrive in dirty environments which were encountered in some farms during the study and hence frequent cleaning of goat houses is recommended.

In the present study, parity (p=0.047) was found to be a risk factor for sub-clinical mastitis. This is in agreement with studies undertaken by others Boscos *et al.,* (1996), Meinzies and Ramanoon (2001), Bergonier *et al.*, 2003; Paape *et al.,* (2007), Ali *et al.,* (2010) and Mbindyo (2014). In this study, the prevalence of subclinical mastitis increased with increasing parity. The increasing prevalence with parity may be due to udder damage caused by frequent and vigorous suckling by the kids (Meinzies and Ramanoon, 2001). Monitoring somatic cell counts on a regular basis give an indication of the success of good animal husbandry and hygiene practices. It forms

an integral part of mastitis control strategies and assists in diagnosis and treatment (Pieterse and Todorov, 2010).

The most frequently isolated bacteria pathogen was Coagulase Negative *Staphylococci* (CNS). These results are in agreement with other studies by, Manser (1986), Contrares *et al.,* (2007), Sanchez *et al.*, (2009), Bourabah *et al.*, (2013), Mbindyo (2014), Salaberry *et al.*, 2015, Dore *et al.,* (2016), Makau, (2017). The members of the genus *Staphylococci* are the most important mastitis causing agents involved in all forms of mastitis even in other ruminants including goats (Radostits *et al.*, 2000). *Staphylococci* are known to cause all forms of mastitis ranging from subclinical, clinical, acute to gangrenous mastitis (Contrares *et al.*, 2007) and are the major cause of culling in domestic ruminants. Some frequently isolated Coagulase Negative *Staphylococci* include *S. epidermidis* and *S. caprae, S. pasteuri* and *S. haemolytica*. There is need to characterize the CN*S* using PCR to determine the exact species responsible for subclinical mastitis.

In the current study, a large proportion (10.65%) of sub-clinical mastitis was due to *S. aureus*. Enterotoxin secreting *Staphylococcus aureus* intramammary infections are associated with mastitis in dairy ruminants (Contrares and Rodriguez, 2011). These bacteria, apart from reducing milk yield, can develop into the clinical form and is the mostly isolated pathogen in clinical mastitis of small ruminants (Bergonier *et al.*, 2003). The clinical form of mastitis responds poorly to antibiotics because of the development of a tissue barrier that prevents penetration of antibiotics to the site of infection and it is estimated that only 70% of these staphylococcal infections responds to therapy (Quinn *et al.*, 1994). Further, *S. aureus* secretes thermostable

toxins, which enhances the zoonotic role of these pathogens in causing foodborne diseases (Manser, 1986; Vyetelova, 2011; Dore *et al.,* 2016).

The other commonly isolated bacteria in this study were coliforms which included *Serratia*, *Citrobacter*, *Klebsiella*, *Enterobacter* and *E. coli*. These results are in agreement with other studies (Radostits *et al.*, 2000) which highlighted that coliforms bacteria are the main cause of environmental mastitis in domestic ruminants. Previous studies have also reported that environmental mastitis accounted for over 50% of bacteria isolated from milk of goats having mastitis (Oliver and Mitchell, 1984). Coliforms thrive in unsanitary housing and living conditions of the dairy animals which were highly prevalent in the study area (unpublished). Some strains of *E.coli* such as the *E.coli* 0157:H7 cause bloody diarrhea in human beings (Quinn *et al.*, 1994).

In the present study, the majority of CMT positive milk samples yielded growth on bacterial culture. A high sensitivity (99%) of CMT in diagnosis has been reported (Bourabah *et al.*, 2013). A positive correlation between CMT and the presence of mastitis pathogens in CMT positive milk samples have also been reported (Mbindyo, 2014). This means that CMT is a reliable screening tool in the detection of sub-clinical mastitis and can be used to investigate sub-clinical mastitis in the dairy goat farms. The test can be used by farmers to screen for subclinical mastitis since it is a simple, field based test which is less costly and is easy to be carried out even by the farmers themselves.

Most bacteria were sensitive to Streptomycin and Gentamycin. The sensitivity of bacterial isolates in this study to Streptomycin and Gentamycin has been attributed to the rare use of these antibiotics in mastitis treatment in the study area.

A study in Nigeria (Egwu *et al.*, 1994), reported a high resistance of bacterial isolates to Streptomycin. This could have been due to the overuse of the antibiotic in the treatment of goat diseases in the study area. Studies in Bangladesh reported high resistance of *S. aureus* to Streptomycin (Razi *et al.*, 2012). In another study in Ethiopia, (Wakwoya *et al.*, 2006) reported varying degrees of resistance of bacteria to Chloramphenicol, Gentamycin and Streptomycin. These studies shows that there are differences in sensitivity to antibiotics based on the region and the use of that antibiotics in different areas and also who administers them in the correct quantities for the required period of time. Tetracycline is mostly used on dairy farms and this may account for the resistance levels observed in this study.

All of the tested bacteria were resistant to Penicillin G. Similar resistance was reported in India (Priya, 2016) and in Brazil (da Silva *et al.*, 2004). In Pakistan (Ali *et al.*, 2010) resistance of bacteria to Penicillin G was found to be 57.7%. In South Africa, Akondilire *et al* (2015) reported a 100% resistance of *S. aureus* isolated from milk to Penicillin G. The current study shows an increased resistance pattern of bacteria to Penicillin G. and this is probably due to the long time and extensive use of penicillins in the treatment of mastitis (Priya, 2016).

Multi drug resistance of bacteria to Tetracycline, Chloramphenicol and Penicillin G found in this work are comparable to earlier reports for dairy goats (Ndegwa *et al.*, 2001; Mbindyo, 2014; Makau, 2017). Recently, Makau (2017) reported resistance to

tetracyclines in Machakos County, Kenya. The resistance to beta lactams and tetracyclines is due to the fact that these antibiotics are mostly used in dairy farming (Makau, 2017). Results of this study also found that most bacteria are still susceptible to antibiotics as reported by others (Ndegwa *et al.*, 2001; Mbindyo, 2014; Makau, 2017) hence they can still be used in the treatment of subclinical mastitis.

In the present study, the antibacterial activity of bromelain was tested using the agar well diffusion method (Bansode *et al.*, 2013 Shweta, 2014. The results show that extracted bromelain may be more potent against bacteria than commercial bromelain. Similar results were reported by others (Hunduza *et al.*, 2018) which reported that extracted bromelain worked better than commercial bromelain on *Haemonchus*. The difference in potency has been attributed to the loss of activity that happens in the production and purification of commercial bromelain. The high temperatures used in commercial bromelain manufacture are able to disrupt the secondary structure of the bromelain, leading to its reduced efficacy.

There is a need to harness bromelain extraction to minimise losses in the pineapple industry by extracting bromelain from pineapple stems, leaves and crowns which are usually agricultural and industrial waste (Castel *et al.*, 2010). Bromelain has been shown to be notably potent against both gram positive and gram negative bacteria (Eshamah *et al.*, 2013; Bansode *et al.*, 2013; Shweta, 2014; Ali, 2014; Praveen *et al.*, 2014, Ashik *et al.*, 2016; Zharfan *et al.*, 2017).

The exact mechanism by which bromelain inhibits the growth of bacteria is not completely understood (Eshamar, 2013; Praveen *et al.*, 2014). However, it is hypothesised that bromelian works on the bacterial membrane which is made up of

proteins. Proteolytic enzymes, or proteases, are a class of hydrolytic enzymes capable of cleaving the peptide bonds of protein chains and are essential in physiological processes. Bromelain works as a proteolytic enzyme once it is bound to the bacterial cell membrane, causing damage and inducing cell death (Zharfan *et al.*, 2017). The number of amino acids in the bacterial cell is thought to determine the antibiotic activity of proteolytic enzymes.

In this study, bromelain was potent against both gram positive and gram negative bacteria. Most of the research on bromelain has focused on gram negative bacteria. Eshamah (2013) reported sensitivity of *E. coli* to bromelain at concentrations of 1-4mg/ml. Zharfan *et al.,* (2017) reported the sensitivity of bromelain to multidrug resistant *Pseudomonas aeruginosa,* Bansode *et al.,* (2013) on *E.coli, Salmonella paratyphy B,* and *Shigella sonnei* and reported that the fresh crude pineapple fruit juice produced the highest antimicrobial activity against *E.coli* followed by *Shigella sonnei* and *Salmonella para.B*. In another study, Ajibade *et al.,* (2015) tested crude bromelain on *Staphylococcus aureus*, *Pseudomonas aureginosa*, *Escherichia coli* and *Streptococcus pneumonia* and found it to be effective against them. Ali *et al.,* (2014) tested bromelain on *E. coli*, and *Proteus* and reported that these bacteria were susceptible to bromelain though a specific strain of *E. coli*, *Streptococcus pyogenes*, and *Bacillus subtilis* were resistant to crude bromelain. Different authors have carried out studies using bromelian and found it effective, including against *Acinetobacter* spp and *S. aureus* respectively (Shweta, 2014; Loon *et al.*, 2018). The above shows that bromelain is potent against both gram positive and gram negative bacteria and can be used as an antibacterial agent and is effective against some bacterial isolates.

The biggest challenge in drug development includes finding compounds with sufficiently lower minimum inhibitory concentrations (MICs), little toxicity, and ease bioavailability for efficient and safe use (Malhaire *et al.*, 2016). In the current study, the MIC of both extracted and commercial bromelain could not be determined as there was growth in all the microtitre plate wells. Higher concentrations of bromelain must be used to effectively determine the MIC. Bromelain was tested bromelain against *Streptococcus mutans*, *Enterococcus fecalis*, *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* using the serial broth dilution method (Praveen *et al.*, (2014). *S. mutans* showed sensitivity at the lowest concentration of 2 mg/ml as compared to *E. fecalis* (31.25 mg/ml) while *P. gingivalis* showed sensitivity at the lowest concentration of 4.15 mg/ml as compared to *Aggregatibacter actinomycetemcomitans* (16.6 mg/ml) and concluded that the MICs obtained were low enough for bromelain to be used as an antibacterial agent.

In this study, most of the bacterial isolates (71.4%) were sensitive to nanoencapsulated bromelain up to 25µg/ml using the agar well diffusion method. There was no difference in sensitivity of gram negative bacteria and gram positive bacteria to nanoencapsulated bromelain. The MIC of nanoencapsulated bromelain ranged from 25µg/ml to 200µg/ml for different isolates.

Nanoparticles can offer a novel strategy to challenge multidrug-resistant bacteria strategy to challenge multidrug-resistant bacteria (Ma *et al.*, 2017). Chitosan nanoparticles have been reported to show toxic effects against *P. aeruginosa*, *Burkholderia cepacia*, methicillin-resistant *S. aureus*, multidrug-resistant *Acinetobacter baumannii*, and *Klebsiella pneumonia* (Cheung *et al.*, 2015). In micro-

or nano-scale experiments, chitosan nanoparticles have shown antimicrobial activity (Ma *et al.*, 2017). Chitosan nanoparticles are engineered from chitosan by cross-linking. The commonly used cross-linkers are sodium tripolyphosphate (TPP) (Ma *et al.*, 2017). In this study, the ionic gelation method was used to make chitosan-TPP nanoparticles which were later used to encapsulate bromelain.

Although, the exact mechanisms of action of nanoparticles toxicity against various bacterial strains are not explored completely; it is hypothesized that antimicrobial nanoparticles tackle multiple biological pathways found in broad species of microbes (Bilal *et al.*, 2017). The advantage of using nanoparticles for chemotherapy is that the synthesis process is easy to scale up, cost effective and produce stable formulations with adjustable sizes and shapes (Bilal *et al.*, 2017).

In the present study, there was no marked difference on the effect of chitosan on gram positive and gram negative bacteria. Sensitivity of all isolates to chitosan was as low as 8µg/ml. The MICs differed with each bacterial isolate. The results of this study are in agreement with a study by Liu *et al.,* (2006), which reported that there was no difference in sensitivity of gram negative bacteria compared to gram positive bacteria to chitosan. However, No *et al.,* (2002) reported that gram-negative bacteria were more resistant than gram- positive bacteria to chitosan. It is impossible to compare the effects of chitosan in different studies because of the use of chitosan with different molecular weight and degrees of acetylation (Ma *et al.*, 2017). The low MIC of chitosan in this study shows that chitosan can be used as an antibacterial agent, as its efficacy had comparable results to those of Streptomycin, which was used as a positive control in the study

Chitosan has a broad spectrum of antimicrobial activity, but it displays different efficiency against microbes (Ma *et al.*, 2017). Chitosan has shown antibacterial activity against both gram positive and gram negative bacteria (Ma *et al.*, 2017). The antimicrobial activity of chitosan is thought to be due to the positively charged amino groups that interact with negatively charged cell membranes of microbes. This results in leakage of intracellular material of the microbe and leads to cell death (Tachaboonyakiat, 2017).

Chitosan is a fully biodegradable and biocompatible natural polymer, and can be used as a broad spectrum antibacterial (Dutta *et al.*, 2004; Ma *et al.*, 2017; Al-Remawi, 2012; Yang *et al.,* 2014). Chitosan has also been used as antibacterial agents, gene delivery vectors and carriers for proteins and drugs (Li *et al.*, 2011). Chitosan has wide spectrum of activity and high killing rate against gram-positive and gram-negative bacteria, but lower toxicity toward mammalian cells (Kong *et al.,* 2010).

The antibacterial activity of chitosan was tested and it was observed that gram-negative bacteria were less resistant to chitosan while the effect on gram-positive bacteria varied (Devlieghere *et al.*, 2004). It was also found that 0.8% of chitosan inhibited the growth of *Aeromonas hydrophila*, while 0.4% of chitosan was needed to inhibit the growth of *Edwardsiella ictaluri* and *Flavobacterium columnare* *(*Yildirim-Aksoy and Beck, (2017). It was proposed that the different responses of bacteria to chitosan were caused by the varied hydrophilicity and negative charge distribution on the bacterial surface (Ma *et al.*, 2017).

In another study, the MIC of nanoparticles made with tripolyphosphate against *E. coli*, *S. enterica* serotype *Choleraesuis* and *S. aureus* were 117, 117, and 234µg/ml respectively (Du *et al.*, 2009) . The differences in the MIC of bromelain and nanoencapsulated bromelain show that nanoencapsulation can increase the antibacterial activity of bromelain.

In the study, chitosan showed the highest antibacterial activity as compared to bromelain and nanoencapsulated bromelain. Chitosan nanoparticles usually have the challenge of poor stability and wide particle size distribution (Fan *et al.*, 2012). The antimicrobial activity of chitosan and its nanoparticles are affected by microbial, environmental and intrinsic factors (Ma *et al.*, 2017).

As the MIC of bromelain was assumed to be higher than that used in the study, bromelain might have had little effect in the nanoencapsulated bromelain. The antibacterial activity expressed might have been solely due to chitosan and the encapsulation process might have reduced the activity of chitosan. The concentration of chitosan solution/ tpp solution, pH and temperature of the chitosan solution, the stirring speed, concentration of the acetic acid and the ambient temperature during crosslinking all affect the properties of the chitosan /tripolyphosphate nanoparticles (Fan *et al.*, 2012). The various conditions should be varied to determine the optimum conditions for nanoencapsulation of bromelain with chitosan nanoparticles.

In the present study, the antibacterial activity of the chitosan/tripolyphosphate nanoparticles was not ascertained. Nanoparticles made by mixing sodium trypolyphosphate and chitosan should be included in the study to ascertain whether

conjugation reduced activity of chitosan and to what extent. Nanoencapsulation should be carried out using different concentrations of bromelain and chitosan and the effect noted. Chitosan nanoparticles and chitosan to be used as controls and the differences noted. The zeta potential of the nanoparticles were not ascertained in the study and this should be done to determine the best conditions and concentrations for efficient encapsulation of bromelain.

It was noted that *Citrobacter* spp was the most resistant bacteria in the study, both using existing antibiotics and the test drugs. C*itrobacter* spp. has been found to be partly responsible for food-borne diseases, with the bacteria contaminating food and water (Ifeadike *et al.*, 2012). *Citrobacter* spp are ubiquitous and are usually resistant to multiple antibiotics due to plasmid-encoded resistance genes. They are often resistant to cephalosporins due to over expression of their chromosomal β-lactamase (Pepperell *et al.*, 2002). In a clinical study, it was also found that the largest group of multi drug resistant *Enterobacteriaceae* identified belonged to the genus *Citrobacter* (Pepperell *et al.*, 2002). In this study, milkers with poor personal hygiene could have potential sources of contamination by the *Citrobacter* spp.

A total of 18 isolates which had been identified as *S. aureus* using the PCR amplification method of *Staphylococcus aureus* specific 16s rRNA in this study gave the same identification results as the conventional method, but took less time, was sensitive and fast. Similar reports were given by El-Hadedy and El-Nour (2012) in a study which gave similar results. A study also carried out by Ismail (2017) in which *S. aureus* from milk was isolated and identified using *S. aureus* specific 16s rRNA primers as a primary step in identification of *mec*A gene (Haran *et al.*, 2012; Ismail,

2017). This clarifies that before genes are identified, there is need to confirm the identity of the pathogen using PCR.

Identification of *S. aureus* using 16s rRNA has been done in mastitis cases and in human clinical samples (Saruta *et al.*, 1997; Mason *et al.*, 2001; Moatamedi *et al.*, 2007; Haran *et al.*, 2012; Ali, 2014; Akindolire *et al.*, 2015; Ismail, 2017). In all these cases, identification of *S. aureus* has been reliable, with most isolates being identified with a sensitivity of 98-100% (Haran *et al.*, 2012).

Staphylococcus aureus is most frequent cause of mastitis in dairy animals, which is often difficult to cure and is prone to resurgence (Hoque *et al.*, 2018). PCR-based techniques are being increasingly used for identification and typing since they are rapid and easy to test large numbers of strains with a high reliability and differentiation power (Pinto *et al.*, 2015; Hoque *et al.*, 2018). These techniques also have the advantage of being universally accepted and applicable when they are also rapid and reliable (Pinto *et al.*, 2005; Akindolire *et al.*, 2015). The polymerase chain reaction (PCR) method based on 16S rRNA gene for the detection and identification of pathogenic bacteria in food presents a sensitive and fast method (El-Hadedy and El-Nour, 2012). However, the procedure is costly to carry out in routine diagnosis.

In the present study, all the 18 *S. aureus* did not amplify for the *mec*A gene. Since there was no positive control, it could not be concluded whether the gene was truly absent in the isolates. However, the absence of the *mec*A gene in *S. aureus* isolates has been recorded. A study done by Aras *et al.,* (2012) reported there had not been detection of *mec*A gene in *S. aureus* isolated from goat mastitis infection in goats in a study in Turkey. In a study by Virdis *et al.,* (2010), no *mec*A gene was found in

100 *Staphylococci* isolates isolated from milk. In Minnesota, Haran *et al.,* (2012) detected a low prevalence (2/95) of the *mec*A gene in *S. aureus* samples isolated from milk. Several studies on the *mec*A gene has been studied in *Staphylococci* isolated from milk (Virdis *et al.*, 2010; Vyeletova *et al.*, 2011; Aras *et al.*, 2012; Akindolire *et al*,. 2015).

The rise of drug resistant virulent *S. aureus* strains is a problem in treatment and control of *Staphylococcal* infections (Tessema *et al.*, 2016). Methods of detection of *mec*A gene except for PCR technique are prone to errors due to heterogeneous nature of methicillin resistance and dependence on environmental conditions (Leke *et al.*, 2017). Detection of the *mec*A gene by polymerase chain reaction (PCR) is the gold standard for identifying methicillin-resistant *Staphylococcus aureus* (Siripornmongcolchai *et al.*, 2002).

Extensive studies have also been carried out using *S. aureus* isolates for detection of the *mec*A gene in clinical samples (Mason *et al.*, 2001; Franca *et al.*, 2012; Abazar *et al.*,2013; Zhou *et al.*, 2017; Hoque *et al.*,2018). In all the cases, the percentage of *S. aureus* isolates carrying the *mec*A gene has been less than 5%. *S. aureus* is thought to be transmitted to animals during milking (Bradley 2002; Bergonier *et al.*, 2003) and these results suggest that methicillin resistant genotypes associated with hospitals and community infections is isolated from milk at very low rates (Haran *et al.*, 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The current study showed that dairy goats in Thika East sub-county of Kenya had high prevalence of sub-clinical mastitis. Since farmers are not aware of the occurrence of the sub-clinical disease, they could be having major economic losses through reduction in milk quality and quantity. Farmers in the study area should be encouraged to use rapid farm-based screening tests like the California Mastitis Test to screen for subclinical mastitis and treat accordingly.

The present study showed that hygiene plays a big role in the occurrence of subclinical mastitis hence the farmers should ensure good sanitation and maintain strict cleaning schedules for goat houses to reduce the occurrence of subclinical mastitis. The study also showed that the prevalence of subclinical mastitis increases with increased parity. Screening tests for mastitis like the California Mastitis Test should be used vigilantly for such does.

The study also showed that the genus *Staphylococci* was the main etiological agent of subclinical mastitis with CNS being the mostly isolated bacteria. Coliforms were also isolated in the milk from does with mastitis which indicated that the does were living in unsanitary conditions. Most broad spectrum antibiotics were effective in treating subclinical mastitis.

The study showed that bromelain was effective against both gram positive and gram negative bacteria. Higher concentrations of bromelain than those used in the study should be used to determine the MIC.

The study also showed that nanoencapsulated bromelain was more effective against the tested bacterial isolates than bromelain. This shows that encapsulation with chitosan increased the efficacy of bromelain. Chitosan had a very low MIC which was comparable to Streptomycin on both gram positive and gram negative bacteria and thus could be used as an antibacterial agent for treatment of bacterial pathogens.

The study also showed that PCR is a rapid, accurate diagnostic tool for bacterial identification in reduced time. Due to the lack of the positive control for the *mec*A gene, results of the *mec*A gene were not definitive.

6.2 RECOMMENDATIONS

1. Livestock Production Officers should train farmers on improved improved animal husbandry practices such as good housing, maintenance of regular cleaning schedule and proper milking procedures in order to reduce subclinical mastitis cases.

2. Surveillance of sub-mastitis can be done using screening tests like the CMT which can be able to reduce the losses.
3. Farmers need to send milk samples for antibiogram before treatment of subclinical mastitis using antibiotics which should be done by qualified professionals.

4. Susceptibility of bacterial isolates should be tested before using Penicillin G for treatment of sub-clinical mastitis
5. Pineapple waste (peels) from industries and agricultural farms can be utilised for bromelain extraction which can be used for treatment of mastitis
6. Nanoencapsulated bromelain and chitosan should be tested for safety *in vivo* levels with an intention of developing it as drug for management of mastitis in dairy goats

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APPENDICES

Appendix 1: Farm Level Questionnaire

STUDY OF SUBCLINICAL MASTITIS IN DAIRY GOAT IN THIKA EAST SUB-COUNTY

BACKGROUND INFORMATION

1. Farm Location:

   Sub-County…………………Division……………….Location…………….

Farmsize………………………………..Farm name…………………………

2. Owner details: Name……………………..

For how long have you been keeping goats……………………………………

3. Current goat herd size…………………………………………………………

4. Goat Breeds kept (Tick applicable)

Toggenburg ☐ Kenyan Alpines ☐ Galla ☐ Saneen ☐

Anglonubian ☐ German Alpines ☐ Crosses ☐

Other (Specify)..

5. Any other form of livestock kept

a. Cattle ☐ b. Sheep ☐ c. Pigs ☐ d. Poultry ☐

6. Do you wash your hands before milking and after? Yes ☐ No ☐

7. Do you wash the udder and teat before and after milking? Yes ☐ No ☐

If Yes :With water alone Yes ☐ No ☐

Water and disinfectant Yes ☐ No ☐

8. Is the udder dried – Yes ☐ No ☐

If yes what is used? a. Disposable paper towels ☐ b. Reusable towels ☐

c. Others specify………………

9. Do you use teat dips; Yes ☐ No ☐

10. How many times are the goat milked a. Once ☐ b. Twice ☐ c. Thrice ☐

11. Dry of periods –Yes ☐ No ☐
If yes; stop milking at once ☐ Gradually ☐

12. Any treatment performed? Yes ☐ No ☐

All halves? Yes ☐ No ☐

Mastitic halves? Yes ☐ No ☐

13. Have you ever had a case of mastitis Yes ☐ No ☐

If yes how was it treated? …………………………………

14. Name of the antibiotic used…………………

15. Any reports of culling due to mastitis? Yes ☐ No ☐

16. What is the local name given to mastitis………………..

17. What are the clinical signs observed…………………………………

18. Any teat / milk abnormalities observed……………………………………

19. Any rejection of milk due to mastitis in the past 12 months Yes ☐ No ☐

20. Type of housing a. Earthen ☐ b. Raised timbers ☐ Other……………….

21. Maintenance of the structures and hygiene of the structures

Good ☐ . Fair ☐ Bad ☐

22. How often is the structure cleaned?

Daily ☐ Once a week ☐ Twice a month ☐ Other………………………..

23. Feeding system: Zero grazing ☐ . Open grazing ☐ Tethering ☐

27. Is the milk pasteurized before it is sold? Yes ☐ No ☐

Appendix 2: Doe Level questionnaire

Doe ID	**Farm Name**	**Breed**	**Goat Age**	**Parity**	**Litter size**	**Lactation Stage**	**Mean Milk Yield/day**	**Mastitis History (Yes/No)**
1.								
2.								
3.								
4.								
5.								
6.								
7.								
8.								

Appendix 3: California Mastitis Test Scores (Quinn et al, 1994)

CMT Score	Visible Reaction	Interpretation
0	Milk fluid and normal	Negative
T	Slight precipitation	Trace
1	Distinct precipitation but no gel formation	Weak positive
2	Mixture thickens with a gel formation	Distinct positive
3	Viscosity greatly increased. Strong gel that is cohesive with a convex surface	Strong Positive

Appendix 4: Biochemical test results and identity of bacterial isolates from milk of dairy goats with sub-clinical mastitis, Thika East Sub County

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
1.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
2.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
3.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
4.	neg rods	+		-	+	+	+	+	+	-	+	-	+	*Proteus*
5.	neg rods	+		-	+	+	+	+	+	-	+	-	+	*Proteus*
6.	neg rods	+		-	-	+	-	+	+	-	-	-	-	*Serratia*
7.	neg rods	+		-	-	+	-	+	+	-	-	-	-	*Serratia*
8.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
9.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
10.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
11.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
12.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
13.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
14.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
15.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
16.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
17.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
18.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S.aureus*
19.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
20.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
21.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
22.	neg rods	+		-	+	+	+	+	+	-	+	-	+	*Proteus*
23.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
24.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
25.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
26.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
27.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
28.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
29.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
30.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
31.	pos rods	+		-			-	-	-	-	-			*Corynebacterium*
32.	pos rods	+		-			-	-	-	-	-			*Corynebacterium*
33.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S.aureus*
34.	pos cocci	-		-						-				*Streptococcus*
35.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*

Isolate ID	**Gram Test**	**Catalase**	**Coagulase**	**Mannitol**	**MR**	**VP**	**Indole**	**Motility**	**Citrate**	**Oxidase**	**Urea**	**LF**	**H2S**	**ID**
36.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
37.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
38.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E. coli*
39.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E. coli*
40.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E. coli*
41.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
42.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
43.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
44.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
45.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
46.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
47.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
48.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
49.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
50.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
51.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
52.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
53.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
54.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
55.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
56.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
57.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
58.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
59.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
60.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
61.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S.aureus*
62.	pos rods	+		-			-	-	-	-	-			*Corynebacterium*
63.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
64.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
65.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
66.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
67.	pos cocci	-		-						-				*Streptococcus*
68.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
69.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
70.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
71.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
72.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
73.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
74.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
75.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
76.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
77.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
78.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
79.	Neg rods	+					+	+	-	-	+	-	-	*Morganella*
80.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
81.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
82.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
83.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
84.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
85.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
86.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
87.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
88.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
89.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
90.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
91.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
92.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
93.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
94.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
95.	neg rods	+		-	+	+	+	+	+	-	+	-	+	*Proteus*
96.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
97.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
98.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
99.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
100.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CNS
101.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
102.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
103.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
104.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
105.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
106.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
107.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
108.	Neg rods	+					+	+	-	-	+	-	-	*Morganella*
109.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
110.	Neg rods	+					+	+	-	-	+	-	-	*Morganella*
111.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
112.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
113.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
114.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
115.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
116.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
117.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
118.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
119.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
120.	Neg rods	+		-	+	-	+	+	+	-	-	-	-	*Providencia*
121.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
122.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
123.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
124.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
125.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
126.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
127.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
128.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
129.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
130.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
131.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
132.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E. coli*
133.	Pos cocci	+		-						+				*Micrococcus*
134.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
135.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
136.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
137.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*
138.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
139.	neg rods	+		+	-	+	-	+	+	-	-	-	-	*Serratia*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
140.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CNS
141.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
142.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
143.	pos cocci	+	+	+	+	+	-	-	+	-	+		-	*S. aureus*
144.	neg rods	+		-	+	+	+	+	+	-	+	-	+	*Proteus*
145.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
146.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
147.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
148.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
149.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
150.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
151.	Pos cocci	+	+	-		-				-				*S. intermedius*
152.	Neg rods	+		+	-	+	-	+	+	-	-	+	-	*Enterobacter*
153.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
154.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
155.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
156.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
157.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
158.	Neg rods	+		-	-	+	-	-	+	-	+	+	-	*Klebsiella*
159.	pos cocci	+	-	+	+	+	-	-	-	-	+		-	CN*S*
160.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
161.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
162.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
163.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
164.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
165.	Neg rods	+		+	+	-	+	+	-	-	-	+	-	*E.coli*
166.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*

Isolate ID	Gram Test	Catalase	Coagulase	Mannitol	MR	VP	Indole	Motility	Citrate	Oxidase	Urea	LF	H2S	ID
167.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
168.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*
169.	neg rods	+		+	+	-	+	+	+	-	+	+	-	*Citrobacter*